Pre- and Pro-biotics May Improve Mineral Absorption and Retention in the Growing Male Rat

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Abstract

Probiotics are bacteria, which reside in the large intestine and concur beneficial health effects on their host. Their abundance can be selectively-stimulated by prebiotics, such as fructo-oligosaccharide (FOS); prebiotics are oligosaccharides, which are not digested in the small intestine, but pass into the large intestine where they are fermented into short-chain fatty acids. Several studies have suggested that prebiotics may improve mineral absorption. This study aimed to determine the effects of pro- and pre-biotic supplementation on mineral absorption and bone quality in growing male rats.

Sixty three-week old male Sprague-Dawley rats were randomised into five groups and fed either a high-calcium milk powder (HCMP) with or without a probiotic added (groups were subsequently named HCMP – and HCMP + respectively), or HCMP and vitamin K with or without the probiotic (HCMPK – and HCMPK +), or the HCMP with FOS replacing the sucrose in other diets, and the probiotic (the dietary group was named FOS). Animals were maintained on diets for 10 weeks.

Balance studies were carried out during weeks 3 – 4 and 8 – 9 of the study. The earlier balance study suggested that dietary interventions may affect mineral absorption. The latter balance study, however, showed no discernable differences between groups. Several reasons were postulated for this. Active-absorption may have been down-regulated as a result of long-term supplementation, or an increased abundance of probiotics could cause an elevation of nutritional demands. Alternatively, supplementation may not prove beneficial once animals had passed their period of peak absorption. Bone resorption and formation did not appear to have been altered as a result of dietary intervention, when measured after 10-weeks. Bone mineral density and content, calcium, magnesium, zinc and ash contents and bone biomechanical testing also showed no significant differences between dietary groups. Further research is required to determine whether results obtained were due to long-term supplementation and / or due to the joint-supplementation of pre- and pro-biotics.

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Abbreviations

$1,25(OH)_2D$	1,25-dihydroxyvitamin D
αvβ3	Vitronectin receptor
AKT	Serine-threonine kinase
BMC	Bone mineral content
BMD	Bone mineral density
BMP	Bone morphogenic protein
BMU	Basic multicellular unit
CAII	Carbonic anhydrase II
CaBP	Calbindin
Cbfa-1	Core binding factor α-1
CFU	Colony-forming unit
CTR	Calcitonin receptor
CTx	C-telopeptides of Type I collagen
DEXA	Dual Energy X-ray Absorptiometry
DP	Degree of polymerisation
E	Oestrogen
ERK	Extracellular regulated kinase
FDCR1	Follicular dentritic cell receptor 1 (OPG)
FGF	Fibroblast growth factors
FOS	Fructo-oligosaccharide
GH	Growth hormone
GOS	Galacto-oligosaccharides
HBSS	Hanks Balanced Salt Solution
HCMP	High calcium milk powder
HCMPK	HCMP with added vitamin K
HSH	Hypomagnesemia with secondary hypocalcemia
I	Inulin
ICP-OES	Individually coupled plasma-optical emission spectrometer
IFN- γ	Interferon- γ
IGF	Insulin-like growth factor
IGFBP	IGF-binding protein
IL	Interleukin

JNK	Protein kinase c-Jun N-terminal kinase
LPS	Lipopolysaccharide
M-CSF	Macrophage colony-stimulating factor
MagT1	Magnesium Transporter protein
Mi	Microphthalmia
MNC	Mononuclear cell
NDO	Non-digestible oligosaccharide
OCIF	Osteoclastogenesis-inhibitory factor (OPG)
ODF	Osteoclast differentiation-inducing factor (RANKL)
OF	Oligofructose
OPG	Osteoprotogerin
Osf-2	Osteoblast-stimulating factor 2 (*Cbfa-1*)
OVX	Ovariectomised
p38 MAPK	Mitogen activated protein kinase
PBS	Phosphate buffer solution
PTH	Parathyroid hormone
PTHrP	Parathyroid hormone-related protein
RANK	Receptor activator of the NF-κB
RANKL	Receptor activator of the NF-κB ligand
ROS	Reactive oxygen species
RS	Resistant starch
SCFA	Short chain fatty acid
SEM	Standard error of the mean
T_3	Triiodothyroxine
T_4	Tetraiodothyroxine
TGF-β	Transforming Growth Factor β
TNF-α	Tumor necrosis factor-α (cachectin)
TR1 or TNFr1	TNF-receptor-like molecule 1 (OPG)
TRAF6	TNF-receptor associated factor-6
TRANCE	RANKL
TRAP	Tartrate-resistant acid phosphatase

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Introduction

Nutritional recommendations were once based on the amount required by the majority of a population to prevent symptoms of deficiency (within the population one would expect the exact amount required to differ slightly between individuals). More recently, however, the importance of producing recommendations based on the ability of a nutrient to cause or prevent illness in a population has become more established. This has been paralleled by an increase in affluence and hence understanding the effect of an excess intake of a nutrient has also become more significant. As a result, realisation of the potential of nutrition to maximise health and well-being is better appreciated.

One such area in which nutrition may prove useful is in the prevention of osteoporosis. Approximately one in four women, and one in eight women over the age of fifty in New Zealand have low bone density (Sainsbury and Richards, 1997); osteoporosis can lead to a significant reduction in quality of life for sufferers, and high financial costs. Prevention of the disease has so far appeared better than cure; attempts at restoring bone loss have so far proved relatively unsuccessful (Sainsbury and Richards, 1997). Maximising peak bone mass early in life has the potential to reduce the likelihood of osteoporotic fractures later in life. Peak bone mass is, in turn, affected by several factors, such as nutrient availability, exercise and hereditable elements (Sainsbury and Richards, 1997). Improving the efficiency with which the minerals deposited in bone, such as calcium, magnesium and zinc, are absorbed and used in bone has the potential to reduce the morbidity of osteoporosis.

Chapter 1. Literature Review

Section 1. Bone

1.1.1. Function

Bones have five main functions in the body. They provide a frame of support and protect organs and bone marrow. By acting as an attachment site for muscles, bones also permit movement. They are an important storage site of minerals such as calcium and phosphate, whilst the haematopoietic tissue in bone marrow produces blood cells.

1.1.2. Structure

Bones can be classified morphologically into long, short, flat, irregular or round bones. Long bones are long and narrow. Short bones have lengths and widths approximately equal. Flat bones have broad surfaces, and round bones are spherical. The remainder are irregular bones, which have varied shapes.

Each bone is encased in a layer of dense connective tissue that contains blood vessels known as the periosteum. The bone can be divided into three areas, the epiphyses (at the extremities), diaphysis (the shaft), and metaphysis (the growing section, which lies between the epiphysis and diaphysis). A diagram of a long bone showing these anatomical distinctions is shown in Figure 1.

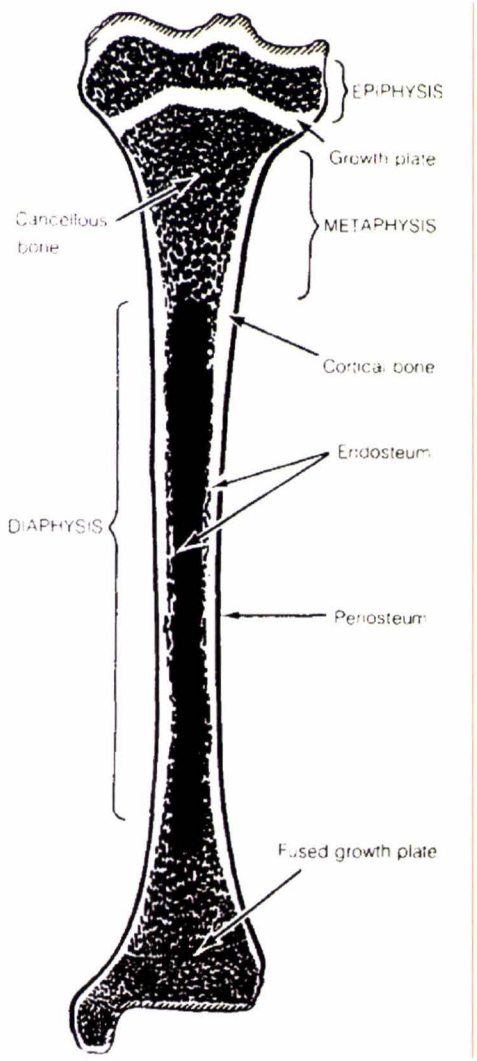

Figure 1: Anatomical features of a long bone, showing epiphysis, metaphysis and diaphysis. Taken from Baron (1999).

Rather than being uniformly solid, bone contains spaces, which provide channels for blood vessels, and reduce the weight of the skeleton. Sections are classified as trabecular (spongy) or cortical (compact), depending on the size and distribution of the spaces they contain, as shown in Figure 2.

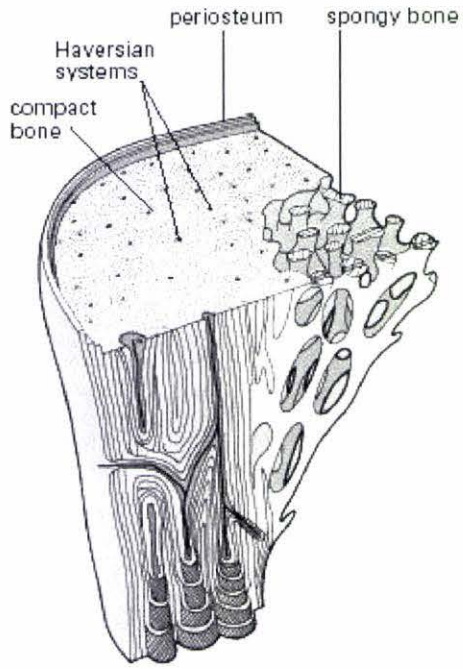

Figure 2: Cortical (compact) and trabecular (spongy) bone. Taken from Oxford University Press (2003).

The relative composition of these two types of skeletal bone differs throughout the body, as shown in Figure 3.

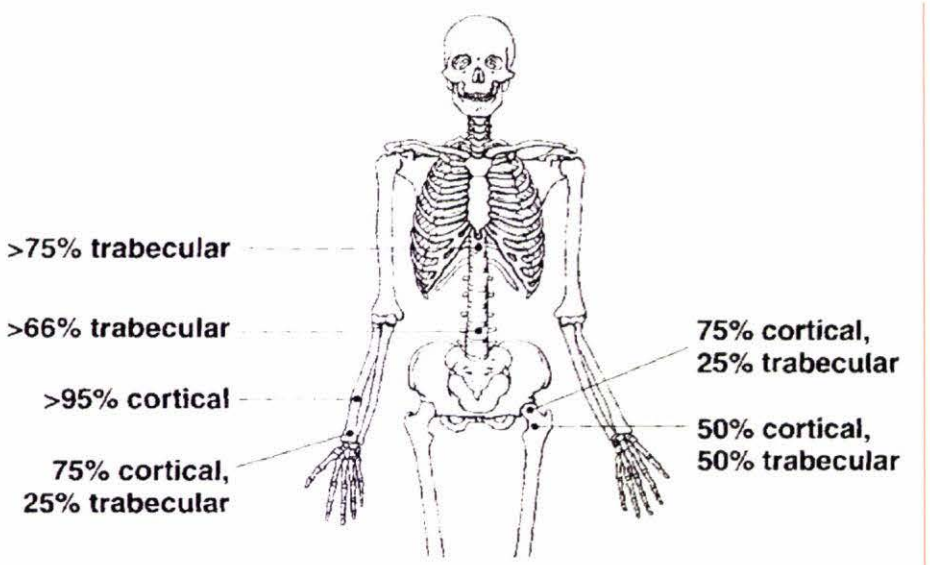

Figure 3: Relative distribution of cortical and trabecular bone in different parts of the skeleton. Taken from Mundy (1999).

1.1.3. Chemical Composition of Bone

There are three main constituents of bone; an organic matrix, inorganic salts, and cells. The bone matrix represents about 30% of the total skeletal mass (Pocock and Richards, 2004). Its major component is collagen; hyaluronic acid and chondroitin sulphate are also present. Bone matrix proteins include osteocalcin, osteonectin, osteopontin, bone sialoprotein, matrix-Gla-protein, fibronectin and alkaline phosphatase. Bone salts are predominately made up of calcium and phosphate in a form known as hydroxyapatite ($Ca_{10}(PO_4)_6(OH)_2$). There are three major bone cell types; osteoblasts, osteoclasts and osteocytes.

1.1.3.1. Osteoclasts

Osteoclasts act to resorb bone by demineralisation and degradation (discussed further in 1.1.4.3). A scanning electron micrograph of an osteoclast is shown in Figure 4.

Figure 4: Scanning electron micrograph of an osteoclast. Taken from Shalhoub *et al.* (1999).

Osteoclasts are giant cells formed from mononuclear precursors in the monocyte / macrophage lineage. The presence of certain factors are necessary to signal these precursors to differentiate into the multinucleated osteoclasts rather than other cells in the family, such as erythrocytes, granulocytes, mast cells, megakaryocytes, lymphocytes and macrophages. Some of the factors involved in osteoclast differentiation are shown in Figure 5.

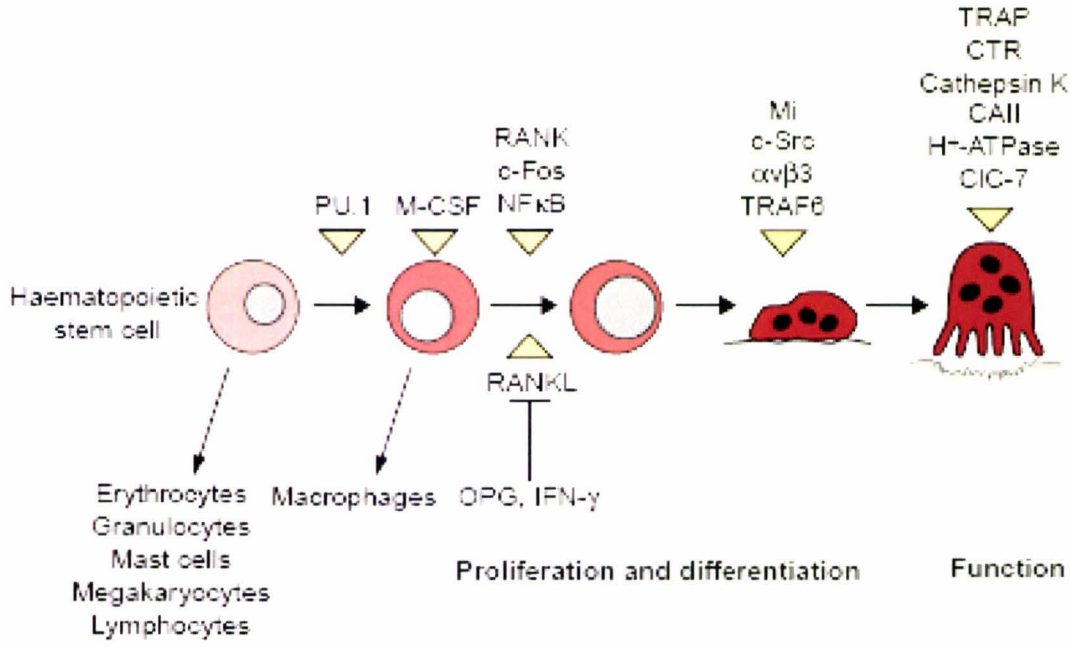

Figure 5: Signals required for osteoclast differentiation and function. αvβ3, Vitronectin receptor; CAII, Carbonic anhydrase II; CTR, Calcitonin receptor; IFN- γ, Interferon- γ; M-CSF, Macrophage colony-stimulating factor; Mi, Microphthalmia; OPG, Osteoprotogerin; RANK, Receptor activator of the NF-κB; RANKL, Receptor activator of the NF-κB ligand; TRAF6, Tumor necrosis factor (TNF) receptor associated factor-6; TRAP, tartrate-resistant acid phosphatase. Taken from Wagner and Karsenty (2001).

PU.1 is a transcription factor that encodes an ETS-domain containing protein required for lymphoid and myeloid differentiation (Wagner and Karsenty, 2001); mice lacking PU.1 are devoid of osteoclasts and macrophages and are osteopetrotic (Tondravi *et al.*, 1997). Osteopetrosis is characterised by increased bone mass and obliteration of bone marrow cavity; bones are dense, brittle and fracture easily (Wagner and Karsenty, 2001; Oxford Reference Online, 2002).

Macrophage-stimulating factor (M-CSF; also known as colony-stimulating factor 1, CSF-1) binds to its receptor c-fms, and, under the influence of the receptor activator of NF-κB ligand (RANKL), signals cells to differentiate into osteoclasts (Zaidi *et al.*, 2005). *op / op* mice are deficient in M-CSF; animals are osteopetrotic, with osteoclasts present in reduced numbers (Chambers, 2000).

RANKL (also known as TRANCE, osteoclast differentiation-inducing factor (ODF), or osteoprotogerin (OPG) ligand) is a member of the tumour-necrosis factor (TNF) family. It is produced as a membrane-bound protein by osteoblasts, and then

cleaved into a soluble form by metalloproteins (Nakashima *et al.*, 2000). RANKL is a protein of 317 amino acids; *OPGL* mRNA is predominately expressed in bone, bone marrow and lymphoid tissues (Steeve *et al.*, 2004). *opgl* mutant mice lack osteoclasts, and show severe osteopetrosis (Kong *et al.*, 1999). RANKL binds to the receptor activator of the NF-κB (RANK), a transmembrane protein of 616 amino acids (Steeve *et al.*, 2004). After binding of RANKL to RANK on osteoclast precursors, the complex interacts with TNF receptor-associated factors (TRAFs) 1 – 6, of which TRAF6 appears to be essential. Mice defective in TRAF6 show osteopetrosis; osteoclasts differentiate, but are unable to resorb bone due to a lack of contact with the bone surface (Lomaga *et al.*, 1999). TRAFs activate several downstream signalling pathways including the NF-κB, AKT (serine-threonine kinase), JNK (protein kinase c-Jun N-terminal kinase), p38 MAPK (Mitogen activated protein kinase) and ERK (extracellular regulated kinase) pathways, which result in osteoclastogenesis, or bone resorption or survival.

Osteoprotogerin (OPG; also known as osteoclastogenesis-inhibitory factor (OCIF), or TNF-receptor-like molecule 1 (TR1 or TNFr1), or follicular dentritic cell receptor 1 (FDCR1)) is a decoy receptor for RANKL. It is also in the TNF-receptor family, and is produced by osteoblasts. OPG is synthesised as a protein of 401 amino acids, and subsequently cleaved to 380 amino acids (Steeve *et al.*, 2004). Its binding to RANKL neutralises the cytokine, inhibiting osteoclastogenesis (Aubin and Bonnelye, 2000; Abu-Amer *et al.*, 2004). OPG deficient mice show increased bone resorption, irrespective of the presence or absence of bone-resorbing factors such as parathyroid hormone (PTH) (Udagawa *et al.*, 1999).

The production of OPG and RANK allows a point of control for osteoclastogenesis. Factors such as Interleukin (IL)-1β, IL-6, IL-11, and TNFα can

promote this process, through increasing expression of RANKL and decreasing expression of OPG, whilst other factors (e.g. IL-13, IL-γ, and TGF-β) can suppress RANKL and / or promote OPG expression, inhibiting osteoclastogenesis (Nakashima *et al.*, 2000).

1.1.3.2. Osteoblasts

Osteoblasts have four main roles in the body; synthesis of components required for the bone matrix, synthesis of factors required for bone formation, regulation of osteoclast activity (through synthesis of factors such as RANKL and CSF-1) and differentiation into osteocytes. A scanning electron micrograph of an osteoblast is shown in Figure 6.

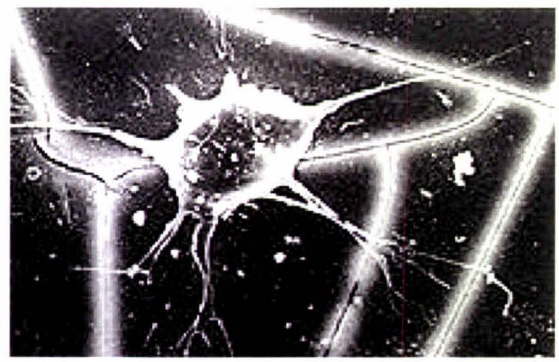

Figure 6: A scanning electron micrograph of an osteoblast. Taken from Loty *et al.* (2001).

Osteoblasts are derived from multipotent mesenchymal stem cell precursors; these precursors also give rise to bone marrow stromal cells, chondrocytes, muscle cells and adipocytes (Manolagas, 2000; Wagner and Karsenty, 2001). The signals required for osteoblast differentiation are shown in Figure 7.

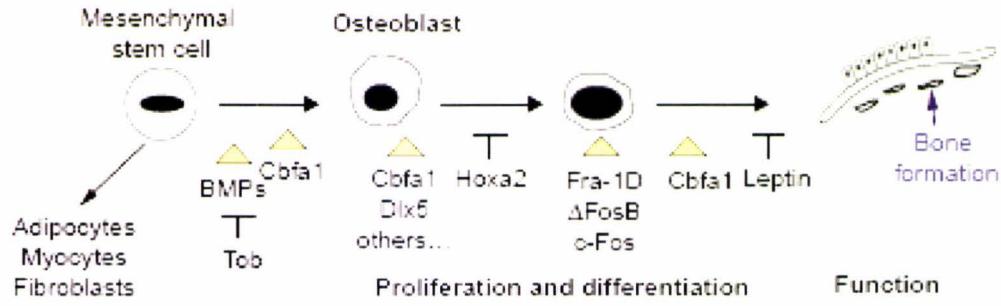

Figure 7: Signals required for osteoblast differentiation and function. Cuboidal osteoblasts are shown on the newly formed bone, together with some osteocytes embedded in the bone matrix. Adapted from Wagner and Karsenty (2001).

Cbfa-1 (Core binding factor α-1; also known as *Runx-2*; or osteoblast-stimulating factor 2, *Osf-2*) is required early in the signalling pathway for osteoblast differentiation. It activates osteoblast-specific genes such as osteopontin, bone sialoprotein, type I collagen and osteocalcin (Ducy *et al.*, 1997; Ducy and Karsenty, 1998; Manolagas, 2000). Deletion of *cbfa-1* results in a complete lack of osteoblasts (Komori *et al.*, 1997; Otto *et al.*, 1997).

Bone morphogenic proteins (BMPs) are members of the Transforming Growth Factor β (TGF-β) superfamily; seven BMPs exist, known as BMPs 1-7, whose roles may overlap (Blair *et al.*, 2002). BMP-2 and BMP-4 are thought to be of particular importance in the differentiation of osteoblasts from their precursors. BMP-4 induces a homeobox-containing gene, distal-less 5 (*Dlx5*), which may act as a transcription factor, regulating the expression of osteocalcin and alkaline phosphatase, as well as regulating mineralisation (Manolagas, 2000). Signalling by BMPs, as with other members of the TGF-β family, involves serine / threonine receptor kinase types I and II. Type I is the signal receptor. To be active it must be associated with the constitutively active type II receptor kinase (Blair *et al.*, 2002). Binding of BMP to types I and II BMP receptors phosphorylates Smad 1, 5 and 8 proteins. These proteins then form a complex with Smad 4 and are translocated to the nucleus, where they interact with other transcription factors such as Cbfa-1 (Chen *et al.*, 2004).

Preventing the downstream signalling caused by BMPs can preclude osteoblast formation (Ghosh-Choudhury *et al.*, 2002); signalling may be regulated at several different levels (Chen *et al.*, 2004).

Insulin-like growth factor (IGF) and fibroblast growth factors (FGF) are also involved in osteoblast differentiation; their role may be limited to acting only on osteoblast progenitor cells that are already committed to this differentiation pathway (Manolagas, 2000). IGF-binding proteins (IGFBPs) can bind to IGFs; some, such as IGFBP-4 are inhibitory, whilst others, such as IGFBP-5 have stimulatory effects (Lian *et al.*, 1999).

1.1.3.3. Osteocytes

Osteoblasts differentiate into osteocytes when the bone matrix surrounds them. The matrix around the osteocytes does not calcify, but forms a lacunocanalicular network between osteocytes, and the surface bone cells, allowing them to remain in contact (Burger and Klein-Nulend, 1999; Tate, 2003), as can be seen in Figure 8. This network is the largest pool of fluid in the bone (Tate, 2003).

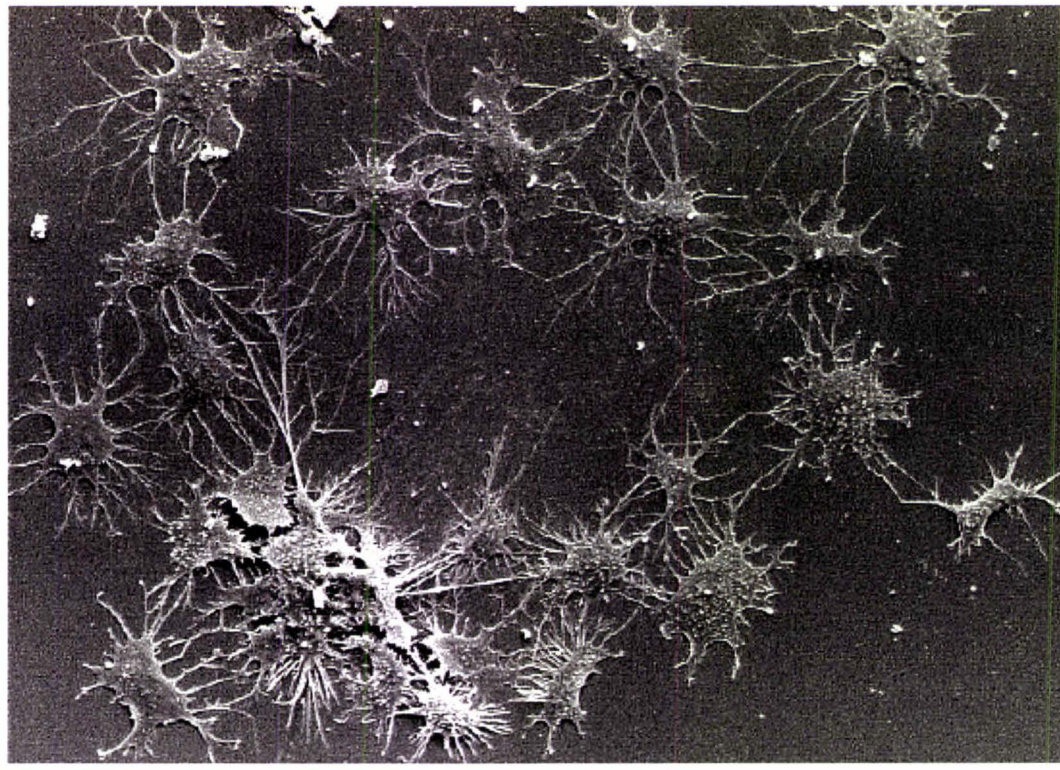

Figure 8: Scanning electron micrograph of osteocytes, isolated from embryonic chicken calvariae, following three days of culture as a monolayer. The lacunocanalicular network can be seen between the osteocytes. Taken from Burger and Klein-Nulend (1999).

1.1.4. Bone Metabolism

1.1.4.1. Ossification

Ossification, or bone modelling, refers to the formation of new bone during embryonic development. There are two types, intramembranous (flat bone) and endochondral (long bone) ossification. In the former, bones are formed directly in connective tissue, whilst the latter also involves cartilage deposition (Oxford Reference Online, 2004).

1.1.4.2. Bone Remodelling

In the adult, bone is continually being broken down and reformed in a process called bone remodelling. Remodelling has two main purposes. It allows bones to become adapted to different levels of stress, in terms of shape and strength, and the replacement of old, degenerating material with new organic matrix. Bone remodelling consists of two processes; bone resorption and bone formation. These procedures occur in the same area of bone, in temporary anatomic structures known as basic multicellular units (BMUs), which has lead to their description as being "coupled". The BMU is approximately 1 – 2 mm long, and 0.2 – 0.4 mm wide. Osteoclasts degrade bone at the front of the unit, and are followed by osteoblasts, which lay down new bone material (Manolagas, 2000). BMUs exist for approximately 6 – 9 months, a period which is split into origination (BMUs commence functioning), progression (advancement of BMUs towards another area of bone requiring replacement) and termination (cessation of BMU functioning) (Manolagas, 2000). In this time, each BMU replaces approximately 0.025 mm^3 of bone (Manolagas, 2000). 3-4 million BMUs are formed each year in the bones of a healthy human, with about 1 million functioning at any one time (Manolagas, 2000).

1.1.4.3. Bone Resorption

The process of bone resorption can be divided into five main stages. These are migration to the resorption site, attachment to the bone surface, establishment of cell polarity, degradation and removal of the bone matrix components, and either osteoclast apoptosis, or their return to the non-resorbing stage (Vaananen *et al.*, 2000; Rousselle and Heymann, 2002). A tightly sealed compartment is formed between the osteoclast and bone surface, isolating the resorption area from the extracellular fluid, which requires $\alpha v\beta 3$ integrin (Blair *et al.*, 2002). Cell polarity is established through the formation of a ruffled border, a specific membrane domain with finger-like extensions that penetrate the bone matrix (Vaananen *et al.*, 2000). Vacuolar H+-ATPases in the ruffled border secrete the H+ ions generated by carbonic anhydrase II (CAII) into the resorption area beneath the osteoclast, facilitating dissolution of the bone matrix (Manolagas, 2000; Rousselle and Heymann, 2002; Martin and Sims, 2005). Matrix metalloproteinases and cathepsin K, secreted by the osteoclast, are also involved in bone matrix degradation (Bossard *et al.*, 1996; Vaananen *et al.*, 2000). The resulting calcium and collagen fragments are then transported by vacuolar transcytosis into the osteoclast (Nesbitt and Horton, 1997; Salo and Lehenkari, 1997). Tartrate-resistant acid phosphatase (TRAP) has been found in these transcytotic vesicles, which generate reactive oxygen species (ROS) able to degrade collagen (Halleen *et al.*, 1999); hence bone matrix degradation may not occur solely extracellularly. This is summarised in Figure 9.

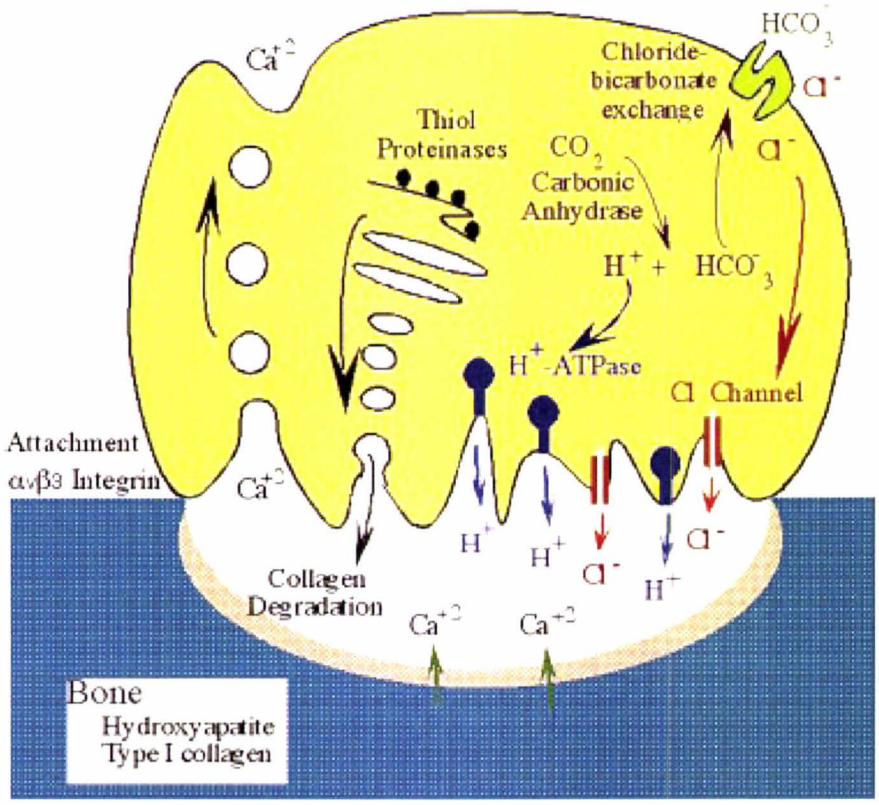

Figure 9: The osteoclast dissolving bone. Taken from Blair (2002).

1.1.4.4. Bone Formation

The activity of osteoblasts in bone formation is less well understood. Bone formation commences with the secretion of the precursor of type I collagen, procollagen, from osteoblasts. These are subsequently cleaved at both the amino- and carboxy-terminal ends, before being subjected to further extracellular processing. The end result are mature three-chained type I collagen molecules that then assemble themselves into a collagen fibril, forming pyridinoline crosslinks with other collagen molecules. Osteoblasts also secrete other proteins that are incorporated into the bone matrix, such as osteocalcin and osteonectin (Manolagas, 2000). They are also responsible for mineralisation (the deposition of hydroxyapatite). This process is not fully understood, but there are two theories as to how it is initiated. The first suggests that the osteoblast produces small vesicles,

which act as nucleation sites for mineralisation, whilst the second suggests that the nucleation site may be the collagen fibril (reviewed by Caverzasio *et al.* (1996)).

1.1.5. Regulation of Bone Metabolism

BMU activity is controlled by the complex interaction of a number of factors. Bone metabolism can be altered in response to lifestyle factors, systemic factors, and local factors, some of which are catabolic, others anabolic. This allows adaptation to environmental conditions and stresses experienced by the individual.

1.1.5.1. Lifestyle factors

Diet and nutrition are the two main lifestyle factors that regulate bone metabolism. Cigarette smoking and high alcohol consumption may be detrimental to bone (World Health Organisation, 2003a). Physical activity can improve bone strength and structure (Marcus, 1999; Brown and Josse, 2002; Kohrt *et al.*, 2004). Overactivity can, however, be detrimental (Brown and Josse, 2002).

Certain nutrients are required for bone metabolism to occur; these include the vitamins A, C, and K, calcium, magnesium and zinc. Vitamin A is involved in the differentiation of osteoblasts; it also decreases collagen synthesis and increases its degradation (Gabbitas and Canalis, 1997; Song *et al.*, 2005). Vitamin C is required for the formation of pyridinoline – deoxypyridinoline crosslinks in collagen molecules (Kipp *et al.*, 1996; Tsuchiya and Bates, 2003; Takamizawa *et al.*, 2004).

Vitamin K is required for the post-translational modification of matrix-Gla-protein and osteocalcin (Yagami *et al.*, 1999; Takeuchi *et al.*, 2000); glutamyl groups on these proteins can be converted to γ-carboxyglutamic acid residues by a vitamin K

dependent γ-carboxylase. These proteins are involved in regulation of bone mineralisation; the carboxylation mediated by vitamin K helps promote calcium binding (Lian *et al.*, 1999). Matrix gla-protein is expressed in many connective tissues, but osteocalcin is more specific to bone. Thus matrix gla-protein and osteocalcin are able to promote cartilage mineralisation and bone mineralisation respectively (Yagami *et al.*, 1999); the proteins may also be involved in the regulation of chondrocyte and osteoclast activity (Lian *et al.*, 1999; Yagami *et al.*, 1999).

Calcium is required for hydroxyapatite crystals in bone mineralisation. Mineralisation provides mechanical rigidity and load-bearing strength to the bone (Lian *et al.*, 1999). Other minerals, such as magnesium and strontium can be incorporated into the crystals should calcium intake be insufficient, but this results in smaller, less perfect crystals, and reduces bone strength (Lian *et al.*, 1999).

Magnesium deficiency has been shown to reduce bone growth, osteoblast number, increase osteoclast number, cause loss of trabecular bone and stimulate productivity or activity of TNF-α, IL-1 and substance P (Creedon *et al.*, 1999; Rude *et al.*, 2003; Rude *et al.*, 2005). Bone quality is reduced, and may even result in osteoporosis (Stendig-Lindberg *et al.*, 2004). Magnesium deficiency is also thought to impair PTH secretion or cause PTH end-organ resistance (Rude *et al.*, 1976) and, therefore hypocalcaemia (Rude *et al.*, 1998). It may also cause reduced serum $1,25(OH)_2D$ (Rude *et al.*, 2005); this may be a result of reduced levels of PTH.

Zinc may improve bone through stimulating bone formation and mineralisation, and inhibition of bone resorption. Zinc may stimulate bone formation and mineralisation through increasing production of alkaline phosphatase, collagen and osteocalcin (Brandaoneto *et al.*, 1995; Cui *et al.*, 1995; Naber *et al.*, 1996). It is

required for DNA and RNA replication, and hence the production of chondrocytes, osteoblasts and fibroblasts (Brandaoneto *et al.*, 1995). Zinc has been shown to inhibit the formation of osteoclast-like cells *in vitro* using mouse and rat bone marrow cells (Yamaguchi and Kishi, 1995; Yamaguchi and Kishi, 1996; Kishi and Yamaguchi, 1997). Zinc supplementation has been shown to improve bone strength in the femoral neck and diaphysis of growing rats (Ovesen *et al.*, 2001).

1.1.5.2. Hormonal factors

1.1.5.2.1. Parathyroid Hormone

Parathyroid hormone (PTH) is a peptide hormone produced by the parathyroid gland in response to a lowering of blood calcium levels. PTH affects both bone resorption and formation (Martin and Sims, 2005). It prevents osteoblast apoptosis and promotes osteoblast differentiation (Dobnig and Turner, 1995; Jilka *et al.*, 1999), but may also act indirectly to increase osteoclast activity (Yu *et al.*, 1996; Greenfield *et al.*, 1999; Swarthout *et al.*, 2002). PTH stimulates the kidney to promote calcium reabsorption and convert inactive 25-hydroxy vitamin D to the active form, 1,25-dihydroxy vitamin D ($1,25(OH)_2D$) (Wood, 2000). Parathyroid hormone-related protein (PTHrP) has effects on osteoclasts identical to those of PTH (Mundy, 1999).

1.1.5.2.2. Vitamin D

$1,25(OH)_2D$ promotes the absorption of calcium from the intestine by increasing the production and activity of several proteins such as calbindin, alkaline phosphatase, low-affinity calcium-dependent ATPase, calmodulin, and brush border actin (Holick, 2000). If dietary availability of calcium is too low to

maintain calcium homeostasis, the vitamin increases bone resorption, by stimulating osteoclastogenesis (Holick, 2000). This ensures that calcium is maintained at a level that allows its passive deposition into hydroxyapatite in bone mineralisation. $1,25(OH)_2D$ can increase transcription of vitamin D-specific genes in osteoblasts, such as osteocalcin, alkaline phosphatase and osteopontin (Holick, 2000).

1.1.5.2.3. Oestrogen

The post-menopausal decrease in circulating oestrogen is well recognised to be responsible for bone loss, and hence potentially cause osteoporosis. The decrease in oestrogen increases the number and activity of osteoclasts, thus increasing bone resorption (Manolagas, 2000; Riggs, 2000). Oestrogen may act to inhibit bone resorption by altering levels of various cytokines, as shown in Figure 10. The increased level of the steroid is also responsible for terminating bone growth at puberty (Gertner, 1999).

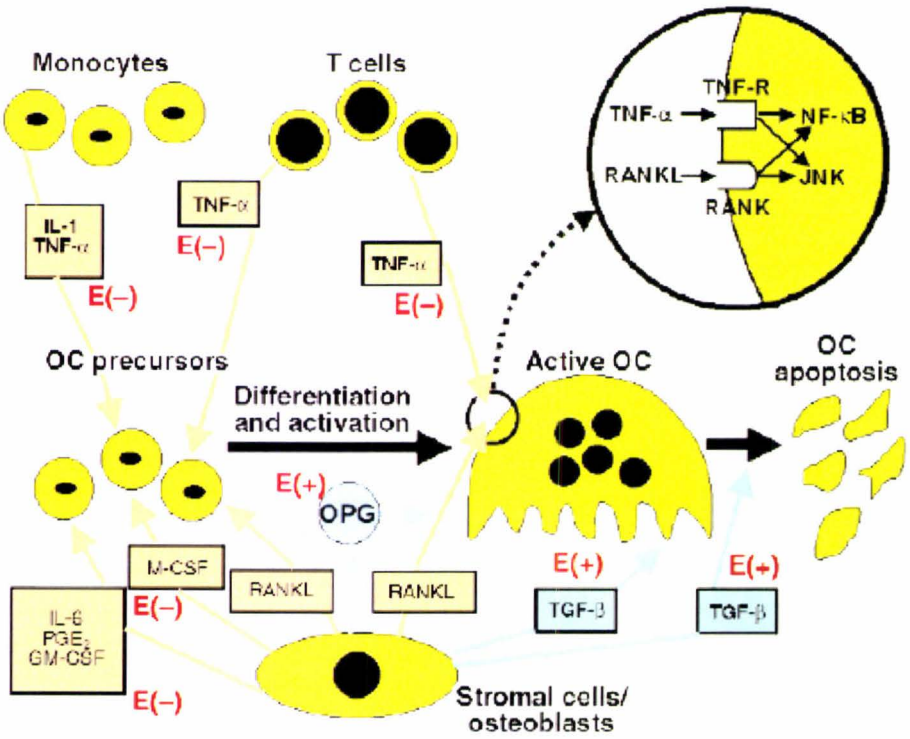

Figure 10: Actions of oestrogen (E) on cytokines in bone. Stimulatory (+) factors are shown in blue and inhibitory (-) effects are shown in orange. Taken from Riggs (2000).

1.1.5.2.4. Growth Hormone

Growth hormone (GH) is a peptide hormone secreted from the pituitary gland, which stimulates release of Insulin-like Growth Factor 1 (IGF-1) from the liver. It stimulates osteoblast proliferation and differentiation both directly, and through IGF-1 (Langdahl *et al.*, 1998; Olsen *et al.*, 2000); this may also indirectly stimulate osteoclast differentiation and activity (Olsen *et al.*, 2000). The overall effect, however, is that of promotion of bone formation.

1.1.5.2.5. Thyroid Hormones

Thyroid hormones are produced in the thyroid gland; predominately in the inactive Tetraiodothyroxine (T_4) form (although some of the active Triiodothyroxine, T_3, is also produced). T_3 promotes long bone growth during development; in adults, excess can cause accelerated bone loss (Bassett and

Williams, 2003). T_3 may stimulate osteoblastic activity both directly and indirectly, through growth factors and cytokines (Bassett and Williams, 2003).

1.1.5.2.6. Insulin

Although insulin is predominately known for its effects on glucose metabolism, it also affects bone metabolism. Insulin promotes bone formation through its actions on osteoblasts, such as alteration of collagen synthesis (Thomas *et al.*, 1998; Ahdjoudj *et al.*, 2001).

1.1.5.2.7. Calcitonin

A high blood calcium level stimulates the release of the peptide hormone calcitonin from the thyroid gland. It acts to decrease the formation and activity of osteoclasts, inhibiting bone resorption (Wood, 2000).

1.1.5.2.8. Glucocorticoids

Glucocorticoids have a multitude of effects on bone. They inhibit calcium absorption from the intestine (thus increasing PTH secretion), and promote calcium secretion from the kidneys, inhibit osteoblast function, and the formation and action of $1,25(OH)_2D$. By decreasing gonadal hormone secretion, and elevated PTH secretion, the number of remodelling sites on bone is increased. Glucocorticoids also stimulate RANKL production, and inhibit RANK production in osteoblasts, promoting osteoclastogenesis and bone resorption (Hofbauer *et al.*, 1999). For example, Cushing's syndrome is characterised by an excess of glucocorticoids, and therefore osteoporosis.

1.1.5.3. Autocrine / Local Factors

As well as the paracrine / systemic factors involved in bone metabolism described above, autocrine / local factors are also produced. Some of these are listed in Table 1.

Table 1: Catabolic and Anabolic Local Factors Involved in Bone Metabolism. Summarised from Watkins *et al.* (2001).

Catabolic Factors (Increase Bone Resorption / Decrease Bone Formation)	
Growth Factors	e.g. EGF, bFGF, FGF-2, PDGF
Cytokines	e.g. TNF, IL-1, IL-4, IL-6, IL-11, M-CSF
Prostaglandins	Particularly PGE2 at high concentrations
Leukotrienes	e.g. LTC4, LTD4, 5-HETE, LTB4 and 12-HETE
Anabolic Factors (Increase Bone Formation / Decrease Bone Resorption)	
Growth factors	e.g. IGF-I, IGF-II, TGF-α, PDGF
Prostaglandins	Particularly PGE2 at low concentrations

TNF-α (tumour necrosis factor-α; cachectin) is a cytokine released by activated macrophages, which stimulates bone resorption and bone cell replication (Lian *et al.*, 1999; Mundy, 1999; Idriss and Naismith, 2000). It also has other roles in the body, including anti-viral, cytostatic and cytolytic actions; it can cause cachexia, suppress erythropoiesis, and may cause signalling events in cells, ultimately resulting in cell apoptosis or necrosis (Lian *et al.*, 1999; Mundy, 1999; Idriss and Naismith, 2000). It appears to be important for resistance to infection and cancers (Idriss and Naismith, 2000). Cenci *et al.* (2000) demonstrated that oestrogen may reduce TNF-α production by T-cells, hence inhibiting bone resorption; oestrogen-deficiency after the menopause results in non-supression of TNF-α levels and ensuing bone loss (Nanes, 2003).

IL-6 is a pro-inflammatory cytokine, which stimulates bone resorption through increasing production of osteoclast precursors (Wang *et al.*, 2003; Xing and Boyce, 2005). IL-6 is also involved in other processes in the body, including immune responses, haematopoiesis, and acute-phase reactions (Simpson *et al.*, 1997). IL-1,

PTH and $1,25(OH)_2D$ promote the expression and release of IL-6 from bone cells (Lian *et al.*, 1999; Mundy, 1999; Riggs, 2000).

IL-10 is an anti-inflammatory cytokine produced by T-cells and macrophages; it induces a wide range of biological activities in the body, such as preventing cytokine production, increasing survival of T- and B-cells and the activity of NK cells (Pestka *et al.*, 2004). Mice deficient in IL-10 show chronic inflammation of the intestine, as they are unable to control immune responses to intestinal flora (Kuhn *et al.*, 1993). Production of IL-10 may be induced by TNF-α in macrophages, lipopolysaccharide (LPS), and IL-6 and IL-12 in T-cells (Daftarian *et al.*, 1996). The cytokine also has anabolic effects on bone (Daftarian *et al.*, 1996; Watkins *et al.*, 2001; Stenvinkel *et al.*, 2005). These effects are due to its inhibition of osteoclast formation, achieved through a direct action on osteoclast precursors (Hong *et al.*, 2000).

SUMMARY

Bones provide support, protection, permit movement, produce blood cells, and act as a storage reservoir for certain nutrients. There are two types of bone, cortical and trabecular; the body is composed of different percentages of these two types of bone allowing greater adaptation in terms of weight, strength, and resource allocation. Alternatively, bones can be classified chemically; bones are composed of an organic matrix, inorganic salts and bone cells (osteoblasts, osteoclasts and osteocytes). Differentiation of these bone cells is controlled by several different factors. In order to develop and function correctly, osteoclasts require PU.1, M-CSF, RANK, and RANKL, amongst other factors. OPG can bind RANKL, a decoy receptor, preventing

osteoclastogenesis. Osteoblasts, however, require *Cbfa-1*, BMPs, IGFs and FGFs in order to function correctly.

There are two processes of bone growth that occur; bone modelling (ossification) and bone remodelling. Ossification allows bones to grow in size, and occurs from the start of life up until the end of adolescence. Bone remodelling, however, occurs throughout life, allowing bones to become adapted to different levels of stress, and the replacement of old material. Remodelling consists of two processes, bone resorption and bone formation; these are regulated by the complex interaction of several hormones and cytokines on activity of BMUs. PTH, Vitamin D, oestrogen, GH, insulin, and calcitonin promote bone formation, whilst thyroid hormones and glucocorticoids promote bone resorption. Several local factors are also produced, including the cytokines IL-6 and TNF-α, which increase bone resorption, and IL-10, which increases bone formation. An imbalance of these factors may have serious consequences, resulting in bone overgrowth, causing problems for nerve and blood supply, or bone weakening, increasing fracture risk

Section 2. Mineral Absorption

1.2.1. Absorption

Through the examination of the kinetics of transport of a mineral, it is possible to determine to some extent, how it may be absorbed. A graph showing the kinetics of a hypothetical mineral is shown in Figure 11.

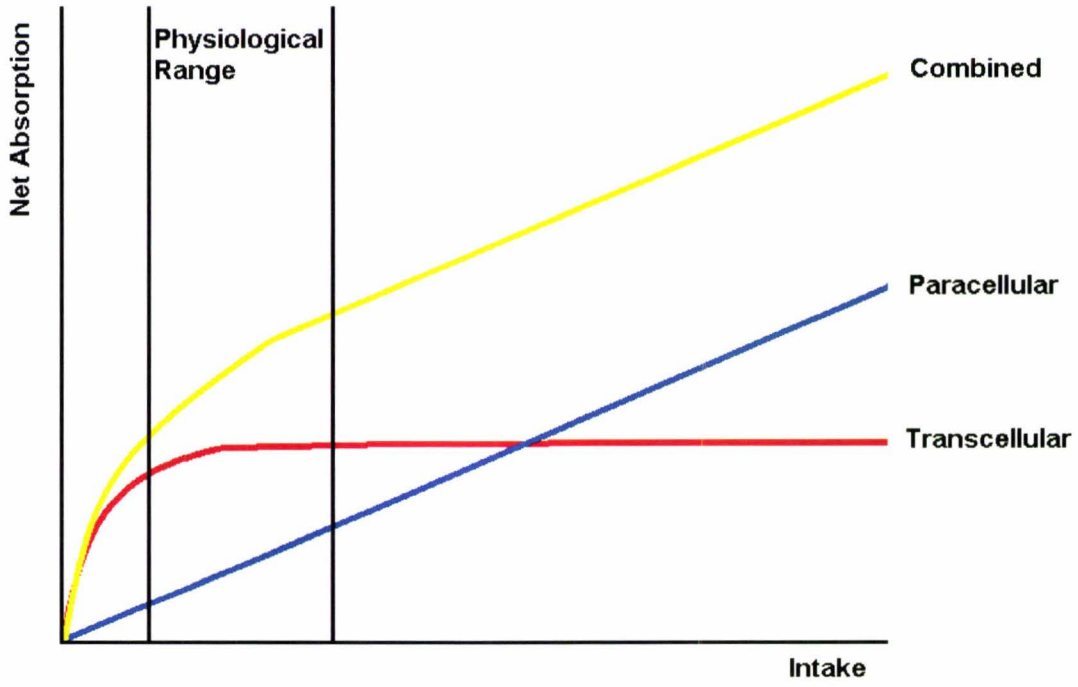

Figure 11: Graph showing net absorption against intake of a hypothetical mineral. Kinetics of absorption are those that could would be expected if absorption took place using solely a transcellular active pathway (shown in red), a paracellular passive pathway (shown in blue) or a combination of the two (shown in yellow). The normal physiological range of magnesium intake (represented by the grey bar) is shown to allow a reference point for the importance of each transport mechanism. Adapted from Konrad *et al.* (2004) and Fleet (2000).

Absorption of a mineral that takes place using only a transcellular pathway could be expected to become saturated at a low level of intake (shown by the red line in Figure 11); an additional increase would not result in any further improvement of net absorption (Fleet, 2000). A paracellular pathway (shown by the blue line), however, might be expected to show a rate of absorption that correlates to intake (Fleet, 2000). A mineral may also be absorbed by a combination of transcellular and paracellular mechanisms (yellow line in Figure 11); the former would be more responsible for

absorption at a lower level of intake, whilst the latter may be more important at a higher level of intake (Fleet, 2000). Net absorption for a mineral absorbed by such a combination is the sum of the absorption rate of its components, the transcellular and paracellular pathways (Fleet, 2000).

1.2.1.1. Calcium

Calcium is taken up across intestinal mucosal cells by active and passive absorption (Bronner *et al.*, 1986; Peacock, 1991; Bronner and Pansu, 1999; Slepchenko and Bronner, 2001). Active transport requires the presence of 1, 25-dihydroxyvitamin D (1, 25$(OH)_2$D), the active form of vitamin D, and its receptors (Freund and Bronner, 1975a). It is a saturable transcellular process, which occurs in the upper duodenum and colon (Bronner *et al.*, 1986). Active transport occurs in three steps; entry of calcium ions into the epithelial cell from the lumen, their intracellular diffusion from the apical to basolateral membrane and extrusion from the cell, across the basolateral membrane. Calcium enters the epithelial cell down a steep concentration gradient, by means of the calcium channel ECaC1 (CaT2 or TRPV5), and calcium transport protein, CaT1 (ECaC2 or TRPV6) (Hoenderop *et al.*, 1999; Peng *et al.*, 1999; Slepchenko and Bronner, 2001); the former is 1, 25$(OH)_2$D dependent, whilst the latter appears to be unresponsive to the vitamin (Freund and Bronner, 1975a; Bronner, 2003a). A dual-transport mechanism may exist to maximise efficiency over a range of calcium concentrations. An animal with low calcium intake would be expected to show low expression levels of CaT1. If calcium intake were to increase suddenly, the channel would prove advantageous. Whilst the transport protein would become saturated at a low level, the channel allows calcium absorption at an amount relative to luminal concentrations. Low calcium causes an

increase in circulating 1, 25$(OH)_2$D. Since CaT2 is 1, 25$(OH)_2$D-dependent, high calcium levels will result in a reduction of CaT2 activity; a calcium channel is less essential, and may even prove detrimental (summarised from Bronner (2003a)).

The calcium binding protein calbindin (CaBP) facilitates calcium transport across the cell; its biosynthesis is dependent on 1, 25$(OH)_2$D (Freund and Bronner, 1975a; Freund and Bronner, 1975b; Buckley and Bronner, 1980; Bronner *et al.*, 1986). This is thought to be the rate-limiting step in the calcium absorption pathway (Bronner *et al.*, 1986). Calbindin may increase calcium diffusion across the cell by a factor of about 60 (Bronner *et al.*, 1986). By binding to calcium, calbindin also ensures that intracellular calcium ion concentrations remain low, thus helping maintain an electrochemical gradient (Bronner, 2003a).

CaATPase is predominately responsible for calcium extrusion from the cell; an Na^+/Ca^{2+} exchanger also appears to be involved, albeit to a smaller extent (Bronner *et al.*, 1986; Wasserman and Fullmer, 1995). Calcium extrusion by CaATPase is energy-requiring and 1, 25$(OH)_2$D dependent (Bronner, 1998; Bronner and Pansu, 1999). Enzyme activity may be increased by two to three times by the vitamin (Bronner *et al.*, 1986). Calcium binds to the transmembrane sections of the CaATPase on the basolateral membrane; phosphorylation of the CaATPase causes a conformational change and creation of a channel, through which the calcium ion is extruded (Bronner, 1998; Bronner and Pansu, 1999).

Passive diffusion involves transport in a paracellular fashion, and movement of calcium ions down a concentration gradient (Bronner *et al.*, 1986; Bronner and Pansu, 1999); absorption rate is, therefore, dependent on the relative level of calcium in the luminal and serosal tissue. High calcium, and hence low 1, 25$(OH)_2$D levels, down-regulate the active-transport mechanism, which explains the increased

importance of passive diffusion at high levels of calcium (Buckley and Bronner, 1980; Bronner and Pansu, 1999). This transport system is non-saturable, occurs independently of 1, 25$(OH)_2$D levels and takes place throughout the length of the small intestine (Pansu *et al.*, 1983a; Bronner *et al.*, 1986; Bronner and Pansu, 1999; Bronner, 2003b). Absorption of calcium also occurs in the large intestine, but is only thought to account for 10% of total absorption (Bronner and Pansu, 1999).

The routes of calcium absorption in active and passive transport are summarised in Figure 12.

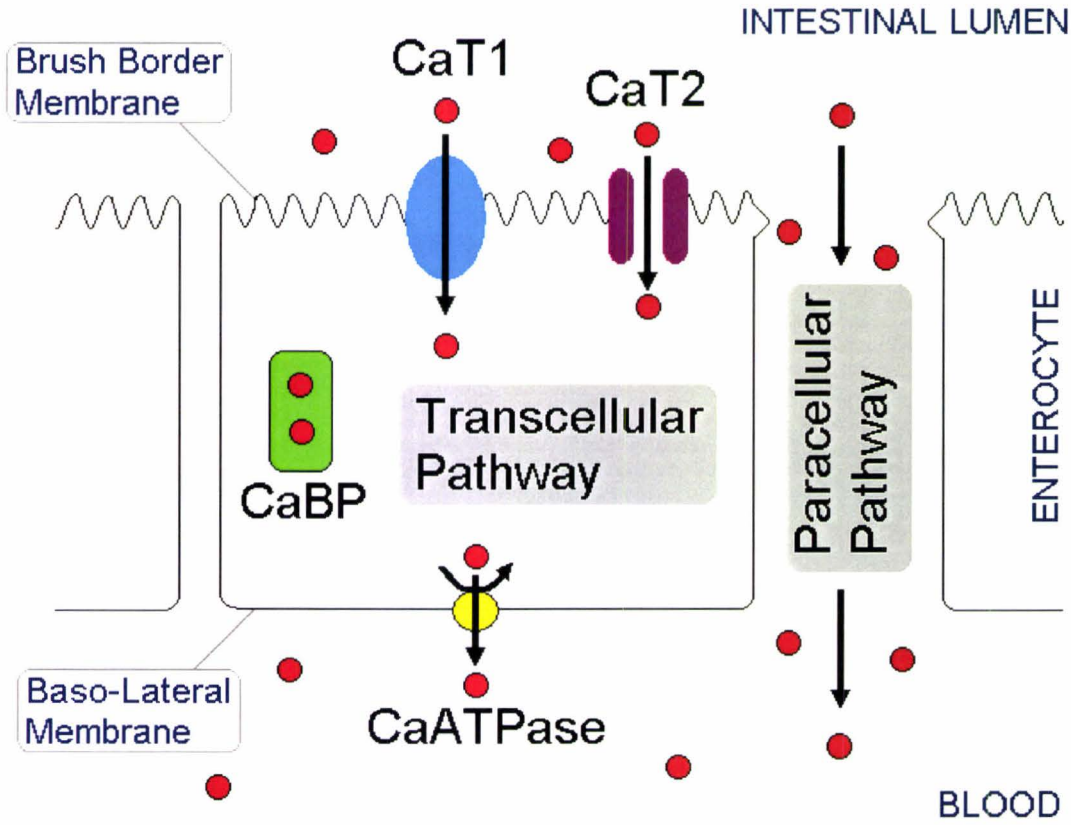

Figure 12: Routes of calcium absorption. Calcium is represented by red circles. The channel CaT2, and transport protein CaT1 absorb calcium from the intestinal lumen. Intracellular transport occurs using CaBP. Calcium is extruded from the cell by CaATPase. This is known as the transcellular pathway. Alternatively, calcium can be absorbed by the paracellular pathway, passing directly into the blood.

1.2.1.2. Magnesium

The mechanisms of magnesium absorption are not completely understood, although it is thought that a non-saturable paracellular passive system and a saturable transcellular active system are involved (Rude, 2000; Konrad *et al.*, 2004). Magnesium is thought to be predominately absorbed from the small intestine, with a small amount absorbed from the large intestine (Konrad *et al.*, 2004).

Hypomagnesemia with secondary hypocalcemia (HSH) is an autosomal recessive condition, manifested in early infancy, characterised by muscular convulsions, and very low serum magnesium and calcium levels (Konrad *et al.*, 2004). Research as to its cause is ongoing (Konrad *et al.*, 2004). Levels of TRPM6 are reduced in HSH patients (Schlingmann *et al.*, 2002). TRPM6 is a member of the transient receptor protein (TRP) family of cation channels, and is expressed along the entire length of the small intestine (Schlingmann *et al.*, 2002; Konrad *et al.*, 2004; Voets *et al.*, 2004). However, administration of high doses of magnesium can restore subnormal levels (Walder *et al.*, 2002), suggesting that there may be at least two transport mechanisms present. A high intake would cause high intraluminal concentrations of magnesium, and hence increased absorption by a passive, paracellular pathway. Research into magnesium transport is ongoing; only this year oligonucleotide microarray analysis identified a putative magnesium channel expressed in the intestine and other tissues, called Magnesium Transporter protein (MagT1) (Goytain and Quamme, 2005). Further research is required to determine the importance of this transporter.

An Mg^{2+} / Na^{+} antiporter has been proposed as the method by which magnesium is extruded from the basolateral membrane; conclusive evidence still remains to be found (Schweigel and Martens, 2000). Magnesium absorption routes are summarised in Figure 13.

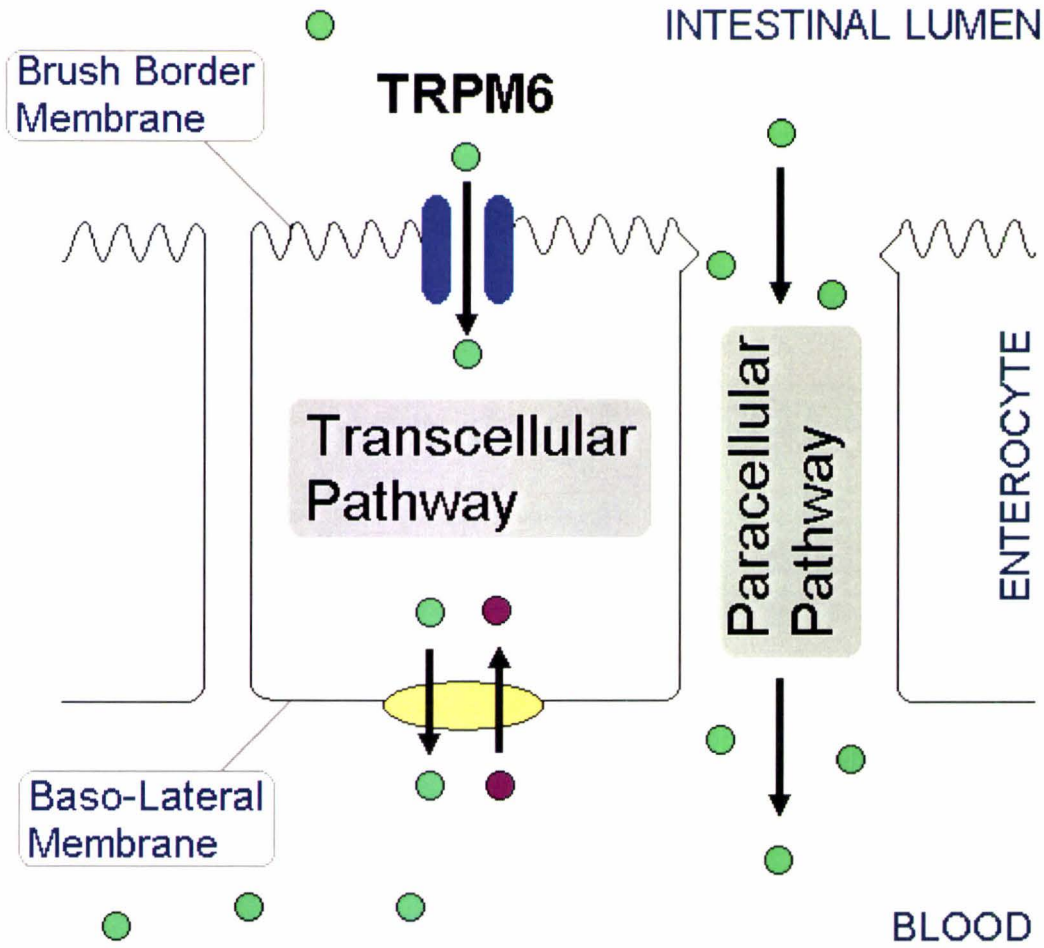

Figure 13: Routes of magnesium absorption. Magnesium is represented by green circles; sodium by purple circles. Intracellular transport is thought to occur through the magnesium channel TRPM6. The method of extrusion remains to be determined, although it has been suggested that a magnesium / sodium antiporter may be involved. This constitutes the transcellular pathway. The paracellular pathway absorbs magnesium directly into the blood. Adapted from Konrad *et al.* (2004).

1.2.1.3. Zinc

Zinc absorption is thought to occur by both passive paracellular and active transcellular routes. At present, two mammalian zinc transporters have been identified. They are the Zinc-Transporter (ZN-T; solute-linked carrier 30, SLC30) proteins, and the ZIP (solute-linked carrier 39, SLC39) proteins. They appear to have opposing roles in the cell; ZN-T exudes zinc from cells, whilst ZIP transporters promote the influx of zinc into cells (Liuzzi and Cousins, 2004).

Nine zinc transporters have been cloned (ZN-T1 – 9), whose tissue expression is summarised in Table 2. It should be noted, however, that this list encompasses only the tissues in which each has been found to date. Further research is needed for a more conclusive list.

Table 2: Tissue expression of the nine zinc transporters found in research carried out so far. Where tissues are followed by (?) more research is needed. Summarised from Liuzzi and Cousins (2004).

Zinc Transporter	Tissue expression
ZN-T1	Wide; more highly expressed in tissues involved in zinc acquisition, recycling or transfer, such as the small intestine, kidney cells and placenta
ZN-T2	Intestine, kidney, testes, placenta, pancreas, mammary gland
ZN-T3	Brain, testes
ZN-T4	Mammary gland, brain, small intestine, kidney
ZN-T5 (hZTL1)	Pancreas, ovaries, testes, prostrate, small intestine (?), brain (?)
ZN-T6	Liver, brain, small intestine
ZN-T7	Small intestine, liver, spleen, kidney, lung
ZN-T8	Pancreas and liver
ZN-T9	Lung

Expression of ZN-T1, 2 and 4 have been shown to increased in response to the presence of zinc (Davis *et al.*, 1998; McMahon and Cousins, 1998; Liuzzi *et al.*, 2001), albeit to different extents. The response is not thought to be unilateral; ZN-T2 may be up-regulated to a greater extent than ZN-T1, which in turn may be up-regulated more than ZN-T4 (Liuzzi *et al.*, 2001).

Acrodermatitis enteropathica is a disease characterised by an impaired absorption of zinc. This allowed identification of the ZIP protein, hZIP4 (SLC39A4) (Wang *et al.*, 2002). Eight ZIP proteins have been identified to date (ZIP1 to 8); tissue expression for these proteins still remains to be determined (Ford, 2004; Liuzzi and Cousins, 2004).

Whilst the influx and efflux mechanisms have been identified to some extent, the method by which zinc is transported across the cell remains uncertain. Also, the possible role of metallothionein remains undetermined. Davis *et al.* (1998) produced

mice that overexpressed metallothionein, expressed no metallothionein, or expressed normal levels. They found serum zinc to be inversely related to metallothionein levels. The high intestinal zinc content in the knockout mice suggests that metallothionein was not acting to sequester zinc in the mucosa. However, the exact role and action of metallothionein remains to be determined. Current understanding of the method of zinc absorption is summarised in Figure 14.

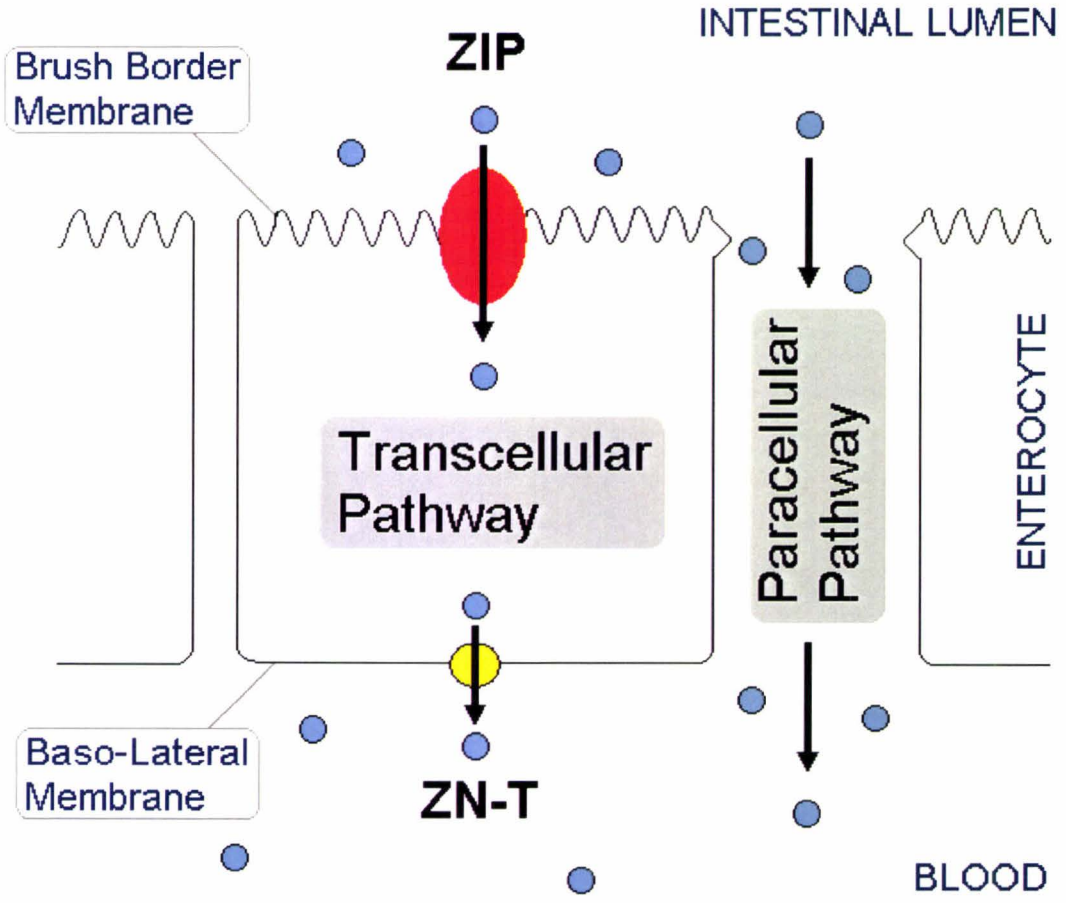

Figure 14: Current understanding of the method of zinc absorption from the small intestine. Zinc is represented by blue circles. Zinc is thought to enter enterocytes by the ZIP transporters, and be extruded by the ZN-T transporters. Method of transport between the apical and basolateral membranes remains to be determined. This constitutes the transcellular pathway. The paracellular pathway absorbs zinc directly into the blood.

1.2.2. Mineral Balance

Minerals in the body can be thought of as a series of entry and exit routes into / out of a "pool"; balance is maintained by altering rate of flow through these systems. The

pool is comprised of mineral present in solution in blood plasma, extracellular fluid and bone (Bronner and Pansu, 1999). Routes of entry into the pool consist of absorption from the small intestine and resorption from bone. Calcium, magnesium and zinc are removed from the pool by urinary excretion, endogenous faecal loss, deposition in bone, and losses in sweat, semen, menstrual fluid, and breast milk. This is summarised in Figure 15.

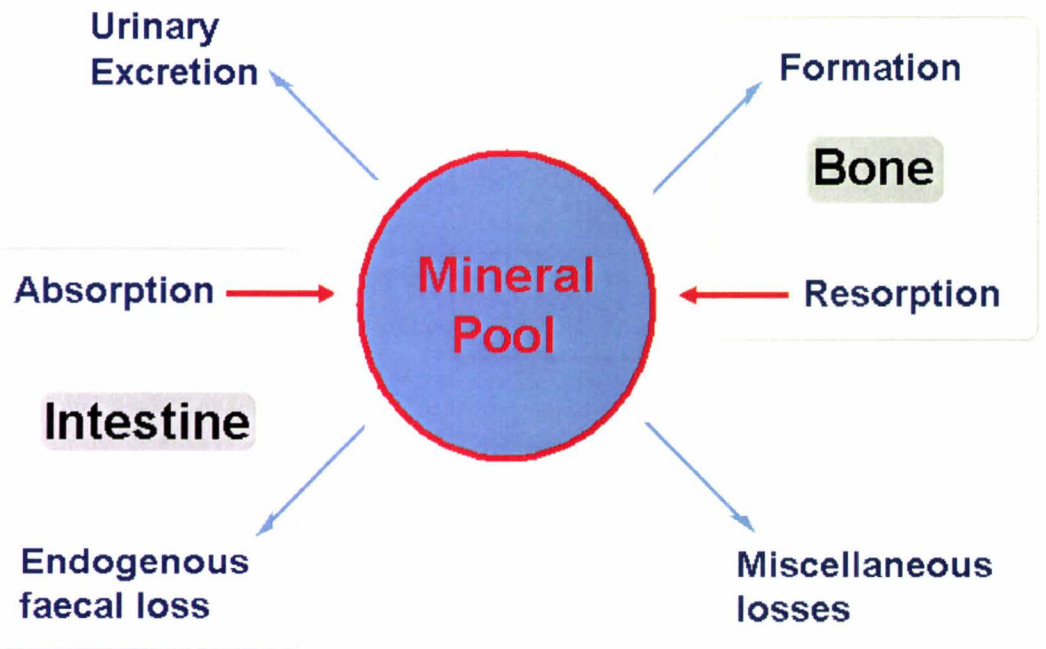

Figure 15: Routes of entry into and exit from mineral pool in the body. The pool consists of the mineral in solution of blood plasma, extracellular fluid and bone. Entry is from absorption from food and resorption from bone. Calcium, magnesium and zinc are excreted in urine, used in bone formation or excreted in faeces. Miscellaneous losses comprise those in sweat, semen, menstrual fluid and breast milk. Adapted from Bronner and Pansu (1999).

In order to maintain a balance of mineral levels, rate of entry into and exit from the pool can be altered. This may be achieved by the complex interaction of a number of factors. As discussed previously, rate of bone resorption and formation can be altered by various hormones and cytokines, which act locally and systemically (see 1.1.5.3). Miscellaneous losses are thought to be minimal (Bronner and Pansu, 1999). Endogenous faecal losses are due to sloughed intestinal cells and digestive secretions, and occur at a rate proportional to mineral intake (Abrams *et al.*, 1994).

Urinary excretion of minerals may exhibit large diurnal fluctuations (Wood, 2000). Several factors contribute to this. For example, the amount of each mineral absorbed may lead to an increase in its urinary excretion (King *et al.*, 2000; Wood, 2000). PTH increases calcium reabsorbed by the kidney. The presence of calciuretic factors in food, such as simple sugars and protein may increase urinary calcium losses (Wood, 2000). Pathological illnesses that affect the small intestine have the potential to affect mineral absorption, as can age and a number of dietary factors, discussed below (Zornitze and Bronner, 1971; King *et al.*, 2000).

1.2.3. Bioavailability

Bioavailability can be defined as the portion of a nutrient in a food that is absorbed and metabolically available (Wood, 2000). In order to be absorbed, calcium, magnesium and zinc must be ionised and in solution. Certain forms of a mineral may be better absorbed than others. For example, low-molecular weigh organic compounds may bind zinc, and promote its absorption, e.g. the amino acids histidine and methionine (Krebs, 2000; Lonnerdal, 2000). Several factors can affect mineral bioavailability, both beneficially and detrimentally.

Phytic acid may prevent mineral absorption by forming insoluble complexes with the minerals (Benway and Weaver, 1993; Mason *et al.*, 1993; Krebs, 2000; Lonnerdal, 2000; Lopez *et al.*, 2002). Oxalic acid can decrease calcium bioavailability; again due to the insoluble complex it forms with minerals (Weaver *et al.*, 1997). Green leafy vegetables are good sources of calcium and magnesium, but their bioavailability may be reduced due to the presence of these acids (Kamchan *et al.*, 2004). A high gastric pH can cause minerals to precipitate, thus reducing their available for absorption (Wood and Serfaty-Lacrosniere, 1992; Bronner and Pansu, 1999).

Minerals and other nutrients may also affect the bioavailability of other minerals. For example, iron and calcium, when consumed at levels consistent with consumption of dietary supplements, may reduce zinc absorbed; further research is necessary to determine the extent of this interaction (Krebs, 2000; Lonnerdal, 2000). High fibre foods have a short intestinal transit time, which may, therefore, decrease bioavailability (Wolf *et al.*, 2000); many high fibre foods also contain phytic acid (Rude, 2000).

Lactose may increase calcium uptake by a direct action on intestinal cells (Armbrecht and Wasserman, 1976; Brommage *et al.*, 1993). Lactulose is a disaccharide analogue of lactose, which is digested in the large intestine. It has been shown to improve calcium absorption (Brommage *et al.*, 1993); this effect is thought to occur in the small intestine, as calcium absorption was improved in both control and caecectomised rats.

The type and amount of protein present may affect zinc absorption. It has been suggested that an ability of a protein to keep zinc dissolved may explain its promotion of absorption of the mineral (Lonnerdal, 2000). Protein may also affect magnesium absorption; soybean protein was found to inhibit magnesium absorption to a greater extent than casein (Brink *et al.*, 1991; Perez-Llamas *et al.*, 2001). However, soybean protein also contains phytate, which itself reduces magnesium bioavailability. Addition of this compound to casein, at a level comparable to that found in soybean protein, reduced magnesium absorption similarly (Brink *et al.*, 1991).

Mineral bioavailability can also be increased by non-digestible oligosaccharides (Morohashi *et al.*, 1998; van den Heuvel *et al.*, 1999; Wolf *et al.*, 2000; Mineo *et al.*, 2001; Scholz-Ahrens *et al.*, 2002; Zafar *et al.*, 2004). Their role and action will be discussed further in Section 3.

SUMMARY

Calcium, magnesium and zinc are absorbed from the small intestine by both active and passive transport pathways. The former is responsible for the majority of absorption that occurs at low and moderate intakes, whilst the latter is more significant at higher concentrations. Passive diffusion transports mineral ions in a paracellular fashion, directly into the blood stream.

Active transport is of calcium 1, 25$(OH)_2$D-dependent. It, and that of magnesium and zinc, can be divided into three steps – entry, diffusion and extrusion. The calcium channel, CaT2, and the calcium transport protein CaT1 permit calcium entry into the intestinal epithelial cell. CaBP facilitates transport of calcium across the cell. CaATPase (and to a smaller extent an Na^+/Ca^{2+} exchanger) extrude calcium out of the cell into the blood, across the basolateral membrane.

Magnesium is thought to be absorbed into enterocytes via a channel called TRPM6. Recent research has suggested that another magnesium channel may exist, MagT1; further study is required to determine its importance. It has not been determined how magnesium passes from the apical to basolateral membrance, nor the method of its extrusion. It has been suggested that an Mg^{2+} / Na^+ antiporter may be involved, but this has not been confirmed.

Zinc is thought to enter enterocytes through ZIP transporters, and be extruded by ZN-T transporters. Numerous proteins belonging to each class have been cloned. However, which tissues they are expressed in is not fully known, neither is the mechanism by which the mineral passes from the apical to basolateral membranes.

Mineral balance in the body is controlled by a series of factors that alter the rate of entry and exit of ions into the body's pool. The mineral pool consists of ions in solution in the blood plasma, extracellular fluid and bone. Minerals enter the pool by being absorbed from the small intestine, which depends on bioavailability, and rate of

resorption. Exit from the pool occurs due to miscellaneous losses, which are thought to be minimal, endogenous faecal loss, which is thought to occur at a rate proportional to intake, bone formation and urinary excretion. Urinary excretion of minerals can be affected by several factors, such as intake, the presence of inhibitory factors, and the action of PTH on the kidney (in the case of calcium).

The bioavailability of these three minerals is affected by a number of factors, Compounds such as phytic and oxalic acid, can bind them, whilst a high gastric pH can cause precipitation, preventing absorption. Other factors, such as non-digestible oligosaccharides may improve mineral absorption. Improving bioavailability therefore has the potential to increase the amount of minerals that enter the pool, for use in bone formation.

Section 3. Pre- and Pro-biotics

1.3.1. Pre- and Pro-biotics

Probiotics are bacteria that reside in the large intestine and have beneficial health effects on their host. These effects are summarised in Table 3.

Table 3: Beneficial effects of predominant bacteria in the large intestine of humans. Bacteria in light blue may also have pathogenic effects. Summarised from Gibson and Roberfroid (1994).

	Enterococci	*Escerichia coli*	*Lactobacilli*	*Streptococci*	*Eubacteria*	*Bifidobacteria*	*Bacteriodes*
Inhibition of growth of exogenous and / or harmful bacteria	*		*	*	*	*	*
Stimulation of immune function		*	*			*	*
Aid digestion and / or absorption of nutrients			*			*	*
Synthesis of vitamins						*	*

Carbohydrates with β-linkages cannot be digested by microbes in the small intestine; they are known as non-digestible oligosaccharides (NDO). NDOs pass into the large intestine where they are fermented by bacteria into the short chain fatty acids (SCFAs) acetic, propionic and butyric acids.

Prebiotics are NDOs that selectively stimulate the growth and / or activity of probiotics (Gibson and Roberfroid, 1994; Beynen *et al.*, 2002; Minamida *et al.*, 2004); prebiotics provide probiotics with a source of food that in turn provides the host with energy, metabolic substrates and nutrients (Roberfroid, 2000a). Selective-stimulation is important, because the activity of pathogenic micro organisms is not increased; thus the gut-floral balance is altered to the advantage of the host. Prebiotics include inulin, oligofructose (OF), galacto-oligosaccharides (GOS), and resistant starch (RS).

Inulin and OF are fructans; polymers of fructose found in plants and micro organisms (Tungland, 2003). They are the second-most common form of carbohydrate storage in plants (starch being the most abundant) (Tungland, 2003). Inulin is a generic name for

all linear fructans with β(2→1) linkages (Roberfroid, 2005); these compounds have a degree of polymerisation (DP; number of monosaccharide units combined) of 2 to more than 60, whilst OF are short-chain fructans, which have a degree of polymerisation of less than 8 (Franck, 1998; Roberfroid and Slavin, 2000b). There is some disagreement regarding the definitions of these oligosaccharides, and their nomenclature is often used interchangeably. FOS (fructo-oligosaccharide) is generally used to describe all oligosaccharides composed predominately of fructose (Roberfroid and Slavin, 2000b); it can thus also describe a mixture of OF and inulin-type fructans.

GOS contain galactose units, linked with β1-4 and β1-6 bonds; they have been shown to stimulate the growth of *Bifidobacterium* species, and to a lesser extent *Lactobaccillus* spp., *Escerichia coli* and *Streptococcus* spp. (Sako *et al.*, 1999).

RS is a term that describes starch and its derivatives that are not digested in the small intestine of healthy individuals (Brown, 2004; Champ, 2004). RS is found in all starch-containing foods. The level present can be difficult to quantify, as it will vary depending on a number of factors such as the type of starch in the food, processing and storage it was subjected to, composition of the meal, and even the individual consuming it (Brown, 2004; Champ, 2004).

1.3.2. Roles in Mineral Absorption

1.3.2.1. Calcium

Several studies have suggested that prebiotics may promote calcium absorption from the large intestine. These are summarised in Table 4.

Table 4: Summary of previous research investigating the effect of prebiotics on calcium absorption. ns indicates no significance; * P<0.05; ** P<0.005. Abbreviations used: FOS, fructooligosaccharides; GOS, Galactooligosaccharides; I, inulin; OF, oligofructose; OVX, ovariectomised; RS, resistant starch.

Prebiotic	Level (percentage of diet)	Model	Effect	Authors
OF	1%	3-11y dogs (12.8 – 16.4kg) (4 males, one female)	↑*	Benyen *et al.* (2002)
GOS	5%	6wk male rats	↑*	Chonan *et al.* (2001)
I	40g/day	19-25y men	↑*	Coudray *et al.* (1997)
OF I OF + I	10% 10% 10%	Male rats (170g)	↑* ↑* ↑*	Coudray *et al.* (2003)
FOS	10%	Male rats (100g +/- 5g)	↑*	Delzenne *et al.* (1995)
OF I + OF	8g/day	11 – 13.9y girls	ns ↑*	Griffin *et al.* (2002)
I + OF	8g/day	10 – 15y girls	↑*	Griffin *et al.* (2003)
FOS	5%	Male rats (209g +/- 1.69g)	↑*	Gudiel-Urbano and Goñi (2002)
RS	20%	7wk male rats	↑**	Lopez *et al.* (1998)
I	1%	Male rats (~160g)	↑*	Lopez *et al.* (2000)
RS	20%	Male rats (~150g)	↑*	Lopez *et al.* (2001)
FOS	5%	45d male rats	↑*	Morohashi *et al.* (1998)
FOS	5%	6wk male rats	↑*	Ohta *et al.* (1994)
FOS	5% 10%	5wk male rats	ns ↑*	Ohta *et al.* (1998a)
I	5%	30kg male pigs	ns	Rideout (2004)
Short chain FOS	10g/day	50-70y postmenopausal women	ns	Tahiri *et al.* (2003)
I FOS GOS	15g/day 15g/day 15g/day	20-30y men	ns ns ns	van den Heuvel *et al.* (1998)
OF	15g/day	14-16y boys	↑*	van den Heuvel *et al.* (1999)
RS	35%	8wk male rats (~200g)	↑*	Younes *et al.* (1996)
I RS I + RS	10% 15% 12.5%	8wk male rats	↑* ↑* ↑*	Younes *et al.* (2001)
I + FOS	5.5%	6Mo OVX rats	↑**	Zafar *et al.* (2004)

As can be seen in Table 4, numerous studies have been carried out, which examine the effect of several different prebiotics, at various levels, and on a range of different models. Most of these studies suggest that prebiotics increase calcium absorption; several mechanisms have been proposed in explanation, which are summarised in Figure 16.

The production of SCFA results in a decrease in pH in the large intestinal lumen, causing more calcium to become ionised. Calcium must be in this form in order to be absorbed; a greater amount of ionised calcium can, therefore, result in an increase in paracellular transport (Sakuma, 2002). Alternatively, the production of SCFA may alter the electrolyte exchanges that occur across the epithelium; cellular H^+ ions are exchanged for luminal Ca^{2+} ions.

SCFA may form complexes with calcium; if these complexes are more soluble than the ionised calcium, they will be more readily absorbed. Fermentation of carbohydrates in the large intestine has been shown to cause atrophy (Ichikawa and Sakata, 1998); this will, therefore, provide a greater surface area over which calcium can be absorbed.

NDOs may be altering the mechanisms involved in calcium absorption. Ohta *et al.* (1998b) showed that FOS caused an increase of CaBP in the large intestine, and a decrease in the small intestine. This would mean that a greater amount of calcium could be transported across the epithelial cells of the large intestine. Mariadason *et al.* (1999) suggested that both digestible and non-digestible fibre in fact reduced paracellular transport in the large intestine. They thought that this may be due to the cell-differentiation that was induced.

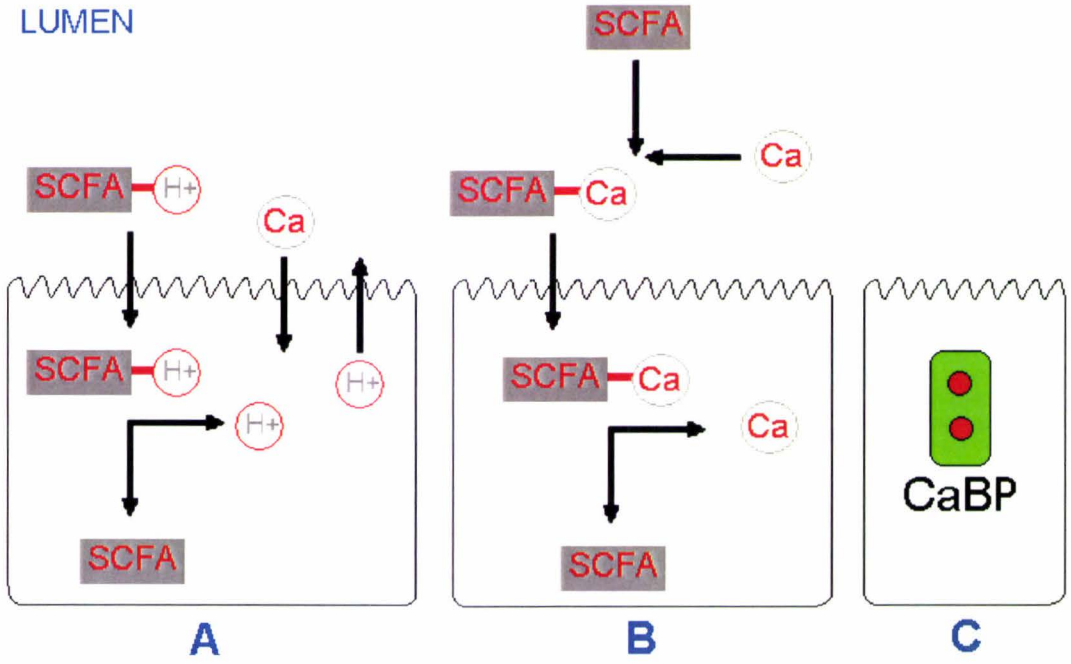

Figure 16: Mechanisms by which NDOs may increase calcium absorption. Cell A shows how short chain fatty acids (SCFAs) can alter electrolyte exchanges. Cell B shows that SCFA may also form complexes with calcium that are more soluble than calcium ions and therefore more readily absorbed. Cell C represents the possible increase in CaBP resulting from NDOs; NDOs may also cause epithelial atrophy, increasing surface area over which calcium can be absorbed. Although not depicted, the increased production of SCFA can cause calcium in the lumen to become ionised, thus making it available for absorption.

It is, however, important to consider the effects that long-term supplementation of pre- and / or pro-biotics may have on the absorption of calcium and other minerals. An increased availability of calcium should result in the down-regulation of the active transport mechanism. The effect that this would have on calcium absorption, particularly if its availability were to suddenly decrease, could be of importance.

1.3.2.2. Magnesium

Several different studies have also been carried out to determine whether prebiotics affect magnesium absorption. These are summarised in Table 5.

Table 5: Summary of previous research investigating the effect of prebiotics on magnesium absorption. ns indicates no significance; * P<0.05; ** P<0.005. Abbreviations used: FOS, fructooligosaccharides; I, inulin; OF, oligofructose; OVX, ovariectomised; RS, resistant starch.

Prebiotic	Level (percentage of diet)	Model	Effect	Authors
FOS	5%	4wk male rats	↑*	Baba *et al.* (1996)
OF	1%	3-11y dogs (12.8 – 16.4kg) (4 males, one female)	↑*	Benyen *et al.* (2002)
GOS	5%	6wk male rats	↑*	Chonan *et al.* (2001)
I	40g/day	19-25y men	ns	Coudray *et al.* (1997)
OF I OF + I	10% 10% 10%	Male rats (170g)	↑* ↑* ↑*	Coudray *et al.* (2003)
FOS	10%	Male rats (100g +/- 5g)	↑*	Delzenne *et al.* (1995)
FOS	5%	Male rats (209g +/- 1.69g)	↑*	Gudiel-Urbano and Goñi (2002)
RS	20%	7wk male rats	↑**	Lopez *et al.* (1998)
I	1%	Male rats (~160g)	↑**	Lopez *et al.* (2000)
RS	20%	Male rats (~150g)	↑*	Lopez *et al.* (2001)
FOS	5%	6wk male rats	↑*	Ohta *et al.* (1994)
FOS	5% 10%	5wk male rats	ns ↑*	Ohta *et al.* (1998a)
Short chain FOS	10g/day	53 – 65y postmenopausal women	↑**	Tahiri *et al.* (2001)
RS	35%	8wk male rats (~200g)	↑**	Younes *et al.* (1996)
I RS I + RS	10% 15% 12.5%	8wk male rats	↑* ↑* ↑*	Younes *et al.* (2001)

Table 5 shows that, as was the case for calcium, studies on the effects of prebiotics on magnesium absorption have been carried out on a range of models, using a variety of NDOs, at different levels. Although fewer studies have been carried out for this mineral, results almost unanimously suggest that prebiotics may increase magnesium absorption.

1.3.2.3. Zinc

Considerably fewer studies have been carried out on the effect of NDOs on zinc absorption. Table 6 lists the findings of research into this subject.

Table 6: Summary of previous research investigating the effect of prebiotics on zinc absorption. ns indicates no significance; * P<0.05; ** P<0.005. Abbreviations used: FOS, fructooligosaccharides; I, inulin; OF, oligofructose; RS, resistant starch.

Prebiotic	Level (percentage of diet)	Model	Effect	Authors
I	40g/day	19-25y men	ns	Coudray *et al.* (1997)
FOS	10%	Male rats (100g +/- 5g)	↑*	Delzenne *et al.* (1995)
FOS	5%	Male rats (209g +/- 1.69g)	↑*	Gudiel-Urbano and Goñi (2002)
RS	20%	7wk male rats	↑*	Lopez *et al.* (1998)
I	1%	Male rats (~160g)	ns	Lopez *et al.* (2000)
RS	20%	Male rats (~150g)	↑*	Lopez *et al.* (2001)

The majority of these studies suggest that NDOs may increase zinc absorption, although this possibly occurs to a lesser extent than calcium. However, it should be noted that not all researchers found this to be the case; further research is needed.

1.3.3. Effects on Bone

Previous studies have suggested that bone mineral density (BMD) and bone mineral content (BMC) may be increased by dietary supplementation with probiotics and / or prebiotics. Supplementation with *Lactobacillus rhamnosus* (DR20™) was found to increase BMD in the growing pig and OVX rat (Kruger *et al.*, 2003a; Darragh *et al.*, 2005). Long-term (14 weeks) supplementation with *Lactobacillus helveticus* increased BMD and BMC in growing male rats (Narva *et al.*, 2004). OF supplementation at 2.5, 5 and 10% increased BMC in OVX rats (Scholz-Ahrens *et al.*, 2002). Ohta *et al.* (2002) showed that FOS supplementation at 5% prevented a decrease in BMD in OVX mice. 5% FOS given to growing male rats resulted in an increase in calcium and magnesium contents, and the trabecular and overall volume of femurs (Takahara *et al.*, 2000).

BMC and BMD may be increased by changes to bone metabolism instead / as well as changes to calcium absorption. Zafar (2004) showed that 5.5% FOS in diets of OVX rats could decrease bone resorption, and increase BMD. Dahl *et al.* (2005) found no change in bone resorption in wheelchair-bound adults, following a three-week crossover study supplying 13g/day of inulin. However, the study-design is not ideal, as the effect that inulin supplementation may have had may be obscured by the subsequent / preceding control period. The study was also fairly small; only thirteen subjects were involved. Wheelchair-bound subjects may have reduced bone content and quality, compared to their more mobile-counterparts (Marcus, 1999; Brown and Josse, 2002;

Kohrt *et al.*, 2004; Dahl *et al.*, 2005); an effect on bone resorption may, therefore, be less apparent.

SUMMARY

Probiotics are bacteria, which reside in the gut and have beneficial health properties. Prebiotics are the oligosaccharides that are not digested in the small intestine, and provide probiotics with a source of energy, metabolic substrates and nutrients, selectively stimulating the growth and / or activity of probiotics.

Previous studies have suggested that prebiotics may improve absorption of calcium, magnesium, and perhaps also zinc. Several mechanisms have been proposed in explanation. When prebiotics are fermented in the large intestine, SCFA are produced. The resulting drop in pH may cause more minerals to become ionised, i.e. converted into the form they must assume in order to be absorbed. Alternatively, the increased SCFA present may alter the electrolyte exchanges that occur across the epithelium; for example, cellular H^+ ions are exchanged for luminal Ca^{2+} ions. SCFA may form complexes with minerals that are more soluble than ionised calcium, thus raising their absorption.

However, another research group has suggested that passive transport of calcium absorption may be decreased. Mariadsaon *et al.* (1999) showed that non-digestible fibre (and also digestible fibre) reduced paracellular transport in the large intestine. The authors commented that this may be due to the cell-differentiation that these compounds induce.

It has been suggested that NDOs may be directly affecting the mechanisms involved in mineral absorption. For example, FOS has been shown to increase the level of CaBP

expressed in the large intestine (Ohta *et al.*, 1998b). Hence, calcium may affect both transcellular and paracellular transport pathways.

Alternatively, prebiotics may be affecting the physical attributes of the intestine. NDOs have been shown to cause atrophy in the large intestine (Ichikawa and Sakata, 1998). By increasing the size and number of the epithelial cells, there will be a greater surface area over which minerals can be absorbed.

Studies have suggested that, as well as mineral absorption being affected, BMC, BMD and bone structure may also be improved by probiotic and / or prebiotic supplementation.

Section 4. Motivation and Objectives

1.4.1. Motivation for the Study

From the literature it appears that pre- and pro-biotics may improve mineral absorption from the intestine, and hence may also affect mineralisation of bone. Whilst many studies have examined the effect of prebiotics on mineral absorption, those that look at probiotic administration are considerably fewer. Only one appears to have examined the effect that joint-supplementation may have (Darragh *et al.*, 2005). The present study aims to examine the effect that probiotics may have on mineral uptake and bone physiology, and the effect (if any) that supplementation with both pro- and pre-biotics has on the same parameters.

1.4.2. Objectives

The objectives of this study were to determine the effects of pro- and pre-biotic supplementation in growing male rats on:

- Mineral absorption
- Bone density
- Bone mineral content
- Rate of bone turnover
- Cytokine status
- Gut microflora

1.4.3. Hypothesis

Supplementation with probiotics will improve bone parameters and mineral absorption; supplementation with pro- and pre-biotics will have a synergistic effect.

Chapter 2. Materials and Methods

2.1. Materials

The high calcium milk powder (HCMP; Anlene™), and the HCMP + Vitamin K (HCMPK) was produced by New Zealand Milk Ltd (Auckland, New Zealand). The probiotic, *Lactobacillus rhamnosus* strain HN001 (DR20™) was obtained from Fonterra (Auckland, New Zealand). The fructo-oligosaccharide (FOS; Frutafit®) was manufactured by Sensus (Rosendaal, Holland). Table 7 lists the carbohydrate composition of FOS; the average chain length was 10 monomers.

Table 7: Analysis of carbohydrates present in FOS (Frutafit®)

Carbohydrate	Chemical Composition (expressed as dry matter) (%)
Inulin (DP2 – DP60)	91.1*
Fibre (AOAC 997.08)	91.1*
Fructose	3.5
Glucose	0.7
Sucrose	4.6

***Chemical composition for inulin and fibre is identical because inulin is a fibre.**

2.2. Methods

2.2.1. Animals

Sixty three-week old male Sprague-Dawley rats were obtained from the Small Animal Production Unit, Massey University. The animals were housed separately in shoebox cages, and kept at 22°C ± 2°C, under a 12 hour light: 12 hour dark cycle in a temperature and light-controlled room in the Small Animal Production Unit. Animals had *ad libitum* access to deionised water. The trial was conducted over 10 weeks. The experiment was conducted under protocol (05/07) as approved by the Massey University Animal Ethics Committee.

2.2.2. Diets

The animals were fed a balanced, semi-synthetic diet for the ten days prior to commencement of trial (weeks -1 and 0) (consisting of 60% starch, 25% HCMP, 5% cellulose, 5% corn oil, 5% sucrose and added vitamins and minerals as needed (Subcommittee on Laboratory Animal Nutrition *et al.*, 1995)). When the trial commenced (week 1), rats were randomised into diet groups based on weight and parentage. Animals were either maintained on the control diet ("HCMP –" (n=12)), the control diet supplemented with vitamin K ("HCMPK –" (n=12)), control diet with DR20™ added ("HCMP +" (n=12)), control diet plus vitamin K and DR20™ ("HCMPK +" (n=12)), or the control diet with added vitamin K, DR20™ and FOS ("FOS" (n=12)). DR20™ was added to appropriate diets at a level of 10^9 colony-forming units (cfu) per rat per day; FOS was supplied at 5% and was substituted for the sucrose present in the other diets. All diets contained calcium at 0.5%. Diet composition is summarised in Table 8.

Table 8: Diet composition expressed as grams per kilogram

	FOS	HCMP –	HCMP +	HCMPK –	HCMPK +
HCMP	250	250	250		
HCMPK				250	250
Sucrose		50	50	50	50
FOS	50				
Amino Acids:					
Alanine	0.5	0.5	0.5	0.5	0.5
Cystine	1.9	1.9	1.9	1.9	1.9
Glutamine	17.8	17.8	17.8	17.8	17.8
Glycine	4	4	4	4	4
Isoleucine	1	1	1	1	1
Leucine	0.6	0.6	0.6	0.6	0.6
Methionine	6.7	6.7	6.7	6.7	6.7
Phenylalanine	5.2	5.2	5.2	5.2	5.2
Threonine	1.5	1.5	1.5	1.5	1.5
Valine	1	1	1	1	1
Mineral Mix:					
Potassium Phosphate	9.8	9.8	9.8	9.8	9.8
Potassium Sulphate	2.3	2.3	2.3	2.3	2.3
Tri-Potassium Citrate	3.5	3.5	3.5	3.5	3.5
Iodised sodium Chloride	3.7	3.7	3.7	3.7	3.7
Magnsium Oxide	1.2	1.2	1.2	1.2	1.2
Trace Salt	2	2	2	2	2
Cellulose	27.4	27.4	27.4	27.4	27.4
Cellulose	50	50	50	50	50
Corn Oil	50	50	50	50	50
Starch	459.8	459.8	459.8	459.8	459.8

Daily intake of food was recorded for each rat. DR20™ was mixed into a weekly-ration, which was subdivided into daily doses for each diet (to ensure that each animal received a constant dose of 1 x 10^9 CFU per day) and stored at 4°C (to prevent oxidation and death of bacteria). Animals were fed the same amount of food, which varied between 5g at the start of trial, and 28g at the end. Table 9 summarises timings of the trial.

Table 9: Summary of trial proceedings, and rat ages.

Week	Rat Age (wks)	Activities
-1 / 0	3 / 4	Rats fed base diet
1	5	Rats randomised into diet groups; started feeding test diet
3 / 4	6 / 7	1st balance study
8 / 9	12 / 13	2nd balance study
10	14	Euthanasia

2.2.3. Balance Studies

At weeks three and eight, animals were placed in individual metabolism cages suitable for rats up to 600g (Techniplast; Buguggiate, Italy) for 8 days (5 days acclimatisation, 3 days collection). Daily food intake was measured. Urine and faeces were collected in pots positioned at the bottom of the cage. 500µl of 1M HCl was added to the urine-collection pots attached to the cage to prevent bacterial growth and nitrogen breakdown. Urine and faeces were pooled in 120ml and 60ml specimen pots respectively each day. The collection pots attached to cages were emptied daily, and samples frozen at 4°C until they were assayed. After 8 days, animals were returned to shoebox cages.

Urine samples were defrosted and filtered through 12.5cm Whatman filter papers (number 4), which had previously been dried in an oven at 105°C overnight and weighed. Filter papers were then returned to the oven at the same temperature, left overnight, and weighed the next morning to determine weight of the urine residue.

Faeces were freeze-dried, weighed, sifted to remove any food particles, and re-weighed. Faeces were homogenised using a coffee-grinder. Plastic bags containing homogenised samples were weighed again, and the recovery coefficient calculated as shown in Figure 17.

$$\text{Recovery coefficient} = \frac{\text{Homogenised sample weight}}{\text{Weight of sample after food particles were removed}} \times 100$$

Figure 17: Calculation of recovery coefficient

Plastic bags were left open for four days to allow samples to equilibrate to air humidity before analysis, and then weighed.

Faecal and urine samples, together with a sample of each diet, were sent to Fonterra Research Centre (Palmerston North) for calcium, magnesium and zinc analysis. Samples were digested in a mixture of 2ml nitric acid and 0.5ml hydrochloric acid for 1 hour at 85°C. They were then diluted in water (containing a small amount of Triton to aid passage through instrument tubing), and run through an ICP-OES (individually coupled plasma-optical emission spectrometer). Two standards were used; one contained calcium, magnesium, potassium, sodium and phosphorus, and the other zinc, iron, manganese and copper. Samples were diluted appropriately to ensure readings lay on the standard curve.

Mineral balance was calculated as shown in Figure 18.

Intake (mg)

$$= \text{Value obtained from analysis of diet (mg/kg)} \times (\text{total food intake (g)} - (\text{diet dust} + \text{residue urine} + \text{cone dust}))$$

Mineral excreted in urine (mg)

$$= \text{Value obtained in analysis (mg/dL)} \times \text{Volume of urine excreted (dL)}$$

Mineral excreted in faeces (mg)

$$= \frac{\left[\frac{\text{Equilibrated faeces weight (g)} \times \text{Value obtained from analysis}}{\text{Recovery coefficient (\%)}}\right]}{1000}$$

Mineral Absorbed (%)

$$= \frac{(\text{Intake} - (\text{Amount excreted in urine} + \text{faeces})) \times 100}{\text{Intake}}$$

Mineral Balance (mg)

$$= \text{Intake} - (\text{Amount excreted in urine} + \text{faeces})$$

Figure 18: Balance calculations

2.2.4. Terminal Heart Puncture

At week 10 animals were weighed and anaesthetised with an appropriate amount of anaesthetic (0.1ml/100g body weight; anaesthetic mixture = 0.2ml Acepromazine (ACP) + 0.5ml Ketamine + 0.1 Xylazine + 0.2ml sterile H_2O), delivered by *intra peritoneal* injection using a 25G x ⅝" needle. A 19G x 1½" hypodermic needle and 5ml syringe were used to extract blood directly from the heart. Blood samples were collected in vacutainers containing heparin and stored on ice. Approximately 2ml whole blood was retained for IL-6 and IL-10 analysis, and the remainder centrifuged at 3000rpm for 10 minutes. Plasma was removed and snap-frozen with liquid nitrogen, and stored at -85°C until assayed. The lumbar spine, left and right femurs and caeca were dissected out. Bones were frozen in phosphate buffered solution (PBS) for later analysis. Caeca were stored at -85°C until assayed.

2.2.5. Dual Energy X-ray Absorptiometry Scans

Dual Energy X-ray Absorptiometry (DEXA) scans were carried out *ex vivo*. Bone mineral measurements were made with a pencil beam Hologic QDR4000 bone densitometer unit (Bedford, USA). A daily quality control (QC) scan was taken to ensure precision met with the required coefficient of variation. The coefficient of variation for the QC was 0.98 – 1.01%. Spines and femurs were stripped of extraneous tissue, leaving about 1cm of flesh attached. These were submerged in a 1.5cm deep dish of PBS, which was positioned on an acrylic platform of uniform 3cm thickness. Spines were scanned from L4 to L1, and femurs from the knee to hip joints. Both femurs were scanned, and the mean used for statistical analysis. Regional high-resolution scans were performed using a 0.15cm diameter collimator with 0.03cm point resolution and 0.06cm line spacing to measure bone mineral density (BMD) and bone mineral content (BMC).

2.2.6. Biochemical Markers

2.2.6.1. Type I Collagen c-Terminal Telopeptides

Plasma samples were analysed for serum C-telopeptides of Type I collagen (CTx), using the "Ratlaps" ELISA kit (Nordic Bioscience Diagnostics A/S, Herlev, Denmark). Plates were read at 550nm using an ELx808 Ultraplate Reader (Bio-tek Instruments Inc, Vermont, USA).

2.2.6.2. IL-6 and IL-10

Whole blood samples were stimulated using the method of Yaqoob *et al.* (1999). 20µl of 100µg/ml lipopolysaccharide in Hanks Balanced Salt Solution (HBSS) was added to each well of a 24-well plate, together with 1.8ml of HBSS.

200µl of heparinised blood was added to each well, thus creating a 10x dilution of the samples. Well plates were incubated at 37°C for 24h. Supernatants were then harvested and stored at -80°C until required. Samples were analysed for IL-6 and IL-10 using "Duoset" ELISA kit (R&D systems, Mineapolis, USA). ELISA plates were read at 550nm using an ELx808 Ultraplate Reader (Bio-tek Instruments Inc, Vermont, USA).

2.2.7. Biomechanics

Right femurs were scraped clean of flesh and incubated at 23 °C for 1 hour before the assay, to ensure they were at room temperature for the test. A three-point bending test was used (Shimadzu Ezi-test, Kyoto, Japan), with a support span of 12mm and a test speed of 50mm/min.

2.2.8. Bone Ash Content

Left femurs were scraped clean of flesh, oven dried for 12 hours at 105°C, measured with callipers and weighed. Bone mineral content was then determined by ashing the bones at 660°C for 12 hours, and then reweighing them.

Ashed bones were then placed in glass universals, and 2ml of 6M hydrochloric acid added; samples were vortexed and left for fifteen minutes until dissolved. They were then mixed, and sent to the Nutrition laboratory, IFNHH (Massey University, Palmerston North) for calcium, magnesium and zinc analysis. Samples were digested in a nitric acid / hydrochloric acid mix, and run through an ICP-OES to quantify minerals.

2.2.9. Gut Bacteriology

Caeca were defrosted and the contents scraped into a sterile container. Caecal contents were weighed, diluted by a factor of 10 with broth, and vortexed until contents were homogenous. Samples then underwent a serial dilution, as depicted in Figure 19. 0.1ml of sample was diluted in 0.9ml broth, and the sample vortexed. 0.1ml of this suspension was then diluted in another 0.9ml of broth, and the process repeated. For each dilution stage, 0.1ml was aliquoted onto plates containing Rogosa media and incubated in anaerobic conditions for 48 hours. The media and incubation conditions allow the selective growth of *Lactobacillus* species (Sutter *et al.*, 1985). An agar plate that contained approximately 30 – 300 visible colonies was used to count colonies. The number of colonies counted was then multiplied by the dilution factor, giving the number of colonies present in the original sample.

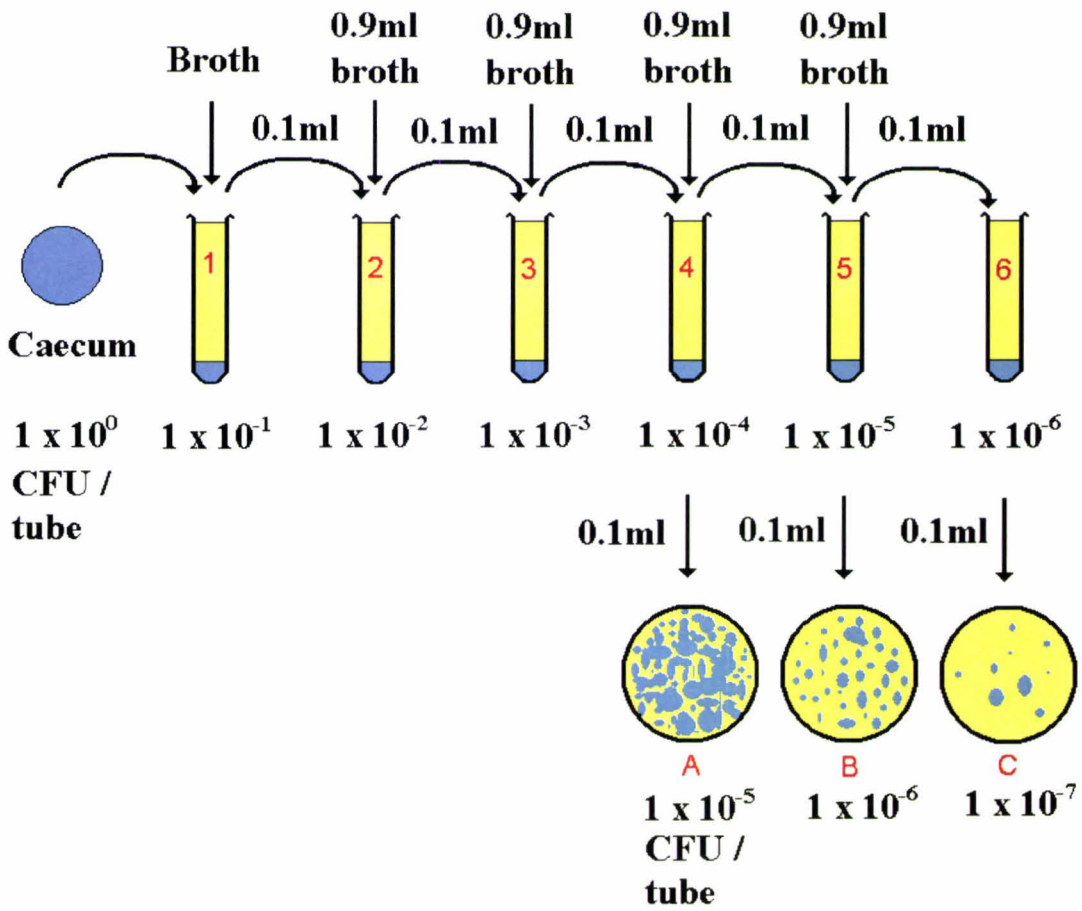

Figure 19: Serial dilution, culture and counting of caecal bacteria. Caecal contents were weighed, and diluted (by a factor of ten) in broth in eppendorf 1. 0.1ml of this solution was then diluted in 0.9ml broth in eppendorf 2. 0.1ml of each dilution was also plated out and incubated in anaerobic conditions. This dilution process was repeated until the approximately 30 – 300 colonies were visible; plate A (and all plates prior to plate A, none of which are shown for ease of viewing) would have too many colonies, and plate C too few. Numbers below the tubes show the concentration at which cells are present, relative to the original sample. By aliquoting only 0.1ml onto each plate, cells are in effect diluted again by a factor of ten. Therefore, since in this example plate B shows 36 CFU, this suggests that the original caecal contents would be 36 x 10^{-6} CFU, or 3.6 x 10^{-5} CFU.

2.2.10. Statistical Analysis

Results were analysed using SPSS version 14.0. Changes in body weight, food intake, faecal and urinal mineral excretion, and mineral balance were subjected to repeated measures analysis, fitted using Systat version 11, and repeated measures graphs plotted using Excel 2003 (to allow error bars representing the standard error of the mean (SEM) to be represented). The remainder of parameters were analysed using one-way ANOVA. Levene's test was used to ensure that groups had equal

variances, in which case they were compared using one-way analysis of variance, followed by *post-hoc* testing (Tukey). If Levene's test showed unequal variances were present, groups of animals were compared using the Kruskal-Wallis test. Where this was necessary it is mentioned in the results section. If the Kruskall-Wallis test indicated a significant effect, Mann-Whitney U independent group comparisons tests were carried out to compare each group. Statistical correlations between different outcome measures were analysed using Pearson Correlation tests. All variables measured were tested in this way to determine whether they were confounding data. Only those that showed a significant relationship were included in the results chapter, and used as covariates during analysis. A p-value of less than 0.05 was considered significant in all statistical tests. Values and graphs are expressed as mean ± SEM.

2.3. The Growing Rat as a Model

Dietary manipulation of bone metabolism is a multifactorial process; an *in vitro* system may not show the same response(s) as an *in vivo* system. Nor would it reflect the physiological process occurring, or allow measurement of the same parameters, BMD and bone biomechanics.

The first three months of a rat's life are characterised by a period of rapid growth and high bone turnover; peak bone mass has been shown to be reached at three months of age, coinciding with the onset of puberty (Sengupta *et al.*, 2005). This reflects the processes occurring in growing humans. Rats display many anatomical similarities to humans, and also undergo bone metabolism (Mosekilde, 1995).

Nutrient absorption is most important for growing animals, as there is an increased requirement for them in bone and other tissues. Figure 20 shows the rate of calcium

deposition and bone mineral content of humans, and changes during age. Both parameters are highest in young, actively growing individuals.

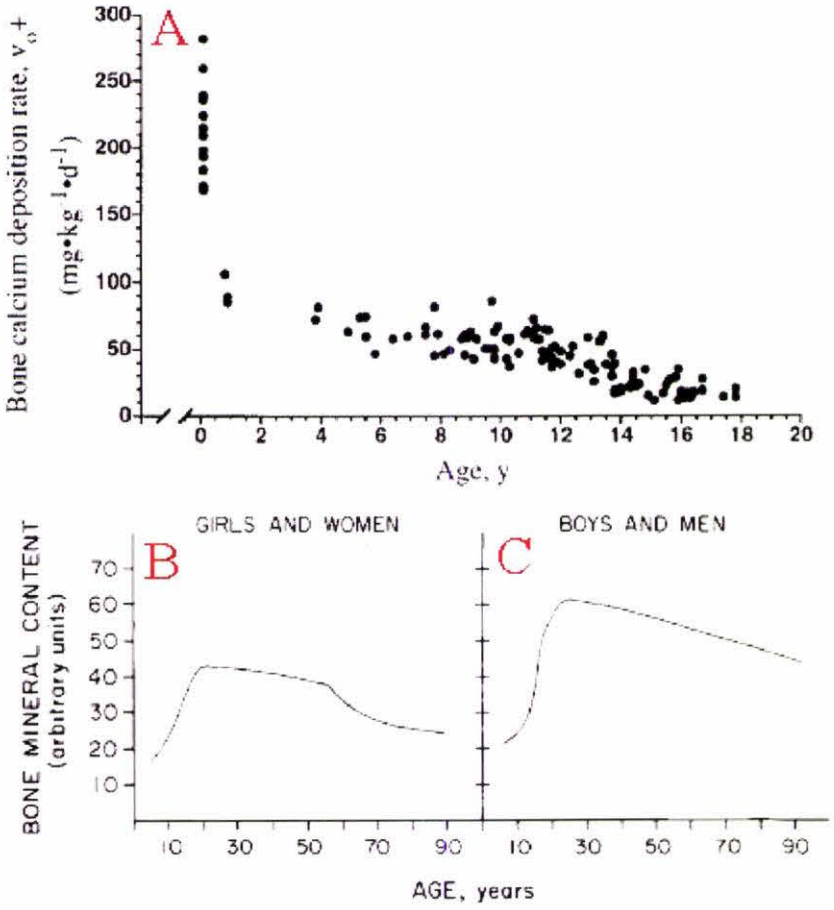

Figure 20: Bone calcium deposition rate (expressed on a body weight basis) changes with age (graph A), and bone mineral content changes with age in females (graph B) and males (C). Taken from Bronner and Pansu (1999).

Bones are growing rapidly; therefore any effect of experimental manipulation should be easily observed. In addition, due to the importance of oestrogen in bone metabolism, the OVX rat shows compromised bone mineralisation and biomechanics.

2.4. Dual Energy X-ray Absorptiometry

DEXA uses X-rays of two different wavelengths, usually 40 and 70kV, to determine amount of bone and soft tissue present. X-rays are attenuated to different extents depending on the density of the tissue through which they pass. This allows provision of

images and calculation of bone mineral density (BMD), bone mineral content (BMC) and area of bone in samples using prediction equations (Ellis, 2000).

Nagy *et al.* (2001) showed DEXA to be an accurate method for assessing BMC and BMD for rats in both *in vivo* and *ex vivo* situations. However, DEXA use may be compromised by the fact that it uses bone area to predict density rather than bone volume. Thus a large bone may be reported as having a higher bone density than a smaller one (Jiang *et al.*, 2000).

DEXA does not record bone quality; there will be no indication of the functional significance of dietary manipulations. Results can be misleading. For example, a bone with high fluoride content will display a high BMC and BMD, but have reduced bone strength (Grynpas *et al.*, 2000). This should not be a concern, as bone biomechanics will be measured at the same time (i.e. at the end of the trial); it may have been more of an issue were DEXA measurements taken at several time points during the study, and used to infer bone quality.

2.5. Biochemical Markers of Bone Metabolism

Several biochemical markers exist for both bone formation and bone resorption, reflecting changes occurring in bone metabolism. These markers make use of the concentration of various molecules produced by the characteristic procedures in both processes. Markers of bone formation allow the synthetic activity of osteoblasts or procollagen metabolism to be assessed (Christenson, 1997). These cause the release of a range of peptides that can be measured in urine and serum, and hence can be used to quantify the rate of bone formation occurring in the whole skeleton. Markers of bone resorption, tend to measure the activity of osteoclasts, or degradation of collagen

(Christenson, 1997). Again, the level of the peptides released can be measured in urine and serum.

Biochemical markers provide a useful tool of analysis. They are highly sensitive, and because they represent occurrences at a molecular level they reflect changes in bone metabolism sooner than other measurements (Adachi, 1996). Equally, however, biochemical markers have limitations, which should be considered when choosing a suitable marker and interpreting the results. Collagen is not solely a product of bone; it is produced in other tissue, such as skin and teeth. Certain markers may, therefore, also represent osteoclast / osteoblast activity at other sites in the body.

2.5.1. Type I Collagen c-Terminal Telopeptides

Type I collagen has two crosslink-forming sites, one in the amino-terminal region, termed N-terminal telopeptides, and the other in the carboxy-terminal region, known as C-terminal telopeptides. Collagen degradation by the osteoclast causes release of C-terminal Telopeptides (CTx), which can be measured in both urine and serum (Christenson, 1997). CTx fragments are thought to be highly bone-specific as osteoclasts are not as actively degrading collagen in other tissues (Christenson, 1997). Plebani *et al.* (1996) showed CTx to be a better marker of bone resorption than others, such as tartrate-resistant acid phosphatase.

2.5.2. IL-6 and IL-10

IL-6 and IL-10 are produced at a level that may be too small to quantify using the size of sample used in this ELISA kit. Samples can be "stimulated", which causes cytokines production to be enhanced at a constant rate, to a level that is quantifiable by ELISAs. Cytokines can be stimulated from either whole blood or the mononuclear

cells (MNCs) from which they are secreted; production has been shown to be similar in these two systems (Yaqoob *et al.*, 1999). Use of whole blood instead of MNCs has several advantages. There is no need for cell culture, and cells remain in the environment in which they normally reside, and may contain factors that control cytokine synthesis. Differences in procedures for MNC culture have been shown to induce differences in cytokine function and potentially production (Yaqoob *et al.*, 1994).

2.6. Biomechanical Testing

Bone fragility describes the ease with which bones fracture (Turner, 2002). As there are many different reasons for bones to fracture, there are also several different methods for assessing fragility. Five biomechanical testing techniques exist; they are compression, tension, bending (three or four point), torsion and fatigue tests. In this instance, a bone bending technique was adopted, as it is suitable for use on small bones, such as rat femurs. A three-point test was used, as it is simpler than the four-point test.

The three-point test involved suspending the dissected rat femurs horizontally between two supports. A downward force was applied gradually, halfway along the length of the bone, and the degree of bone bending and ultimate bone breaking point was recorded. The force / load at which the bones would fracture depends on the cross-sectional shape of the bone, the quantity and distribution of bone mass, and factors such as distance between the plates supporting the bone. The loading that the bones undergo before fracture was presented in a load-deformation curve, similar to the one shown in Figure 21.

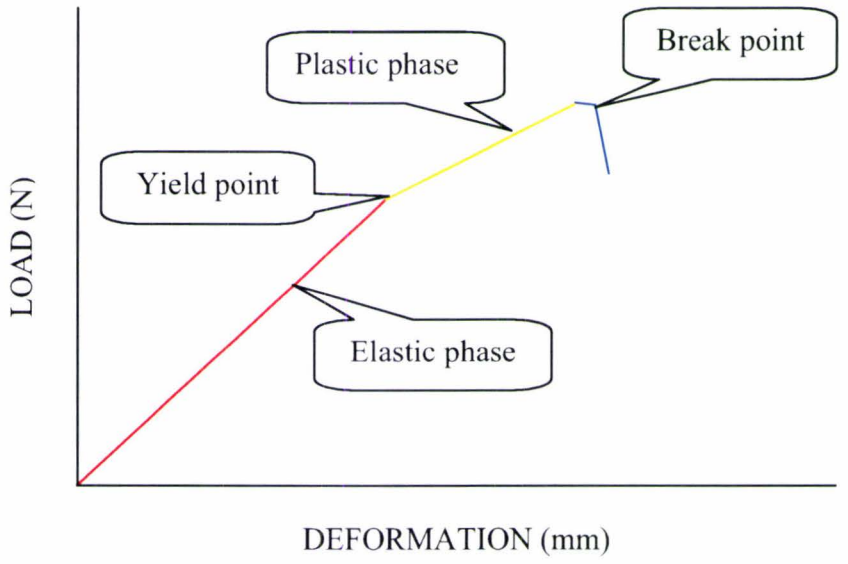

Figure 21: Typical load-deformation curve. Load is a measure of force applied, deformation a measure of amount of bending the bone displays. The red section indicates the elastic phase, the yellow the plastic phase, and the blue section represents the bone break point. Terms are summarised in the text.

As can be seen in Figure 21, there are three stages in bone bending – the elastic, plastic and breakage stages. The elastic phase is a period where load applied is directly proportional to deformation induced; release of the load during this stage causes the bone to restore its original shape. The plastic phase describes the following stage, where deformation increases at a greater rate than force is applied; bone incurs permanent damage. The yield point is the moment at which the applied load causes permanent damage to the bone; it is the transition between elastic to plastic phases. The break point or ultimate point is the maximum load that the bone sustains; bone fractures. The parameters measured in bending tests are summarised in Table 10.

Table 10: Definitions of parameters measured in 3-point bending test.

Parameter	Definition
Maximum load (N)	Highest load to which bone resists deformation
Maximum stroke (mm)	Greatest deformation that bone experiences, before fracture
Break load (N)	Measure of the force needed to cause bone to fracture
Break stress (N/mm^2)	Force applied per unit area of bone
Break stroke (mm)	Quantity of bone deformation at the maximum stroke
Break strain (%)	Deformation relative to bone dimensions
Elastic (N/mm^2)	Measure of stiffness; slope of elastic phase
Energy (j)	Total force needed to induce fracture; area under the load-deformation curve. Indicative of bone collagen content.

SUMMARY

The growing rat is a useful model to study mineral utilisation, as the process is of great importance; results of experimental manipulation should be more easily observed than would be the case in other models. In addition, the OVX model may show comprised bone mineralisation and biomechanics, due to the absence of oestrogen, an important hormone in bone metabolism.

Rats were fed a combination of FOS (a prebiotic) and DR20™ (*Lactobacillus rhamnosus* HN001; a probiotic). These have been used in previous studies in the department (IFNHH, Massey University), and showed the potential to affect mineral absorption.

DEXA and balance studies determine BMC / BMD, trabecular / cortical content and mineral balance respectively. CTx was used as a biomarker for measuring level of bone resorption. IL6 and IL10 were quantified using ELISAs. Gut bacteriology was examined in order to determine how effective each diet was at manipulating flora. The biomechanical and biochemical properties of bones were tested to measure their mechanical properties, and hence the functional significance of experimental manipulations.

Chapter 3. Results

3.1. Animals

There was no significant difference between groups in terms of body weight at week 1 (109.6 +/- 1.2g) or week 10 (409.6 +/- 2.8g). There were no significant differences between body weights of rats in diet groups for any of the trial weeks (data not shown). Figure 22 shows the weekly changes in body weights for rats during the trial.

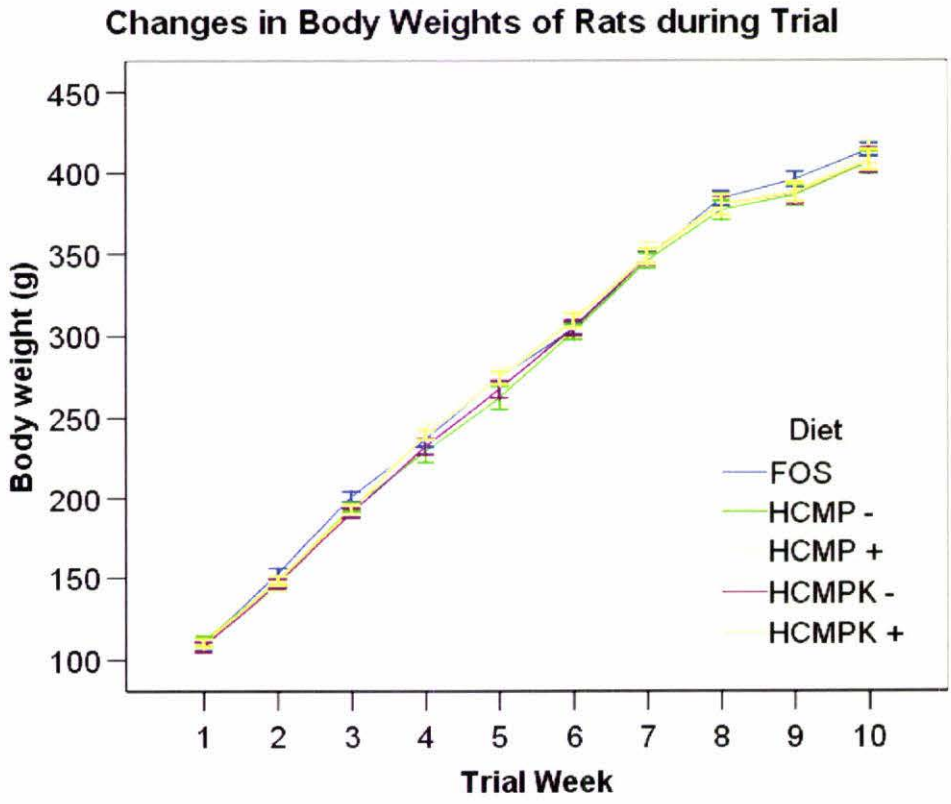

Figure 22: Weekly changes in rat body weights during trial. Animals are classified by dietary groups (twelve animals per group), in order to determine that there was no difference induced as a result of diet, as this may have affected nutrient absorption and / or bone parameters measured. Rats were aged 5-weeks at the start of the trial (week 1), and 14-weeks at the end of the trial (week 10).

At the conclusion of the trial (trial week 10), animals were 14 weeks old. Since puberty, and cessation of growth, does not occur until approximately 12 weeks of age (Sengupta *et al.*, 2005), one would expect to observe weight gain until this time. Figure 22 confirms this occurred during the trial. Weight was highly statistically increased from the previous week for all diet groups throughout the trial ($p<0.01$).

Figure 22 shows an almost linear relationship between body weight and week. Each group displays a slight deviation from the apparent straight line at week 9, although weight increase was comparable to that at week 8, and was still highly significant ($p<0.01$). This was during the second balance study; some rats lost weight or gained it at a slower rate, due to the stress of a new environment. Food intake decreased in some animals during this period (see 3.2.1).

3.2. Diets

3.2.1. Food Intake

Figure 23 shows the mean daily food intake of rats in different diet groups during weeks one to nine. Data from week ten is not included, as rats were fasted overnight prior to euthanisation; therefore daily intake would appear deceptively low.

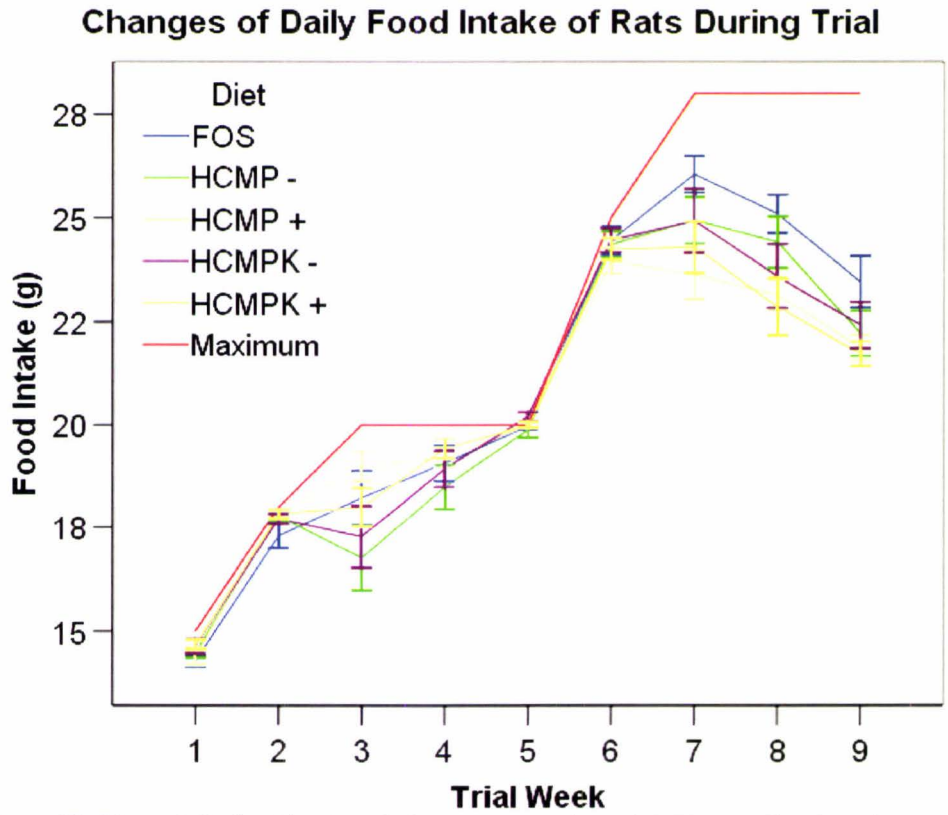

Figure 23: Mean daily food intake of diet groups during trial. The red line (maximum) shows the amount of food that was given to animals each day. Animals are classified by dietary groups; there were twelve rats in each dietary group. Rats were aged 5-weeks at the start of the trial (week 1), and 14-weeks at the end of the trial (week 10).

Balance studies were carried out in weeks 3 / 4 and 8 / 9 of the trial, when they were 8-weeks and 13-weeks old, respectively. Figure 23 shows a slight decrease in food intake during these periods, but this was not significant.

The total food eaten during the trial, by animals in diet groups is listed in Table 11; there appears to be no difference between total food eaten and diet group.

Table 11: Analysis of total food eaten by animals on different diets; there were twelve rats per dietary group. Values with different superscripts (a) denote significant differences between groups at p<0.05.

		FOS (n=12)	HCMP – (n=12)	HCMP + (n=12)	HCMPK – (n=12)	HCMPK + (n=12)
Total Food Eaten (g)	Mean	1315.67[a]	1283.53[a]	1281.71[a]	1287.61[a]	1281.37[a]
	SEM	13.14	17.75	8.75	19.50	13.25

3.2.2. Mineral Content

Calcium, magnesium and zinc levels in the study diets were verified by analysis, shown in Table 12.

Table 12: Analysis of calcium, magnesium and zinc levels of study diets; four samples of each diet were taken. Values with different superscripts (a) denote significant differences between groups at $p<0.05$.

		FOS (n=4)	HCMP (n=4)	HCMPK (n=4)
Calcium (mg/kg)	Mean	5492.5[a]	5490.0[a]	5557.5[a]
	SEM	50.39	87.18	48.20
Magnesium (mg/kg)	Mean	1275.0[a]	1285.0[a]	1297.5[a]
	SEM	15.55	19.37	20.16
Zinc (mg/kg)	Mean	49.1[a]	44.5[a]	45.9[a]
	SEM	3.378	0.616	0.550

There were no significant differences in calcium, magnesium and zinc levels between diets. Levene's test for equal variances carried out on zinc, yielded a statistic of 0.031, suggesting that variances are significantly different, whilst non-parametric testing suggested that mean zinc content did not differ between diets. It is possible that the diet was not mixed sufficiently, thereby leading to differences in zinc content between different samples of FOS. Overall, however, zinc content of the FOS diet was not sufficiently affected to cause a significant difference to diets.

3.2.3. Probiotic Content

Diets were also analysed to determine their DR20™ count. It was intended that the HCMP +, FOS and HCMPK + diets should provide each animal with 1 x 10^9 cell forming units of DR20™ per day. Samples were taken from the original sample, received from Fonterra Research Centre, and from the three diets that contained the probiotic (HCMP +, FOS and HCMPK +), nine weeks into the trial in order to determine the actual level of DR20™ administered. Table 13 lists the results.

Table 13: Probiotic counts of the original sample (given as colony forming units (CFUs) / gram) and the three probiotic diets (given as CFU / day). Four samples of each diet were taken.

Diet / Sample	DR20™ Count (CFU / gram)
Original Sample	5.57×10^{10}
Diet / Sample	**DR20™ Count (CFU / day)**
FOS	2.25×10^{8}
HCMP +	1.45×10^{7}
HCMPK +	1.52×10^{7}

SEMs are not shown in Table 13, due to data loss as a result of computer malfunction. This data loss also meant that probiotic counts in the FOS, HCMP + (nd HCMPK + could not be compared to the required daily dose (1×10^{9} CFU) using statistical tests. However, the DR20™ counts in diets listed in Table 13 appear slightly lower than the required daily dose. This may be due to bacterial cell death, or perhaps insufficient homogenisation of samples (although without analysing for differences in variances this cannot be determined).

3.3. Balance Studies

3.3.1. Mineral Balance

Figure 24 and Table 14 show the effect of three weeks of feeding trial diet on calcium, magnesium and zinc balance. SEMs are reasonably small, except in the HCMP – group, which displays a fairly large error margin in all measures of mineral absorption, particularly those of magnesium and zinc. In the first balance study (Figure 24 and Table 14), there were only eleven animals in HCMP -, HCMP +, HCMPK – and HCMPK + groups due to errors in data collection. All variables were analysed using non-parametric testing.

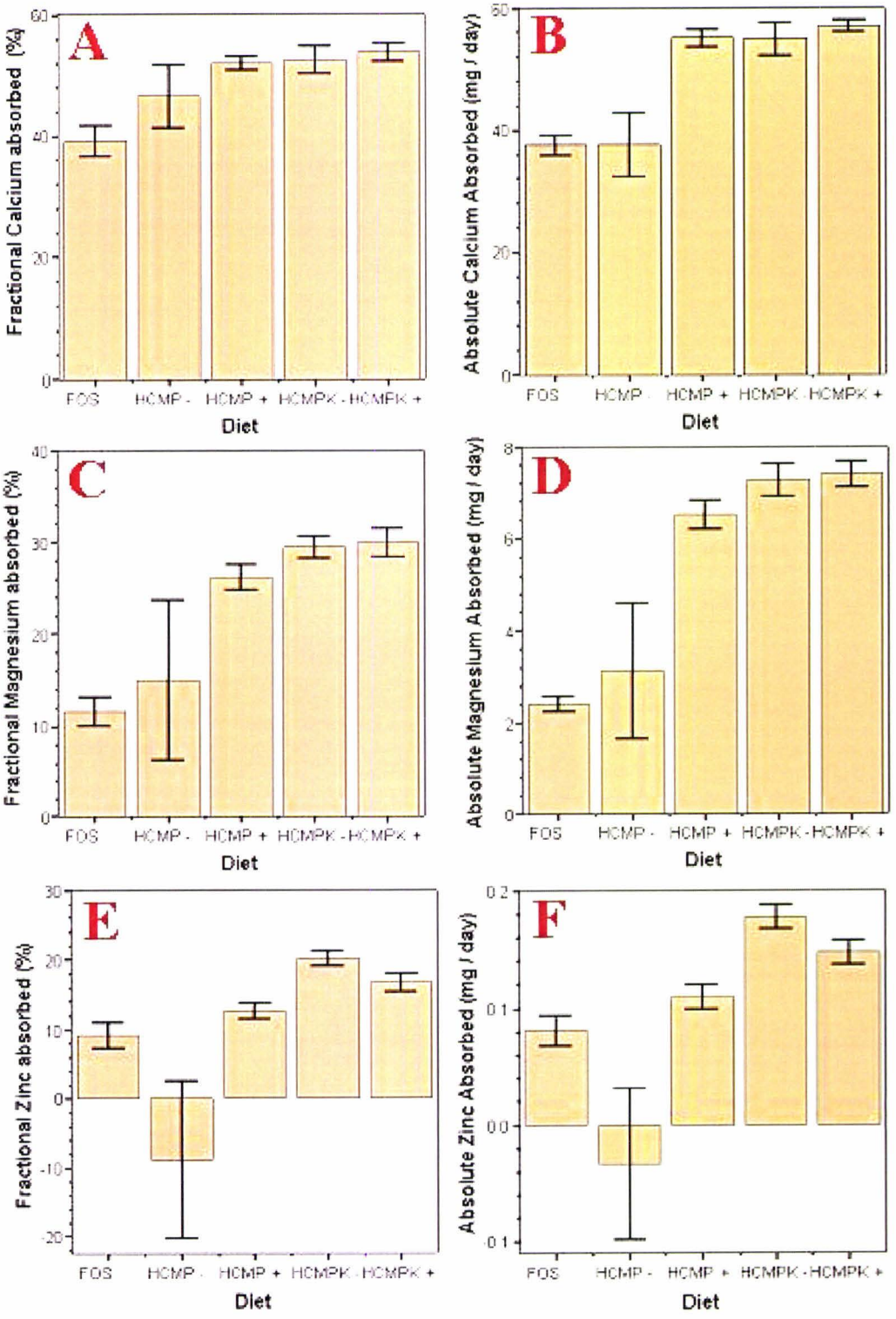

Figure 24: Fractional absorption (graphs A, C and E) and absolute absorption (graphs B, D, and F) of calcium (graphs A and B), magnesium (graphs C and D) and zinc (graphs E and F) in first balance study, following three weeks of feeding trial diets. There were eleven animals in each of the HCMP -, HCMP +, HCMPK – and HCMPK + groups, and twelve in the FOS group.

Table 14: Fractional and absolute absorption of calcium (Ca), magnesium (Mg) and zinc (Zn) in first balance study, following three weeks of feeding trial diet. There were eleven animals in each of the HCMP -, HCMP +, HCMPK – and HCMPK + groups, and twelve in the FOS group. Values with different superscripts (a, b, c) denote significant differences between groups at $p<0.05$ on the same row.

Absorption			FOS (n=12)	HCMP – (n=11)	HCMP + (n=11)	HCMPK – (n=11)	HCMPK + (n=11)
Ca	Fractional (%)	Mean	39.19^{a}	46.60^{ab}	52.14^{b}	52.63^{b}	53.90^{b}
		SEM	2.57	5.34	1.08	2.21	1.37
	Absolute (mg / day)	Mean	37.54^{a}	37.58^{a}	55.1^{b}	55.04^{bc}	57.05^{c}
		SEM	1.56	5.31	1.39	2.70	1.07
Mg	Fractional (%)	Mean	11.62^{a}	15.00^{ab}	26.15^{b}	29.49^{b}	29.91^{b}
		SEM	1.57	8.70	1.40	1.17	1.54
	Absolute (mg / day)	Mean	2.42^{a}	3.14^{a}	6.52^{b}	7.27^{b}	7.42^{b}
		SEM	0.15	1.47	0.30	0.37	0.29
Zn	Fractional (%)	Mean	9.24^{a}	-8.82^{a}	12.73^{a}	20.21^{b}	16.75^{b}
		SEM	1.90	11.42	1.17	1.05	1.34
	Absolute (mg / day)	Mean	0.08^{a}	-0.03^{a}	0.11^{a}	0.18^{b}	0.15^{b}
		SEM	0.013	0.07	0.01	0.01	0.01

FOS rats had the lowest fractional calcium absorption compared to rats in other diet groups; other groups showed similar levels of absorption to one another. HCMP – was not statistically different from FOS. FOS and HCMP – again showed the lowest calcium absolute absorption levels. The highest calcium absorption was observed in animals consuming diets containing vitamin K. Fractional and absolute calcium absorption for groups fed HCMPK – and HCMP + were not statistically different.

Fractional magnesium absorbed was lowest in FOS animals; other groups showed similar levels of absorption to one another. HCMP – was not statistically different from FOS, or any of the other diets. When magnesium absolute absorption was measured, FOS and HCMP – again showed the lowest levels. HCMP +, HCMPK – and HCMPK + showed comparable fractional and absolute absorption levels to one another.

Fractional zinc absorption was lowest in FOS, HCMP – and HCMP + groups, and highest in the diets containing vitamin K (HCMPK – and HCMPK +). This pattern

was also reflected when zinc absolute absorption was measured. HCMP – animals showed negative mean fractional and absolute zinc absorption. They also had the largest SEM, which may indicate that this large value was the result of anomalous zinc balances in a small number of animals. Examination of raw data indicated that four animals displayed a negative zinc balance. However, these animals displayed similar weight gain, food intake, and urinal zinc to other animals in their dietary group. Faecal zinc output was marginally higher in these animals.

Table 15 shows the effects of nine weeks of feeding trial diets on the fractional and absolute absorption of calcium, magnesium and zinc.

Table 15: Absolute and fractional absorption of calcium (Ca), magnesium (Mg) and zinc (Zn) in second balance study, following nine weeks of feeding trial diet. There were twelve animals in each dietary group. Values with different superscripts (a) denote significant differences between groups at $p<0.05$ on the same row.

Absorption			FOS (n=12)	HCMP – (n=12)	HCMP + (n=12)	HCMPK – (n=12)	HCMPK + (n=12)
Ca	Fractional (%)	Mean	26.72[a]	29.264[a]	27.77[a]	29.08[a]	26.84[a]
		SEM	2.021	2.108	1.758	1.828	2.079
	Absolute (mg / day)	Mean	33.61[a]	34.61[a]	34.37[a]	35.34[a]	32.13[a]
		SEM	2.52	2.11	2.71	2.12	2.36
Mg	Fractional (%)	Mean	24.89[a]	25.60[a]	19.90[a]	21.85[a]	21.97[a]
		SEM	1.264	1.868	1.738	1.997	2.254
	Absolute (mg / day)	Mean	7.49[a]	6.99[a]	5.63[a]	6.17[a]	6.11[a]
		SEM	0.40	0.43	0.50	0.58	0.62
Zn	Fractional (%)	Mean	7.873[a]	3.733[a]	3.204[a]	6.379[a]	2.558[a]
		SEM	1.783	3.072	2.412	2.403	3.139
	Absolute (mg / day)	Mean	0.084[a]	0.033[a]	0.034[a]	0.063[a]	0.023[a]
		SEM	0.020	0.031	0.025	0.024	0.032

There appears to be no significant difference between fractional or absolute absorption of the minerals measured after 9 weeks of feeding trial diets. SEMs are reasonably small.

Data from balances were also compared to see whether there was a significant difference on mineral absorption between the two time points. Figure 25 and Table 16 show the results of the analysis.

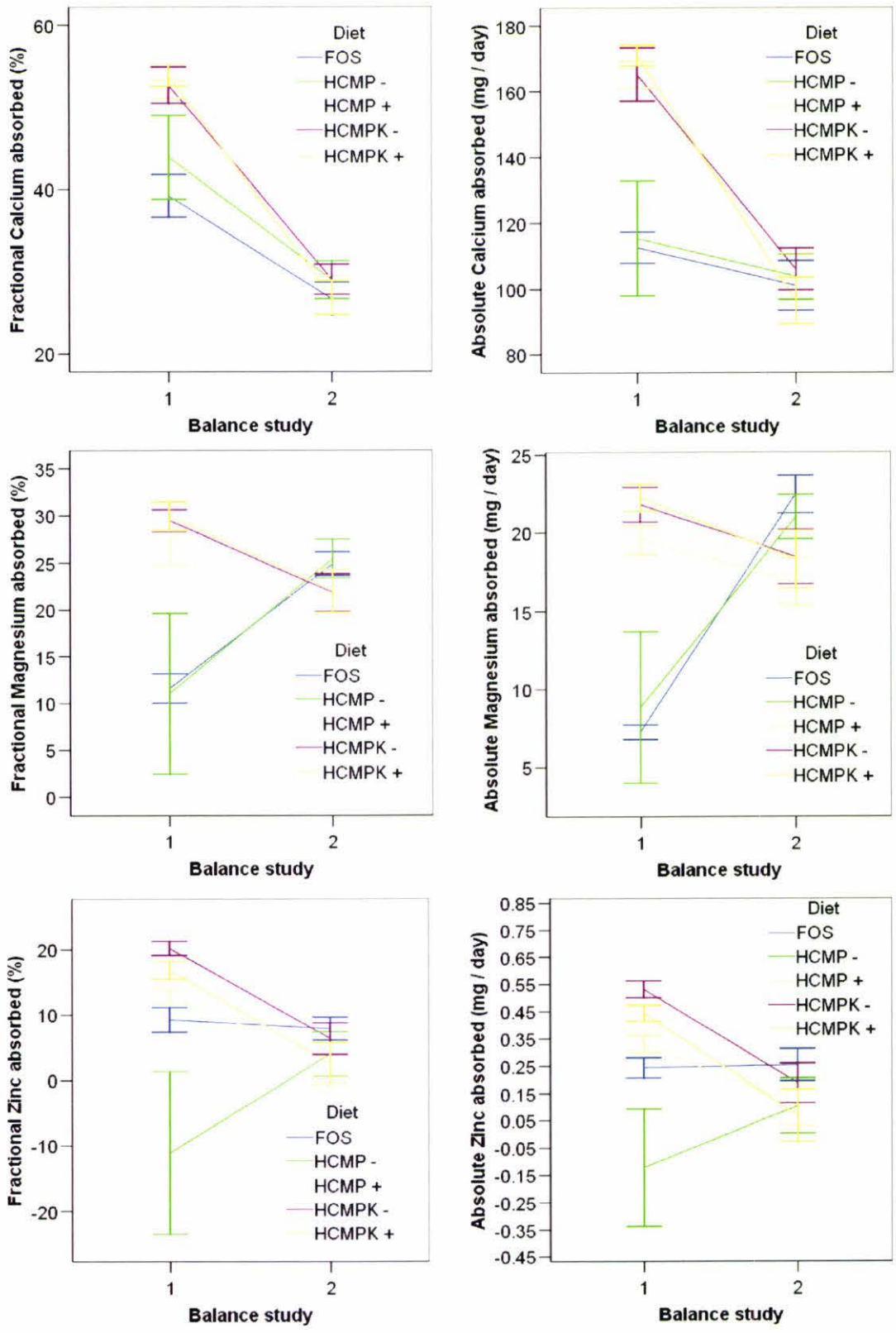

Figure 25: Effect of diet on fractional and absolute absorption of calcium, magnesium and zinc in first and second balance studies. The first balance study was carried out after three weeks, and the second after nine weeks of receiving trial diets. There were eleven animals in each of the HCMP -, HCMP +, HCMPK – and HCMPK + groups, and twelve in the FOS group in the first balance study, and twelve in each dietary group in the second balance study.

Table 16: Comparison of calcium (Ca), magnesium (Mg) and zinc (Zn) absorption in first and second balance studies; the first balance study was carried out after three weeks and the second after nine weeks of receiving trial diets. There were eleven animals in each of the HCMP -, HCMP +, HCMPK – and HCMPK + groups, and twelve in the FOS group. Significance is displayed by sig. NS indicates no significance difference between the two balance studies, * indicates significance of p<0.05 and ** indicates significance of p<0.01.

Absorption			FOS (n=12)	HCMP – (n=11)	HCMP + (n=11)	HCMPK – (n=11)	HCMPK + (n=11)
Ca	Fractional (%)	p-value	<0.01	<0.01	<0.01	<0.01	<0.01
		Sig.	**	**	**	**	**
	Absolute (mg / day)	p-value	0.264	0.384	<0.01	<0.01	<0.01
		Sig.	NS	NS	**	**	**
Mg	Fractional (%)	p-value	<0.01	0.012	0.101	0.038	0.054
		Sig.	**	*	NS	*	NS
	Absolute (mg / day)	p-value	<0.01	<0.01	0.169	0.100	0.096
		Sig.	**	**	NS	NS	NS
Zn	Fractional (%)	p-value	0.806	0.044	0.060	0.013	0.017
		Sig.	NS	*	NS	*	*
	Absolute (mg / day)	p-value	0.931	0.146	0.025	0.003	0.003
		Sig.	NS	NS	*	**	**

Table 16 suggests that there is a highly significant difference between fractional calcium absorbed in all diets between the first and second balance studies. Figure 25 suggests that all diet groups showed a reduction in the second balance. When calcium absolute absorption was measured, however, HCMP – and FOS groups demonstrated no significant difference between balance studies. HCMP +, HCMPK – and HCMPK + showed a highly significant change; Figure 25 suggests animals in these diet groups experienced a decrease in the second balance.

HCMP + and HCMPK + groups showed no significant difference in fractional magnesium absorption. HCMP – and HCMPK – groups showed a significant difference; these were an increase and decrease respectively. Figure 25 and Table 16 suggest that there was a highly significant increase in fractional magnesium absorbed in FOS groups by the second balance study. Magnesium absolute absorption was the same in balance studies for HCMP +, HCMPK – and HCMPK + animals. FOS and HCMP – showed highly significant increases by the second balance.

There was no significant difference observed in fractional zinc absorbed in the HCMP + and FOS groups. HCMP –, HCMPK – and HCMPK + showed a significant difference; Figure 25 suggests the former group experienced an increase, whilst the latter two experienced a decrease in absorption. Where zinc absolute absorption was measured, however, HCMP – and FOS groups showed no difference, with HCMP + showing a significant decrease, and HCMPK – and HCMPK + groups showing a highly significant decrease by the second balance study.

3.3.2. Urinary Mineral Content

Mineral content of urine samples were analysed in order to determine whether there were any differences induced by diet. Data are shown in Table 17. All variables from the first balance study were analysed using non-parametric tests.

Table 17: Calcium (Ca), magnesium (Mg) and zinc (Zn) (mg/day) excreted in urine in first and second balance studies; the first balance study was carried out after three weeks and the second after nine weeks on trial diets. There were eleven animals in each of the HCMP -, HCMP +, HCMPK – and HCMPK + groups, and twelve in the FOS group. Different superscripts (a,b) denote statistical differences between groups on the same row at $p<0.05$.

			FOS (n=12)	HCMP – (n=11)	HCMP + (n=11)	HCMPK – (n=11)	HCMPK + (n=11)
First balance study	Ca (mg/day)	Mean	1.838[a]	0.804[a]	0.929[a]	1.287[a]	1.032[a]
		SEM	0.316	0.134	0.125	0.308	0.222
	Mg (mg/day)	Mean	8.92[a]	6.24[b]	7.15[b]	6.71[b]	6.69[b]
		SEM	0.549	0.716	0.352	0.254	0.381
	Zn (mg/day)	Mean	0.0058[a]	0.0029[a]	0.0042[a]	0.0037[a]	0.0042[a]
		SEM	0.0009	0.0006	0.0008	0.0006	0.0006
Second balance study	Ca (mg/day)	Mean	1.80[a]	1.15[b]	1.49[ab]	1.49[ab]	1.17[b]
		SEM	0.15	0.12	0.19	0.14	0.08
	Mg (mg/day)	Mean	7.53[a]	6.23[a]	7.22[a]	7.78[a]	6.87[a]
		SEM	0.42	0.46	0.52	0.32	0.50
	Zn (mg/day)	Mean	0.0061[a]	0.0049[a]	0.0049[a]	0.0112[b]	0.0064[a]
		SEM	0.0007	0.0004	0.0005	0.0013	0.0007

In the first balance study, no difference was found in calcium excreted in urine as a result of diet. In the second balance study, however, FOS animals showed higher excretion compared to HCMP – and HCMPK + animals (HCMP + and HCMPK –

animals showed statistically similar levels of calcium excretion to all other diet groups). In the first balance study, magnesium excretion was highest in the FOS group compared to other diets. In the second balance study, there was no difference in magnesium excretion between diets. In the first balance study there was no difference in zinc excretion between diets; in the second balance study HCMPK – animals showed statistically higher zinc excretion compared to animals in the other diet groups.

Data were also analysed in order to determine whether there was a difference in urinary mineral excretion between the two balances. Results are shown in Table 18 and Figure 26.

Table 18: Comparison of urinary calcium (Ca) magnesium (Mg) and zinc (Zn) excretion in first and second balance studies; the first balance study was carried out after three weeks and the second after nine weeks on trial diets. There were eleven animals in each of the HCMP -, HCMP +, HCMPK – and HCMPK + groups, and twelve in the FOS group. NS indicates no significance, * indicates significance of $p<0.05$, and ** indicates significance of $p<0.01$.

		FOS (n=12)	HCMP – (n=11)	HCMP + (n=11)	HCMPK – (n=11)	HCMPK + (n=11)
Ca (mg/day)	p-value	0.883	0.190	0.090	0.379	0.548
	Significance	NS	NS	NS	NS	NS
Mg (mg/day)	p-value	0.019	0.898	0.787	0.070	0.789
	Significance	*	NS	NS	NS	NS
Zn (mg/day)	p-value	0.712	0.033	0.394	<0.01	0.011
	Significance	NS	*	NS	**	*

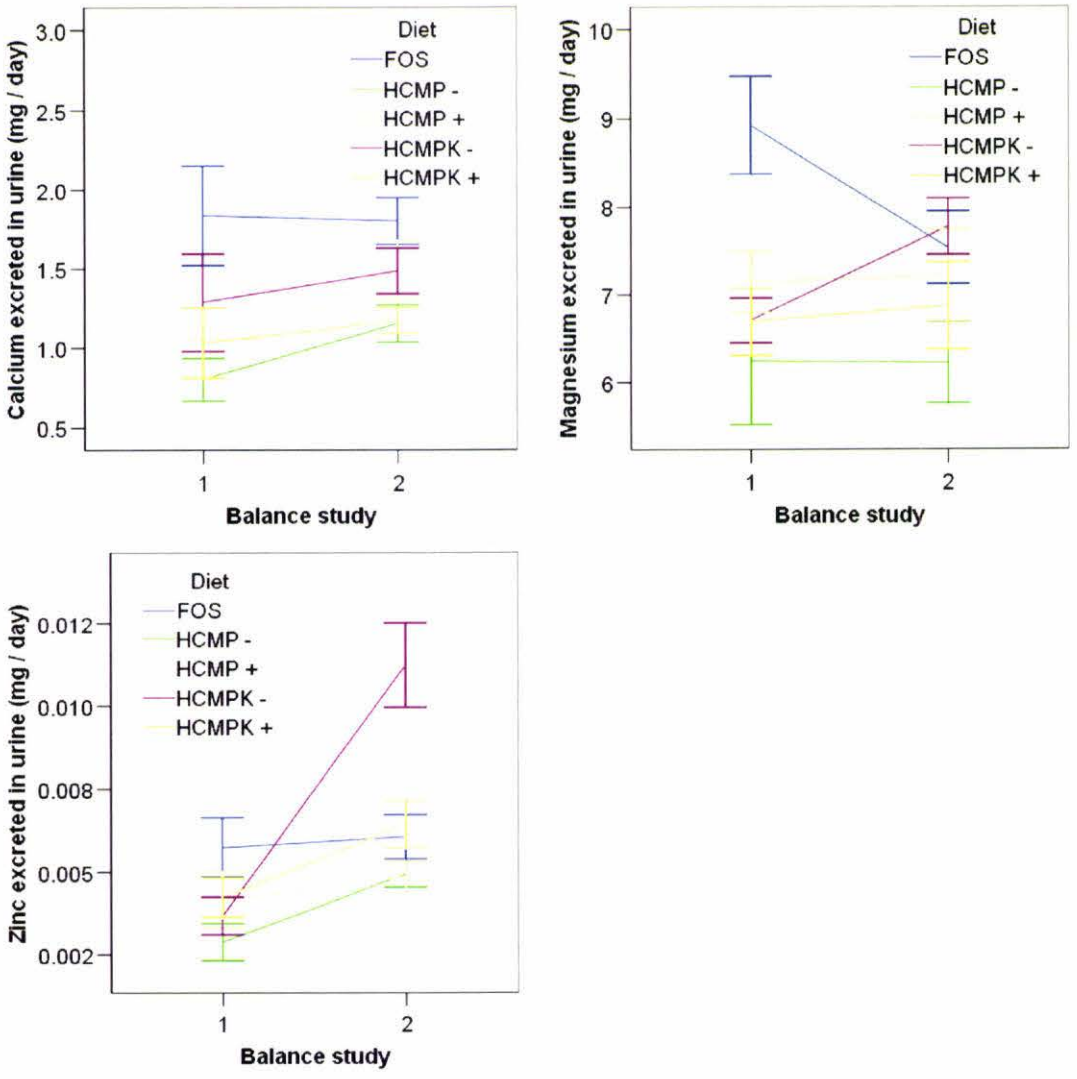

Figure 26: Comparison of urinary calcium (Ca) magnesium (Mg) and zinc (Zn) excretion in first and second balance studies; the first balance study was carried out after three weeks and the second after nine weeks on trial diets. There were eleven animals in each of the HCMP -, HCMP +, HCMPK – and HCMPK + groups, and twelve in the FOS group.

Urinary calcium excretion was not significantly affected by timing of the balance study for any diet. Urinary magnesium excretion was statistically different in only the FOS group between the first and second balance studies; Figure 26 suggests that there was a decrease by the second balance, although magnesium levels were already elevated at the first balance. HCMP – and HCMPK + groups showed a significant difference in urinary zinc excretion between the two balance studies; HCMPK – animals showed a highly significant difference.

3.3.3. Faecal Mineral Content

Mineral contents of faecal samples were analysed in order to determine whether there were any differences induced by diet. Data are shown in Table 19. Faecal weight and magnesium in the first balance study, and zinc from both balances were analysed using non-parametric tests.

Table 19: Calcium (Ca), Magnesium (Mg) and Zinc (Zn) content of faeces, and faecal weight, from animals receiving diets for three weeks (first balance study) and nine weeks (second balance study). There were eleven animals in each of the HCMP +, HCMPK – and HCMPK + groups, ten in the HCMP – group and twelve in the FOS group. Values with different superscripts (a, b) denote significant differences between groups on the same line at $p<0.05$.

			FOS (n=12)	HCMP – (n=10)	HCMP + (n=11)	HCMPK – (n=11)	HCMPK + (n=11)
1st balance study	Faecal weight (g/day)	Mean	2.34[a]	1.76[b]	1.91[b]	1.82[b]	2.00[b]
		SEM	0.15	0.10	0.06	0.08	0.06
	Ca (mg/day)	Mean	59.49[a]	44.45[b]	49.70[ab]	48.38[ab]	48.12[ab]
		SEM	4.69	2.78	1.46	2.81	2.08
	Mg (mg/day)	Mean	11.04[a]	10.15[a]	11.39[a]	10.65[a]	10.87[a]
		SEM	0.896	0.516	0.394	0.411	0.317
	Zn (mg/day)	Mean	0.987[a]	0.719[b]	0.746[b]	0.698[b]	0.739[b]
		SEM	0.052	0.050	0.013	0.021	0.023
2nd balance study	Faecal weight (g/day)	Mean	2.50[a]	2.34[a]	2.38[a]	2.40[a]	2.41[a]
		SEM	0.12	0.12	0.08	0.08	0.09
	Ca (mg/day)	Mean	91.29[a]	85.16[a]	86.71[a]	85.10[a]	87.24[a]
		SEM	3.744	4.871	2.299	3.000	3.464
	Mg (mg/day)	Mean	15.16[a]	14.62[a]	15.46[a]	14.31[a]	14.96[a]
		SEM	0.670	0.878	0.522	0.565	0.592
	Zn (mg/day)	Mean	0.987[a]	0.939[a]	0.951[a]	0.918[a]	0.952[a]
		SEM	0.031	0.055	0.022	0.031	0.042

In the first balance study, when compared on a daily excretion basis, calcium content was significantly higher in the FOS group than the HCMP - group. Magnesium excretion was not significantly altered as a result of dietary intervention. Zinc excreted in faeces was significantly higher in FOS animals. Faecal weight was significantly higher in FOS animals. In the second balance study, there was no difference between daily excretion of calcium, magnesium or zinc. Faecal weight also showed no signicant difference between groups.

Data were also analysed in order to determine whether there was a difference in faecal mineral excretion between the two balances. Results are shown in Figure 27 and Table 20.

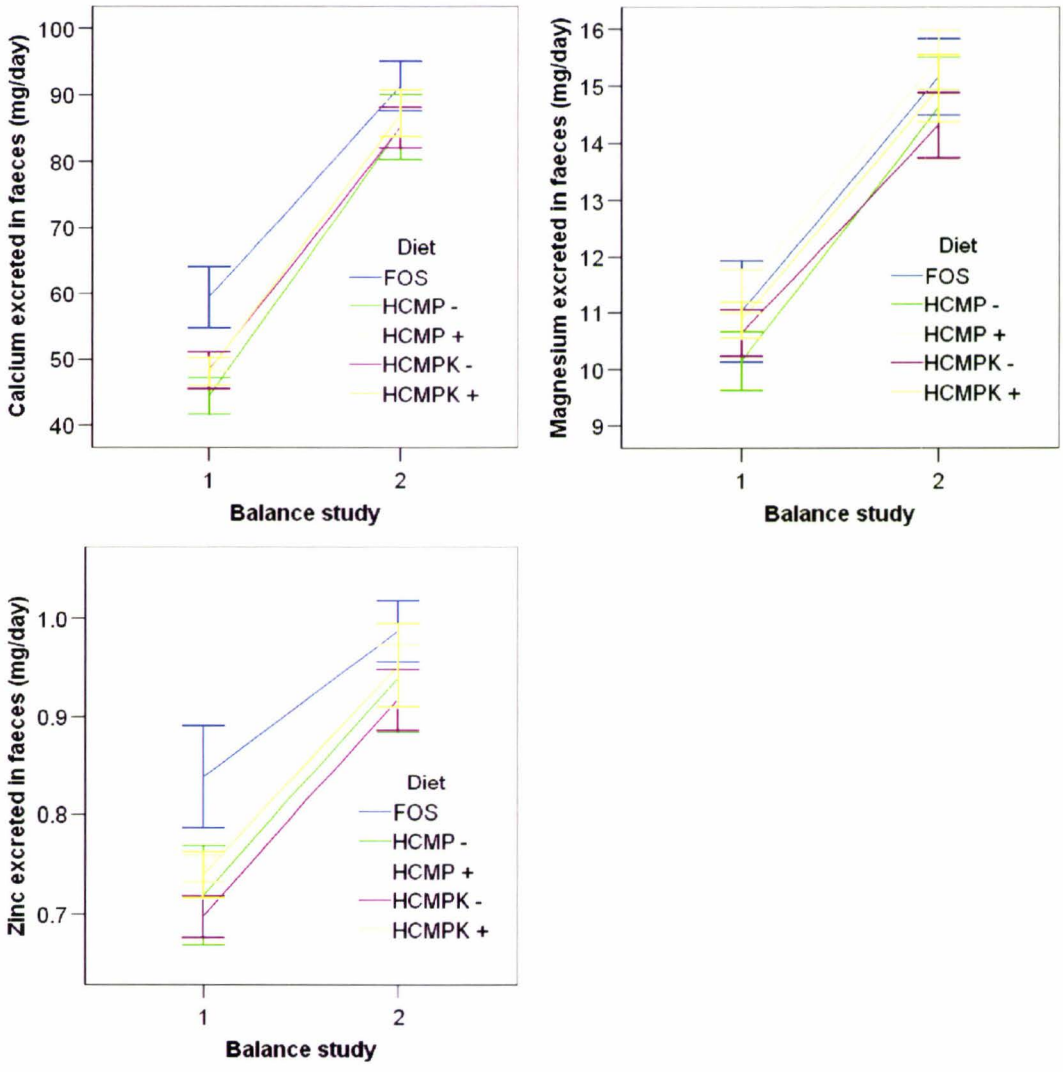

Figure 27: Comparison of calcium (Ca) magnesium (Mg) and zinc (Zn) excretion in faeces, and faecal weight during first and second balance studies; the first balance study was carried out after three weeks and the second after nine weeks on trial diets. There were eleven animals in each of the HCMP -, HCMP +, HCMPK – and HCMPK + groups, and twelve in the FOS group.

Table 20: Comparison of calcium (Ca), magnesium (Mg) and zinc (Zn) faecal excretion, and faecal weight in the first and second balance studies; the first balance study was carried out after three weeks and the second after nine weeks of receiving trial diets. There were eleven animals in each of the HCMP +, HCMPK – and HCMPK + groups, ten in the HCMP - group and twelve in the FOS group. Significance is displayed by sig. NS indicates no significance difference between the two balance studies, * indicates significance of p<0.05 and ** indicates significance of p<0.01.

		FOS (n=12)	HCMP – (n=10)	HCMP + (n=11)	HCMPK – (n=11)	HCMPK + (n=11)
Faecal weight (g/day)	p-value	<0.01	<0.01	<0.01	<0.01	<0.01
	Significance	**	**	**	**	**
Ca (mg/day)	p-value	<0.01	<0.01	<0.01	<0.01	<0.01
	Significance	**	**	**	**	**
Mg (mg/day)	p-value	<0.01	<0.01	<0.01	<0.01	<0.01
	Significance	**	**	**	**	**
Zn (mg/day)	p-value	<0.01	<0.01	<0.01	<0.01	<0.01
	Significance	**	**	**	**	**

All dietary groups showed a highly significant increase in faecal mineral excretion by the second balance study, expressed as mg / day, and faecal weight (g / day). This is as would be expected, as food intake also increased between the two balances (see 3.2.1).

3.3.4. Faecal Weight

Pearson's correlation coefficients were calculated between dry faecal weight, feed intake and rat weight during the balance studies. These are shown in Table 21.

Table 21: Pearson's correlation coefficients and p-values for the effect of food intake and rat weight on faecal weight (g/3days), in first (after two weeks on trial diets) and second balance studies (after nine weeks on trial diets). There were eleven animals in each of the HCMP -, HCMP +, HCMPK – and HCMPK + groups, and twelve in the FOS group. ** indicate significance of p<0.05.

		Food Intake	Rat Weight
Faecal weight 1st balance	Pearsons corr.	0.658**	0.592**
	p-value	<0.01	<0.01
Faecal weight 2nd balance	Pearsons corr.	0.630**	0.363**
	p-value	<0.01	<0.01

In order to determine whether these values were a result of a relationship or erroneous analysis, scattergraphs were plotted, shown in Figure 28.

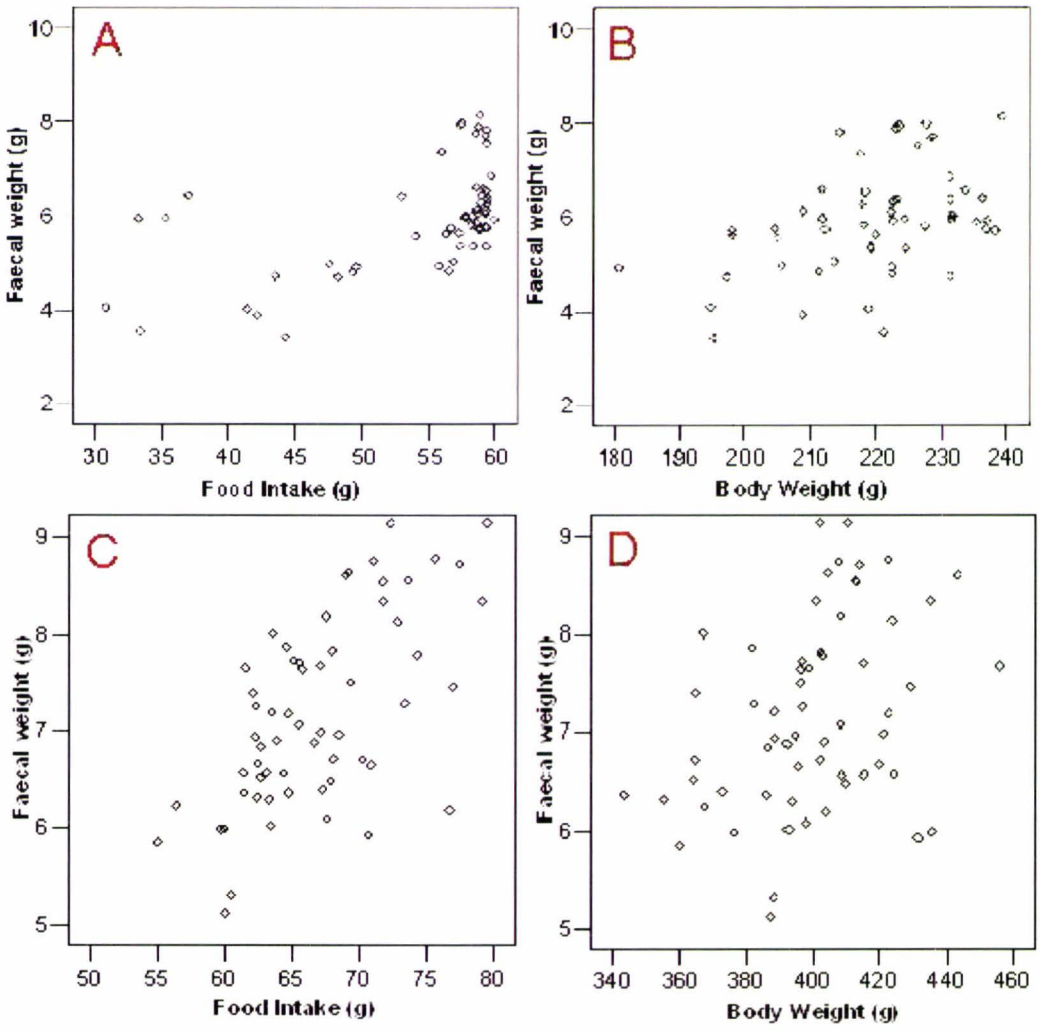

Figure 28: Effect of food intake (graphs A and C) and body weight (graphs B and D) on dry faecal weight. Graphs A and B show data from the first balance study (after three weeks on trial diets); C and D show data from the second balance study (after nine weeks on trial diets). There were eleven animals in each of the HCMP -, HCMP +, HCMPK – and HCMPK + groups, and twelve in the FOS group.

Data from both balance studies appear to show a linear relationship between daily food intake and dry faecal weight, but no clear relationship between body weight and dry faecal weight. Hence dry faecal weights were analysed using food intake as a covariate. Faecal weight from the first balance study was analysed using non-parametric tests. Results of the analyses are shown in Table 22.

Table 22: Analysis of faecal weight in first and second balance study, after three and nine weeks respectively, on trial diets. There were eleven animals in each of the HCMP -, HCMP +, HCMPK – and HCMPK + groups, and twelve in the FOS group. Values with different superscripts (a, b, c) denote significant differences between groups on the same row, at $p<0.05$.

			FOS (n=12)	HCMP – (n=11)	HCMP + (n=11)	HCMPK – (n=11)	HCMPK + (n=11)
Faecal weight (g)	1st Balance study	Mean	7.01[a]	5.28[b]	5.74[b]	5.45[b]	6.01[b]
		SEM	0.45	0.29	0.18	0.23	0.18
	2nd balance study	Mean	7.48[a]	6.92[a]	7.15[a]	7.20[a]	7.22[a]
		SEM	0.35	0.33	0.23	0.25	0.26

Figure 29 shows the dry faecal weights from animals in both balance studies; data are classified according to diet groups.

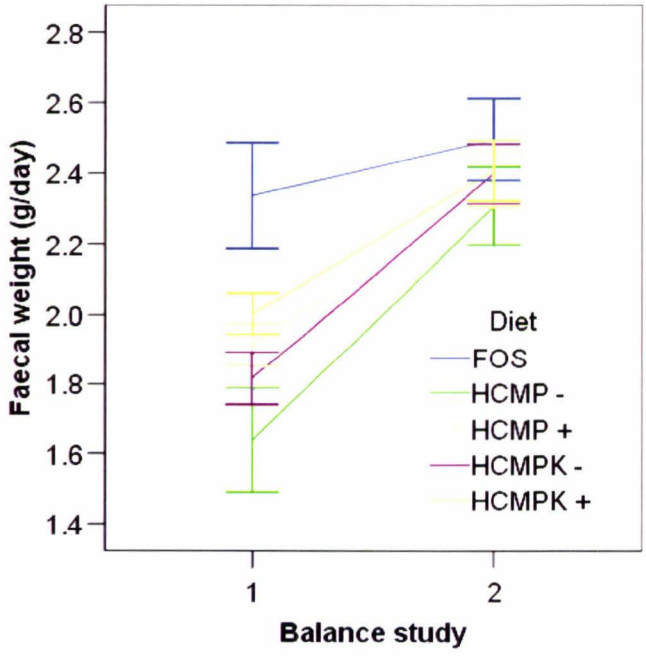

Figure 29: Comparison of effect of diet of faecal weight (g/day) in first and second balance studies. Animals are classified by the diets they received. There were eleven animals in each of the HCMP -, HCMP +, HCMPK – and HCMPK + groups, and twelve in the FOS group. The first balance study was carried out after three weeks, and the second after nine weeks of receiving trial diets.

Table 23 lists the p-values for comparison of faecal weight in first and second balance studies.

Table 23: Comparison of faecal weight in first and second balance studies between animals consuming different diets. The first balance study was carried out after three weeks, and the second after nine weeks of receiving trial diets. There were eleven animals in each of the HCMP –, HCMP +, HCMPK – and HCMPK + groups, and twelve in the FOS group. NS indicates no significance and ** indicates significance of p<0.01.

		FOS (n=12)	HCMP – (n=11)	HCMP + (n=11)	HCMPK – (n=11)	HCMPK + (n=11)
Faecal weight	p-value	0.207	<0.01	<0.01	<0.01	<0.01
	Significance	NS	**	**	**	**

There was no significant difference between faecal weight in balances for animals in the FOS group; all other diet groups showed a highly significant difference ($p<0.01$). Faecal weight increased between the first and second balance studies (Figure 29).

3.4. Blood / Serum Analysis

3.4.1. Type I Collagen c-Terminal Telopeptides

There were no significant differences between groups for serum type I collagen c-terminal telopeptides (CTx) concentrations. Rat weight was not significantly correlated with serum CTx concentrations (data not shown); hence CTx values were analysed assuming there were no confounders. Data are listed in Table 24.

Table 24: Serum Type I collagen c-telopeptide (CTx) concentrations following 10 weeks of feeding trial diets. There were nine animals receiving FOS, eleven receiving HCMP –, HCMP+ and HCMPK +, and ten receiving HCMPK – (due to errors during analysis). Values with different superscripts (a) denote significant differences between groups at p<0.05.

		FOS (n=9)	HCMP – (n=11)	HCMP + (n=11)	HCMPK – (n=10)	HCMPK + (n=11)
CTx (ng/ml)	Mean	17.94[a]	21.46[a]	20.99[a]	18.20[a]	18.53[a]
	SEM	2.47	1.45	2.00	0.84	0.83

3.4.2. IL-6

There were no significant differences between groups in terms of IL-6 levels (data not shown). Most of the results were below the detection limit of 125 pg/ml, except

for samples taken from one HCMP – animal, two HCMP +, four FOS and two HCMPK – animals.

3.4.3. IL-10

There were no significant differences between diet groups in terms of IL-10 levels (data not shown). Most of the data were below the detection limit of 62.5 pg/ml, except for one HCMPK – sample.

3.5. Bone Analysis

3.5.1. Dual Energy X-ray Absorptiometry Scans

Body weight was found to be highly significantly correlated to spine and femur area, BMC and BMD (correlation coefficients shown in Table 25).

Table 25: Pearsons correlation coefficients for *ex vivo* DEXA measurements on femurs of sixty rats, following 10 weeks of feeding trial diets. * indicates significance at the 0.05 level; ** indicates significance at the 0.01 level.

		Rat weight
Spine Area	Pearsons corr.	0.560**
	p-value	<0.001
Spine BMC	Pearsons corr.	0.524**
	p-value	<0.001
Spine BMD	Pearsons corr.	0.402**
	p-value	0.001
Femur Area	Pearsons corr.	0.375**
	p-value	0.003
Femur BMC	Pearsons corr.	0.304*
	p-value	0.018
Femur BMD	Pearsons corr.	0.462**
	p-value	<0.001

In order to determine whether these Pearson's correlation coefficients were indicative of a relationship or due to erroneous analysis, scattergraphs were plotted, shown in Figure 30.

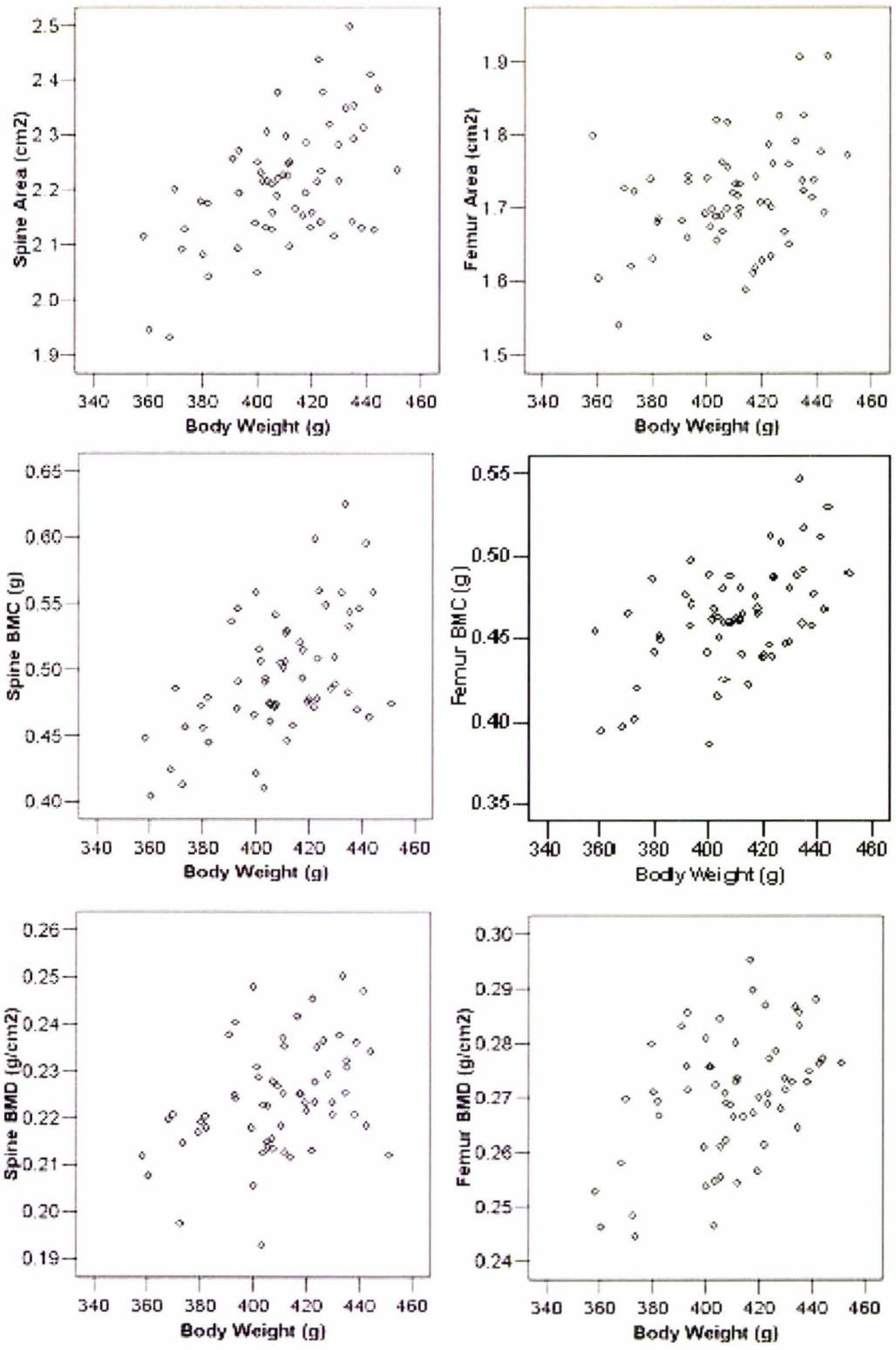

Figure 30: Effect of body weight on spine and femur area, bone mineral content (BMC) and bone mineral density (BMD). Measurements were made using *ex vivo* DEXAs after 10 weeks of receiving trial diets, on sixty rats.

Figure 30 and Table 25 suggest that there is a linear relationship between rat weight and spine/femur area, BMC and BMD. These parameters were therefore corrected

for rat weight during analysis. Femur BMC was analysed using non-parametric testing. There was no significant difference between the study groups for any of the parameters measured. Table 26 lists the results.

Table 26: *Ex vivo* spine and femur area, bone mineral content (BMC) and densities (BMD) of rats, following 10 weeks of feeding trial diets. There were twelve animals per dietary group. Values with different superscripts (a) denote significant differences between groups in the same row at $p<0.05$.

		FOS (n=12)	HCMP – (n=12)	HCMP + (n=12)	HCMPK – (n=12)	HCMPK + (n=12)
Spine Area (cm^2)	Mean	2.216[a]	2.196[a]	2.212[a]	2.202[a]	2.219[a]
	SEM	0.028	0.028	0.039	0.042	0.022
Spine BMC (g)	Mean	0.497[a]	0.490[a]	0.501[a]	0.492[a]	0.499[a]
	SEM	0.014	0.012	0.016	0.016	0.009
Spine BMD (g/cm^2)	Mean	0.224[a]	0.223[a]	0.226[a]	0.223[a]	0.225[a]
	SEM	0.004	0.003	0.003	0.004	0.003
Femur Area (cm^2)	Mean	1.720[a]	1.715[a]	1.708[a]	1.698[a]	1.720[a]
	SEM	0.015	0.018	0.028	0.027	0.021
Femur BMC (g)	Mean	0.465[a]	0.463[a]	0.467[a]	0.455[a]	0.467[a]
	SEM	0.009	0.008	0.011	0.011	0.008
Femur BMD (g/cm^2)	Mean	0.271[a]	0.270[a]	0.272[a]	0.268[a]	0.272[a]
	SEM	0.004	0.003	0.003	0.003	0.004

3.5.2. Bone Biomechanics

Rat weight, and femur width and length were found to be correlated to several of the parameters measured in biomechanical testing; these are listed in Table 27.

Table 27: Correlation between rat weight, femur weight and length, and the biomechanical parameters measured after ten weeks of trial diets being consumed by sixty rats. * indicates significance at the 0.05 level; ** indicates significance at the 0.01 level; ^{ns} indicates no significance.

		Rat weight	Femur weight	Femur length
Max Load	Pearsons corr.	0.279*	0.218^{ns}	0.201^{ns}
	p-value	0.039	0.110	0.142
Max Stroke	Pearsons corr.	0.208^{ns}	0.334*	0.304*
	p-value	0.127	0.013	0.024
Break Load	Pearsons corr.	0.189^{ns}	0.188^{ns}	0.226^{ns}
	p-value	0.166	0.170	0.097
Break Stress	Pearsons corr.	-0.063^{ns}	-0.158^{ns}	-0.106^{ns}
	p-value	0.650	0.248	0.440
Break Stroke	Pearsons corr.	0.287*	0.296*	0.285*
	p-value	0.033	0.028	0.035
Break Strain	Pearsons corr.	0.358**	0.370**	0.361**
	p-value	0.007	0.005	0.007
Elastic	Pearsons corr.	-0.166^{ns}	-0.373**	-0.334*
	p-value	0.224	0.005	0.013
Energy	Pearsons corr.	0.341*	0.291*	0.288*
	p-value	0.011	0.031	0.033

In order to determine whether the correlation coefficients were indicative of a relationship, or due to erroneous analysis, scattergraphs were plotted, and are shown in Figure 31 and Figure 32.

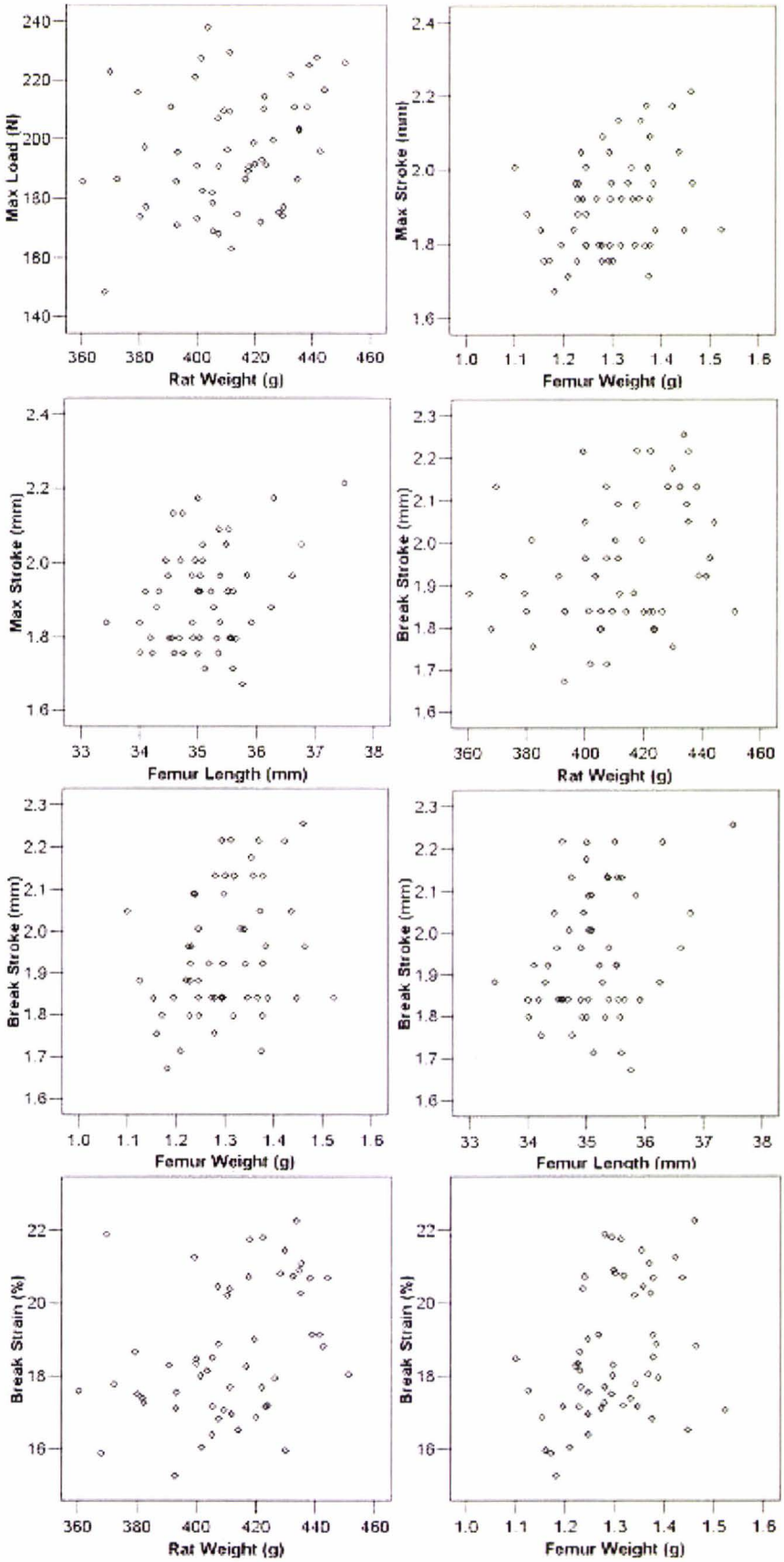

Figure 31: Relationship between rat weight, femur weight and length and bone biomechanical parameters (maximum load, maximum stroke, break stroke and break strain). Measurements were made on femurs of sixty rats who had consumed trial diets for ten weeks. Measurements were made after ten weeks of receiving trial diets.

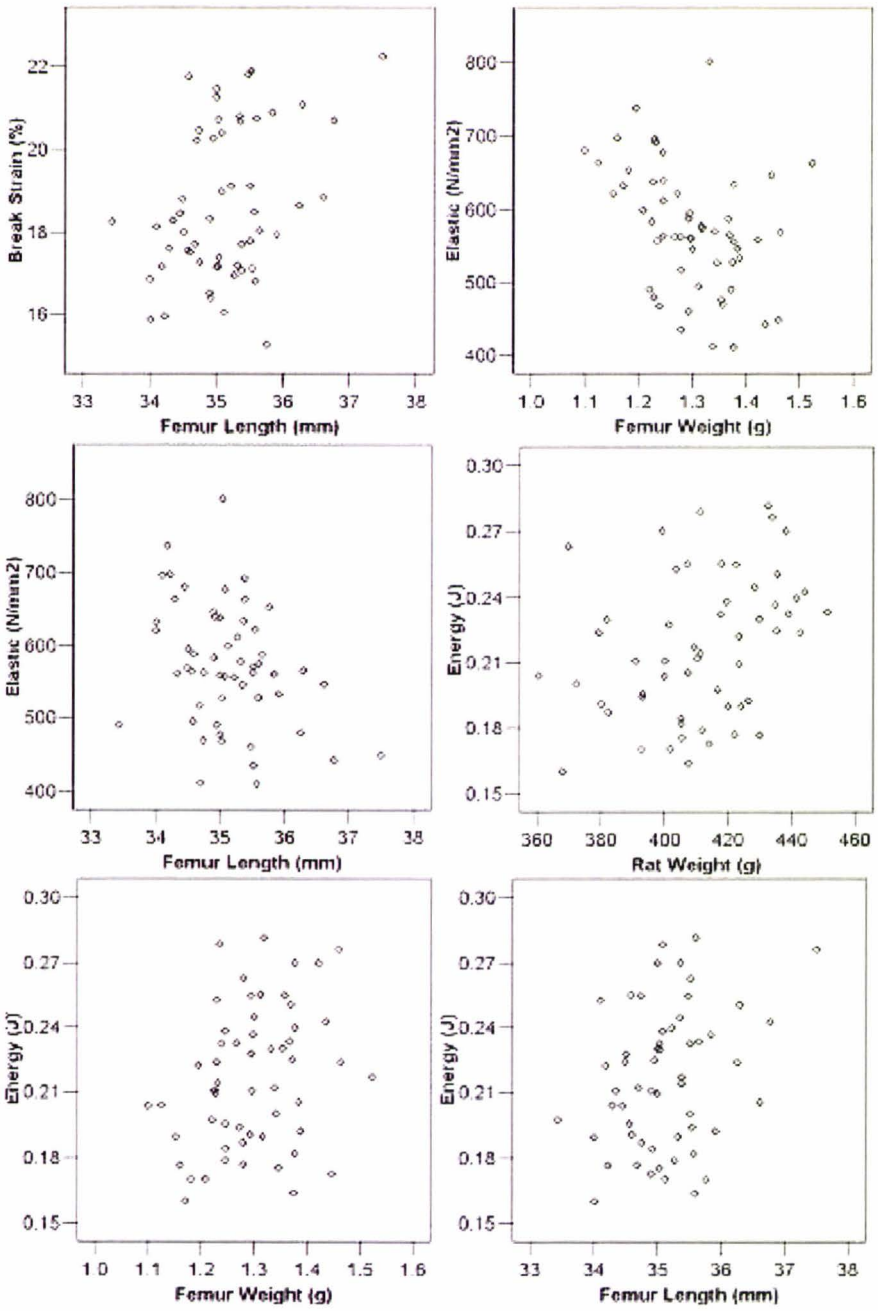

Figure 32: Relationship between rat weight, femur weight and length and bone biomechanical parameters (break strain, elasticity, and energy required to break bones). Measurements were made on femurs of sixty rats who had consumed trial diets for ten weeks.

Figure 31 and Figure 32 suggest that some of the supposed correlations may be incorrect. For simplicity, Table 28 summarises the correlations that may exist.

Table 28: Confounders of biomechanical parameters, used as covariates during analysis of variance. Significance is determined using the Pearson's Correlation coefficients in Table 27, and examination of scattergraphs in Figure 31 and Figure 32. NS indicates no significance, † indicates possible confounder.

	Rat weight	Femur weight	Femur length
Max Load	NS	NS	NS
Max Stroke	NS	†	†
Break Load	NS	NS	NS
Break Stress	NS	NS	NS
Break Stroke	NS	NS	NS
Break Strain	NS	NS	NS
Elastic	NS	†	NS
Energy	NS	NS	NS

Whilst statistical analysis suggested that many of these variables may influence the biomechanical parameters measured, Table 28 suggests that there may only be three confounders. Therefore, femur weight and length were used as covariates in the analysis of maximum stroke, and femur weight used for elasticity measurements. Results of biomechanical tests are shown in Table 29.

Table 29: Results of biomechanical tests on right femurs following 10 weeks of feeding study diets. There were twelve rats per dietary group. Values with different superscripts (a,b) denote significant differences between groups in the same row at p<0.05.

		FOS (n=11)	HCMP – (n=11)	HCMP + (n=11)	HCMPK – (n=11)	HCMPK + (n=11)
Max Load (N)	Mean	194.08[a]	197.22[a]	194.98[a]	198.99[a]	193.69[a]
	SEM	5.26	4.35	5.73	8.30	6.83
Max Stroke (mm)	Mean	1.93[a]	1.91[a]	1.89[a]	1.89[a]	1.90[a]
	SEM	0.05	0.04	0.04	0.04	0.04
Break Load (N)	Mean	189.60[a]	189.82[a]	194.08[a]	198.29[a]	186.86[a]
	SEM	5.07	6.26	5.81	8.17	8.22
Break Stress (N/mm^2)	Mean	73.62[a]	74.70[a]	76.27[a]	80.47[a]	67.83[a]
	SEM	3.13	3.85	2.83	2.47	3.00
Break Stroke (mm)	Mean	1.96[a]	1.97[a]	1.93[a]	1.90[a]	1.98[a]
	SEM	0.06	0.05	0.05	0.04	0.05
Break Strain (%)	Mean	18.85[a]	18.72[a]	18.43[a]	17.89[a]	19.31[a]
	SEM	0.58	0.59	0.52	0.47	0.59
Elastic (N/mm^2)	Mean	561.47[ab]	585.65[ab]	584.11[ab]	621.60[a]	510.75[b]
	SEM	25.61	29.28	25.24	17.12	20.24
Energy (J)	Mean	0.22[a]	0.22[a]	0.21[a]	0.21[a]	0.22[a]
	SEM	0.01	0.01	0.01	0.01	0.01

There was a highly significant difference between HCMPK – and HCMPK + for bone elasticity ($p = 0.017$). There were no significant differences between diets for any of the other biomechanical parameters measured.

3.5.3. Bone Ash Content

Of the parameters measured during analysis of bone ash content, rat weight and bone length were highly significant predictors of dry bone weight, and ashed bone weight ($p<0.01$); bone length was a highly significant predictor of non-mineral bone matter ($p<0.01$). Pearson's correlation coefficients are shown in Table 30.

Table 30: Pearson's correlation coefficients for measurements of wet, dry and ashed femurs following 10 weeks of feeding trial diets to sixty rats. * indicates significance at the 0.05 level; ** indicates significance at the 0.01 level; ns indicates no significance.

		Rat weight	Bone length
Bone length	Pearsons corr.	0.225^{ns}	
	p-value	0.084	
Dry weight	Pearsons corr.	0.471**	0.729**
	p-value	0.000	0.000
Ashed weight	Pearsons corr.	0.514**	0.598**
	p-value	0.000	0.000
Non-mineral bone matter	Pearsons corr.	0.234^{ns}	0.603**
	p-value	0.072	0.000
Ratio non-mineral:mineral bone matter	Pearsons corr.	-0.123^{ns}	0.201^{ns}
	p-value	0.348	0.123

Where Pearson's correlation coefficients suggested a relationship between two variables may exist, scattergraphs were plotted to determine whether this was indeed the case, or whether it was simply the result of erroneous analysis; these are shown in Figure 33.

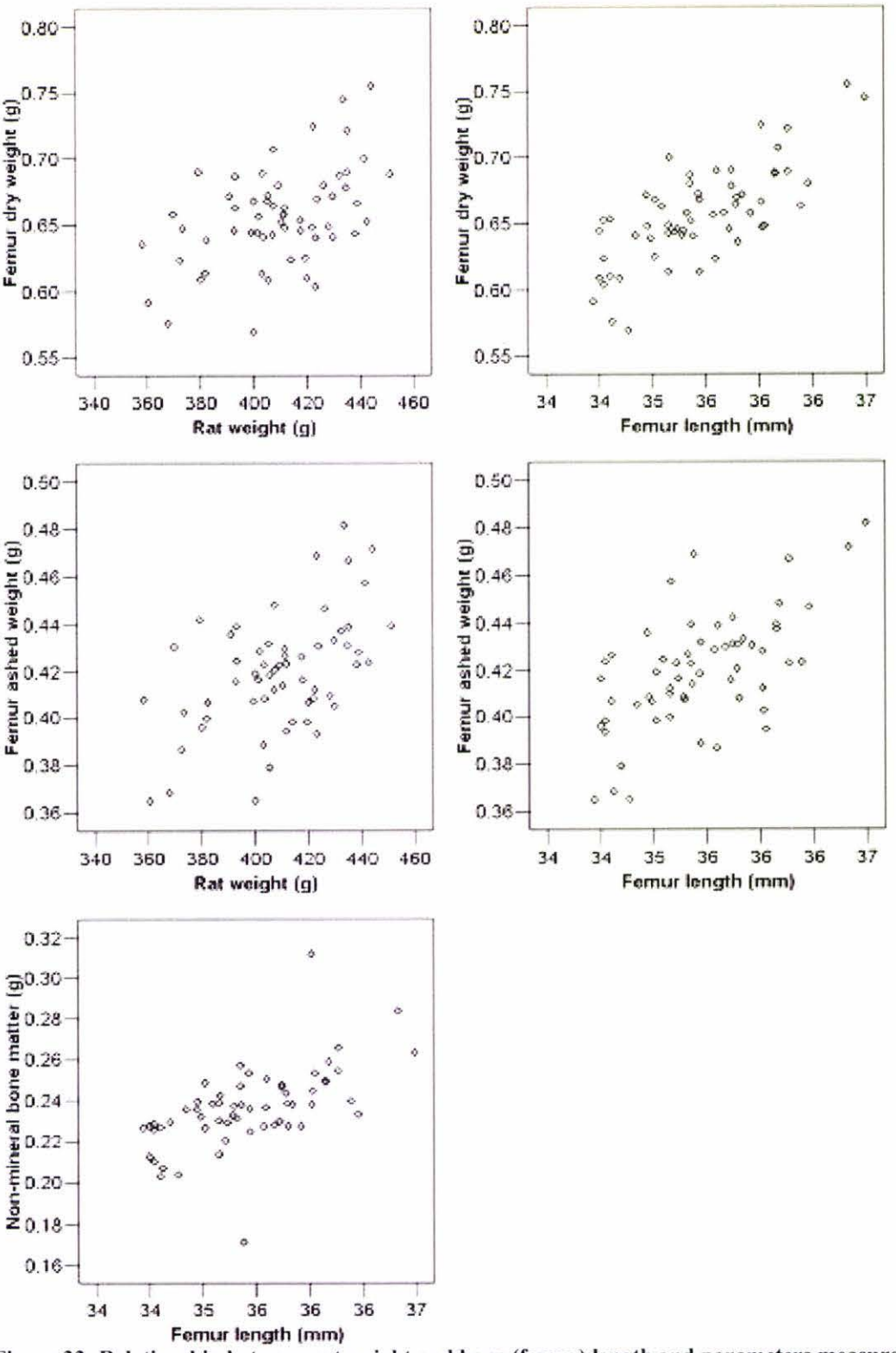

Figure 33: Relationship between rat weight and bone (femur) length and parameters measured during ashed bone analysis (dry weight, ashed weight and non-mineral bone matter). Measurements were made on sixty rats who had consumed trial diets for ten weeks.

Figure 33 confirms that the suspected correlations between the parameters may indeed exist; they were therefore used as confounders during analysis of the effect of the trial diets on bone weight and composition. Table 31 lists the results of analyses.

Table 31: Weight and composition of rat femurs following 10 weeks of feeding trial diets; there were twelve animals per dietary group. Values with different superscripts (a) denote significant differences between groups in the same row at $p<0.05$.

		FOS (n=12)	HCMP − (n=12)	HCMP + (n=12)	HCMPK − (n=12)	HCMPK + (n=11)
Bone Length (mm)	Mean	35.293[a]	35.604[a]	35.408[a]	35.262[a]	35.605[a]
	SEM	0.149	0.149	0.230	0.185	0.177
Dry Bone Weight (mm)	Mean	0.657[a]	0.655[a]	0.650[a]	0.654[a]	0.664[a]
	SEM	0.011	0.009	0.012	0.012	0.011
Ashed Bone Weight (g)	Mean	0.418[a]	0.419[a]	0.423[a]	0.413[a]	0.423[a]
	SEM	0.006	0.006	0.009	0.008	0.007
Non-Mineral Bone Matter[†] (g)	Mean	0.239[a]	0.236[a]	0.227[a]	0.240[a]	0.240[a]
	SEM	0.008	0.004	0.007	0.005	0.005
Ratio Non-Mineral : Mineral Bone Matter[*]	Mean	0.572[a]	0.564[a]	0.537[a]	0.582[a]	0.568[a]
	SEM	0.019	0.010	0.016	0.008	0.009

[†] Non-mineral bone matter calculated by subtracting ashed bone weight from dry bone weight

[*] Ratio calculated by dividing non-mineral bone matter by ashed bone matter

There appears to be no significant difference between diets and any of the ashed bone parameters measured.

3.5.4. Bone Mineral Content

Table 32 shows the effect of diet on calcium, magnesium and zinc content of bone.

Table 32: Effect of diet on bone mineral content of rats fed trial diets for ten weeks; there were twelve animals per dietary group. Values with different superscripts (a) denote significant differences between groups in the same row at $p<0.05$.

		FOS (n=12)	HCMP – (n=12)	HCMP + (n=12)	HCMPK – (n=12)	HCMPK + (n=12)
Calcium (mg)	Mean	119.07[a]	120.47[a]	122.27[a]	119.27[a]	122.20[a]
	SEM	2.31	1.68	2.43	2.57	2.34
Magnesium (mg)	Mean	2.21[a]	2.17[a]	2.20[a]	2.14[a]	2.21[a]
	SEM	0.05	0.04	0.07	0.05	0.05
Zinc (mg)	Mean	1.29[a]	1.30[a]	1.32[a]	1.33[a]	1.33[a]
	SEM	0.03	0.03	0.03	0.04	0.03

There were no significant differences in calcium, magnesium or zinc content of bone after ten weeks of feeding trial diets.

Mineral content ratio to ashed weight was also calculated, and is shown in Table 33.

Table 33: Effect of diet on ratio of mineral to ash in femurs of rats fed trial diets for ten weeks; there were twelve animals per dietary group. Values with different superscripts (a) denote significant differences between groups in the same row at $p<0.05$.

		FOS (n=12)	HCMP – (n=12)	HCMP + (n=12)	HCMPK – (n=12)	HCMPK + (n=12)
Calcium	Mean	0.2858[a]	0.2875[a]	0.2892[a]	0.2883[a]	0.2908[a]
	SEM	0.0031	0.0022	0.0023	0.0021	0.0031
Magnesium	Mean	0.0053[a]	0.0052[a]	0.0052[a]	0.0052[a]	0.0052[a]
	SEM	0.0001	0.0001	0.0001	0.0001	0.0001
Zinc	Mean	0.0031[a]	0.0031[a]	0.0031[a]	0.0032[a]	0.0032[a]
	SEM	0.0001	0.0001	0.0001	0.0001	0.0001

There were no significant differences in the ratio of calcium, magnesium or zinc and ash content.

3.6. Gut Bacteriology

Caecum counts showed a significant difference between levels of *Lactobaccillus* spp.; results are shown in Table 34. Data were analysed using non-parametric tests. It should be noted, however, that the CFUs recorded reflect all *Lactobaccilus* species, and not solely *Lactobaccilus rhamnosus*, let alone DR20™. Therefore counts for animals on the FOS diet will be complicated by the fact that FOS, being a prebiotic, would be

expected to increase the level of all probiotics, including *Lactobaccilus rhamnosus* and DR20™.

Table 34: Caecum counts of Colony Forming Units (CFUs) of animals fed different diets for ten weeks. There were twelve animals per dietary group. Values with different superscripts (a,b) denote significant differences between groups at p<0.05.

Diet	FOS (n=12)	HCMP – (n=12)	HCMP + (n=12)	HCMPK – (n=12)	HCMPK + (n=12)
Mean	$5.5 \times 10^{6\,a}$	$1.1 \times 10^{6\,ab}$	$6.3 \times 10^{5\,b}$	$9.1 \times 10^{5\,b}$	$1.5 \times 10^{6\,ab}$
SEM	3.4×10^{6}	2.0×10^{5}	1.8×10^{5}	2.9×10^{5}	4.4×10^{5}

Rats fed FOS had the highest caecum counts of *Lactobaccilus* CFUs. HCMP +, HCMPK –, HCMP – and HCMPK + had comparably lower counts, although the counts of the latter two diet groups were not statistically different from the FOS diet. Data are displayed in Figure 34.

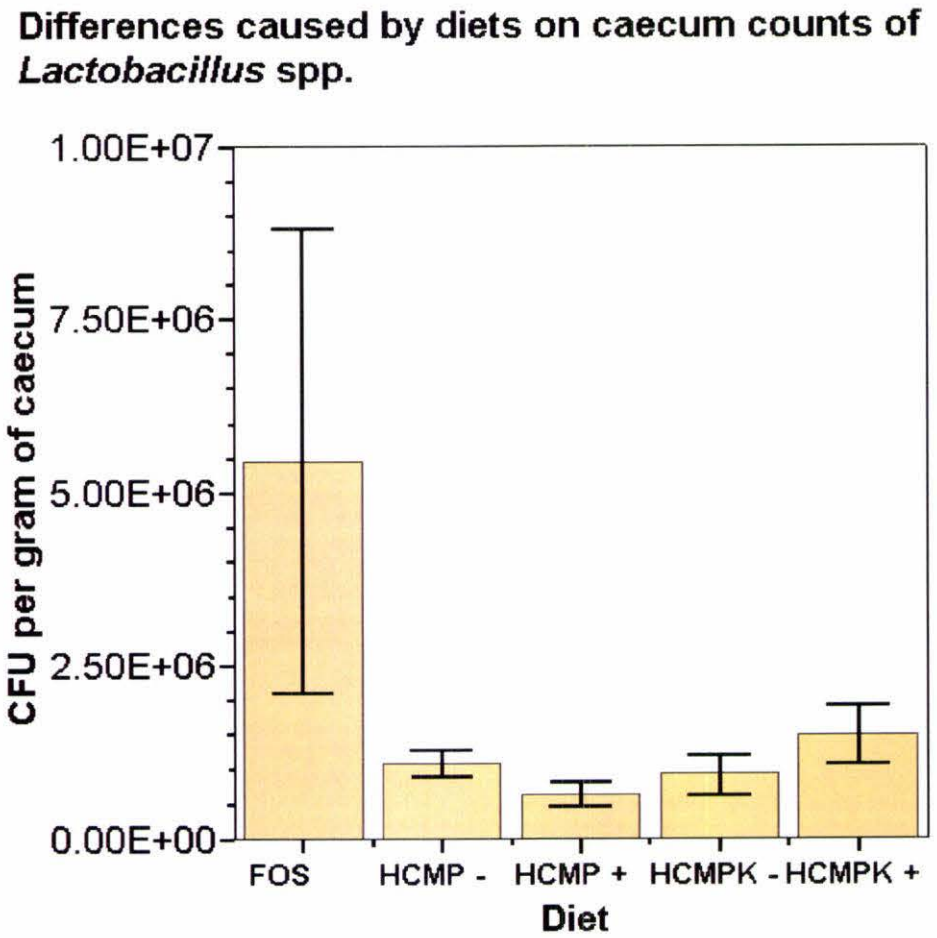

Figure 34: Graph showing the differences caused by diets on the colony forming units (CFUs) per gram of caecum. Caecal counts were made from rats who had been consuming trial diets for ten weeks; there were twelve animals per dietary group. Data are classified by dietary groups of the animals.

Chapter 4. Discussion

The aim of the present study was to test the hypothesis that probiotic supplementation could improve calcium balance and increase bone density in growing animals. The study also hypothesised that, joint pre- and pro-biotic supplementation would have a synergistic effect. The observed effects of pro- and pre-biotic supplementation on the various aspects of bone metabolism measured in the current study are discussed and interpreted in order to ascertain the validity of this hypothesis.

4.1. Mineral Balance

4.1.1. Effect of Probiotic Supplementation

Mineral fractional and absolute absorption in the first balance study was similar to that reported by previous authors who used model animals comparable to that used in this study (Morohashi *et al.*, 1998; Lopez *et al.*, 2000; Chonan *et al.*, 2001; Lopez *et al.*, 2001; Coudray *et al.*, 2003). No differences were observed in zinc fractional or absolute absorption between probiotic diets and their controls in the first balance study. Inclusion of the probiotic increased calcium and magnesium absolute absorption in HCMP diets but not in the HCMPK diets.

The effect of probiotics on fractional and absolute absorption in the second balance study has not been previously reported in a similar animal model. However, efficiency of calcium absorption should decrease with aging (Armbrecht and Wasserman, 1976; Armbrecht *et al.*, 1979; Pansu *et al.*, 1983b), and this did occur in the present study. In the second balance study, there was no significant difference in calcium, magnesium or zinc fractional or absolute absorption between groups that were or were not supplemented with probiotics. This supports the findings of Narva *et al.* (2004) who found no significant difference between calcium excreted in urine

between growing rats receiving *Lactobacillus helveticus* and those that did not. It should be noted, however, that the present study only examines urinary excretion, and not overall absorption or retention. This confirms the results of this study, in which there was no difference in either balance studies for urinary or faecal calcium excretion as a result of probiotic supplementation. There was also no difference in magnesium and zinc urinary excretion as a result of probiotic supplementation in the first balance study. In the second balance study, however, HCMPK – showed a significant increase in urinary zinc compared to HCMPK + animals. Faecal magnesium and zinc excretion was not statistically different in either balance study as a result of probiotic supplementation.

It is possible that a high availability of calcium, magnesium and zinc in the large intestine would result in a reduction of their absorption; long-term supplementation of the probiotic may result in a down-regulation of the active pathways of calcium, magnesium and zinc absorption, and therefore provide no apparent benefit to their absorption at this point (Cashman and Flynn, 1996). Alternatively, there may be no benefit to mineral balance of supplementation to animals of this age. Intestinal transport of calcium declines with age (Armbrecht *et al.*, 1979; Brown *et al.*, 2005). Peak active calcium absorption in the rat is thought to occur at about 35 days old and then decreases to a lower level, whilst passive calcium absorption decreases over the first 35 days and then remains constant (Pansu *et al.*, 1983b), with peak bone mass reached at about 3 months of age (Sengupta *et al.*, 2005). Therefore, probiotic supplementation (long term) may prove more effective in a younger animal, where passive absorption is occurring at a greater rate. In the first balance study, rats were 49 – 52 days old; in the second they were 88 – 91 days old. Therefore, in the first balance study, mineral absorption should have been at about its peak, and would be

expected to decrease up until the second, where no effect of pro- and / or pre-biotics was observed.

Probiotic supplementation may have no effect if levels of CaBP, the rate-limiting step in calcium absorption (Bronner *et al.*, 1986) are not altered. Ohta *et al.* (1998b) found FOS to increase CaBP in the large intestine, suggesting that diet can alter the level of the protein present. To date no work has examined the effect of DR20™ on CaBP, but since FOS was included in the probiotic diet, one might expect that a similar effect might be seen in animals receiving the probiotic in this trial. For an electrochemical gradient to be maintained in the cell, calcium, magnesium and zinc must also be extruded from the cell at an increased rate compared to control animals. The effect of pro- and pre-biotics on the CaATPase, Mg2+ / Na+ and zinc transporters have not been characterised.

Absorption of calcium in the large intestine is thought to only account for about 10% of total absorption (Bronner and Pansu, 1999). Therefore there may have been no observed effect on absorption since most of the minerals may have already been taken up in the small intestine. This may be especially the case in this study, where the minerals were present in forms that are easily absorbed, and not hindered by factors that are known to reduce mineral absorption, such as the presence of phytic acid, or oxalic acid. This would aid their absorption in the small intestine, and reduce the amount that would pass into the large intestine, where probiotics and prebiotics could exert their positive effects on mineral absorption.

4.1.2. Effect of Vitamin K Supplementation

There was no significant difference between the fractional absorption of calcium and magnesium, in response to vitamin K supplementation. However, absolute

absorption of calcium was significantly higher in vitamin K diets (HCMPK – and HCMPK +) compared to their controls (HCMP – and HCMP + respectively), and absolute absorption of magnesium was significantly higher in HCMPK – than HCMP – animals. Absolute absorption of calcium in animals receiving vitamin K but no probiotic (HCMPK –) was comparable to those receiving probiotic but no vitamin K (HCMP +). Certain probiotics and gut bacteria are known to produce vitamin K as menaquinone (Morishita *et al.*, 1999; Parker *et al.*, 2003). These authors tested the ability of three different species of *Lactobacillus* amongst other bacteria, to produce vitamin K; none was detected (Morishita *et al.*, 1999; Parker *et al.*, 2003). However, *Lactobacillus rhamnosus* was not one of those examined, and hence it is still possible that it may produce vitamin K, although available evidence tends towards the suggestion that this may not be the case. Absolute absorption of calcium was similar in vitamin K groups regardless of whether probiotic was supplied or not. This suggests that the probiotic is not using (all) the vitamin itself, therefore some can be absorbed and used by the host. However, the fact that fractional absorption of calcium does not differ with or without vitamin K supplementation means that groups that showed higher retention (absolute absorption level), must be excreting more calcium; therefore, the fractional absorption level remains the same, despite a difference in retention. Faecal and urinary calcium and magnesium, and zinc faecal excretion was unaffected by vitamin K supplementation in either balance study. Urinary zinc excretion was significantly higher in the HCMPK – animals in the second balance study compared to all other diet groups; in the first balance study there was no significant difference in urinary zinc excretion between any diet groups.

In the first balance study, supplementation of vitamin K resulted in significantly higher absolute absorption of magnesium compared to the HCMP – diet, which had

no probiotic and no vitamin K; there was no difference, however, between vitamin K groups (HCMPK – and HCMPK +) and animals receiving probiotic but no vitamin K (HCMP –). Absolute and fractional absorption of zinc was higher in animals supplemented with vitamin K. At present there are no known links between vitamin K and magnesium or zinc balance. It is possible, however, that vitamin K could indirectly affect mineral absolute and fractional absorption through acting on the body's mineral pools. Vitamin K could affect the amount removed from the body's mineral pool for use in bone mineralisation. If an equilibrium were in place then more may be absorbed from the diet, or less excreted in urine and faeces to counteract this change.

The second balance study showed no significant difference in calcium, magnesium or zinc absolute or fractional absorption between vitamin K and non-vitamin K supplemented groups. This suggests that long-term vitamin K supplementation, or to animals at this life-stage or age may not prove beneficial in mineral absorption. Again it is possible that long-term supplementation may result in a down-regulation of the active pathway of calcium, magnesium and zinc absorption, thus apparently creating no difference between supplementation and non-supplementation after eight weeks.

4.1.3. Effect of Prebiotic Supplementation

Prebiotic supplementation of the FOS group did not affect calcium, magnesium or zinc absorption in the first or second balance studies compared to the animals receiving no pro- or pre-biotic (HCMP – group). However, in the first study both fractional and absolute absorption of calcium and magnesium in animals from the pro- and prebiotic group (FOS) were lower than those of animals receiving the probiotic and no prebiotic (HCMP +). This may be because the prebiotic encourages

the growth of probiotics already present in the gut, and those administered. Therefore these bacteria may be utilising minerals for their own needs, decreasing the amount that is available for the host, and hence causing no apparent benefit of supplementation on mineral balance. Fractional and absolute absorption of zinc was not significantly different between FOS and HCMP + groups. Urinary magnesium excretion was higher and faecal magnesium excretion lower as a result of prebiotic supplementation in the first balance study; there was no difference by the second. FOS groups were not significantly different in terms of calcium or zinc urinary or faecal excretion, from the HCMP + group, i.e. combined pro- and pre-biotic supplementation did not significantly affect these parameters compared to probiotic supplementation alone. When FOS groups are compared to HCMP – groups, however, there is a significant increase in urinary calcium excretion in the second balance study, and a significant reduction in faecal zinc excretion as a result of combined supplementation of pro- and pre-biotic.

Whilst numerous studies have suggested that prebiotics may increase absorption of these minerals, there is only one that examines the effect of joint supplementation with a probiotic; Darragh *et al.* (2005) found that a probiotic / prebiotic mix had no effect on nutrient absolute absorption.

Differences observed between this study, and those summarised in Chapter 1 may be due to the type of prebiotic supplied. Kruger *et al.* (2003b) found inulin to be more effective at increasing bone mineral density compared to the shorter-chain oligofructose (OF), or a mixture of the two oligosaccharides, when supplied to 7-week old male rats. Interestingly, there was also no significant difference found in absolute absorption of calcium in the same study, as a result of any of the dietary interventions, compared to the control group. Griffin *et al.* (2002) found that

supplementation with OF at 8g/day to girls, did not significantly affect fractional calcium absorption, whilst supplementation of a mixture of OF and inulin did. Results were confirmed by a later study by the same authors (Griffin *et al.*, 2003).

Oligosaccharides are fermented in the large intestine to short-chain fatty acids (SCFAs), which has been proposed as a mechanism by which mineral absorption may be increased; through complexes it forms with the mineral(s), altering electrolyte exchanges, atrophy of absorptive cells and / or increasing CaBP. Long-chain oligosaccharides are fermented more slowly, and reach further parts of the large intestine than their short-chain counterparts; they are, therefore, converted into SCFAs for a longer length of time and in more distant regions of the intestine and therefore exert a more pronounced effect on mineral absorption (Van Loo, 2004).

It has recently been suggested that age may influence the possible effect that prebiotics may have on mineral absorption. Raschka and Daniel (2005) found that inulin-type fructans only increased calcium, magnesium and zinc absorbed from the diet of rats whose mineral demand is particularly high, such as those actively growing. Having said that, the authors provided animals with two different diets; one group (young adult rats) received 0.9% calcium, 0.2% magnesium and 0.007% zinc, whilst the other (adolescent rats) received 0.5% calcium, 0.05% magnesium and 0.003% zinc. There is no explanation in the paper for this change in diet, except that the former, the synthetic diet, was found to contain naturally occurring fructans, which the later, the semi-synthetic diet, did not. When the synthetic diet was supplied to young adult rats, either with or without 10% inulin added, no difference was found in calcium fractional or absolute absorption. Addition of 10% inulin to the semi-synthetic diet supplied to adolescent rats resulted in a significantly lower faecal calcium excretion, and higher fractional calcium absorption; absolute absorption

remained the same. This data suggests that it is also possible that the level of mineral in the diet may influence the extent to which prebiotics can alter mineral absorption.

Whilst probiotic supplementation may increase mineral absorption short term, combined administration with a prebiotic may not prove beneficial to mineral absorption, nor supplementation with short-chain oligosaccharides. Since the second balance study showed no discernable differences in mineral absorption, long-term supplementation and / or supplementation to animals of this age or life stage may not prove beneficial. Le Blay *et al.* (1999) suggested that supplementation of 9% FOS for two weeks increased *Lactobacillus* spp. numbers, whilst supplementation for eight or twenty seven weeks resulted in no discernable difference from control animals. The same study showed that short-chain fatty acids (SCFAs) production, and caecal wall weight increased at all three time-points as a results of FOS supplementation; SCFAs and physiological changes in the large intestine were one of the proposed effectors for increased mineral absorption. If mineral balance is increased during the two time-points in our study, at which the balance studies were carried out (which would not, therefore, have been measured), it is possible that the prolonged increase in mineral availability may result in a down-regulation of their active pathways of absorption.

4.2. Faecal Weight

4.2.1. Effect of Probiotic Supplementation

Probiotic supplementation did not appear to cause a significant change in faecal weight in the first balance study. Vitamin K supplementation also did not appear to affect faecal weight in the same balance study, despite the fact that this is the predominate excretion route of the vitamin (Suttie, 2000). However, it was present at an extremely low level compared to the recorded food intake (daily intake of vitamin

K averaged approximately 23μg per day, compared to an average daily food intake of approximately 57g), therefore, it may not have had an effect on faecal weight.

There was no difference in faecal weight between groups in the second balance study. This suggests that long-term probiotic supplementation may not affect faecal weight. The latter groups may be experiencing an increase in colonic bacteria later in life; probiotic groups may not show this increase if the large intestine has already been colonised by bacteria, which have been supplied in their diet, since there is only a limited area available for colonisation. No other studies could be found in published literature to confirm or contrast any of these findings or potential explanations for the effect of probiotics and / or vitamin K on faecal weight. Faecal weight was highly correlated with food intake in both balance studies.

4.2.2. Effect of Prebiotic Supplementation

Faecal weight was significantly higher in animals supplemented with FOS in the first balance study; in the second balance study, there was no difference between faecal weight in animals that received and did not receive FOS. Fermentation of NDOs produces biomass, which therefore causes faecal bulking and increases defecation frequency (Van Loo *et al.*, 1999). Chonan *et al.* (2001) fed six-week old male rats 5% GOS for seven days; the animals used were the same age as rats in this study during the first balance, where an increase in faecal weight was also observed. Delzenne *et al.* (1995) found FOS fed at 10% of the diet increased faecal weight in rats smaller than those used here (100±5g), after receiving diets for 24 days. Rats of body weights similar to those in this study (209±1.69g) showed an increase in faecal weight after receiving 5% FOS supplemented diets for four weeks (Gudiel-Urbano and Goni, 2002). Faecal weight was highly correlated with food intake in both balance studies.

In the first balance study, animals received trial diets for 3 – 4 weeks. It is possible that prebiotic supplementation for a longer period may have had no further effect on faecal weight, or that faecal weight of non-supplemented groups was increased at a greater rate than prebiotic groups, resulting in a similar output between all groups. Faecal weight was found to highly significantly increase in all dietary groups between the first and second balance studies. This is as may be expected, as animals also had increased food intake. Benyen *et al.* (2002) found no significant difference in faecal weight of dogs after feeding of a 1% OF diet for three weeks. However, this is at a much lower level than was used in this study, and most other prebiotic trials.

4.3. Type I Collagen c-Terminal Telopeptides

There was no effect of pro- and / or pre-biotic supplementation on plasma type I collagen c-terminal telopeptides (CTx) level in animals, a marker for bone resorption. This is in accordance with the findings of Darragh *et al.* (2005), who found no significant difference between plasma CTx levels of pigs receiving a supplement of *Bifidobacterium lactis* HN019 (DR10™), DR20™ or DR10™ combined with FOS.

Serum C-telopeptide concentrations are indicative of the rate of osteoclastic activity. Osteoclast activity is affected by several parameters as outlined in Chapter 1. Some of these factors, such as PTH and 1,25(OH)2D are affected by calcium levels. The results from the second balance study, and bone calcium content suggest that at this point there was no difference in calcium in animals in different groups; therefore one may also not expect to see a difference in CTx level.

4.4. IL-6 and IL-10

The majority of samples tested for IL-6 and IL-10 contained levels below the limit of detection, even though whole blood cells had been stimulated. This confirms the results of Darragh *et al.* (2005), who also found that even fewer samples showed detectable results for IL-10 and IL-12 when cells had not been stimulated compared to those that had. Roller *et al.* (2004) found no change in cytokine status as a result of dietary supplementation to 12-13 week male rats of 10% inulin and / or *Lactobacillus rhamnosus* and *Bifidobacterium lactis* for one week. It should be noted, however, that this was an extremely short trial; the effect of diet may have taken longer to be seen.

IL-6 is secreted by bone cells in response to hormones such as PTH, $1,25(OH)_2D$ and IL-1, and stimulates osteoclast formation (Mundy, 1999). The CTx results suggest that there may be no difference between bone resorption as a result of pre- and / or pro-biotic supplementation, which is confirmed by the low IL-6 levels measured.

IL-10 inhibits bone resorption (Hong *et al.*, 2000); although at present there is poor understanding of the factors that promote its production and / or activation. Its expression is thought to be induced by TNF-α in macrophages, lipopolysaccharide (LPS), and by IL-6 and IL-12 in T-cells (Daftarian *et al.*, 1996). Again, since the CTx and IL-6 results suggest that there may be no difference in bone resorption as a result of pre- and / or probiotic supplementation, one would also expect to see no difference in IL-10 activity.

It should be noted, however, that the amount of cytokines produced is highly specific to an individual (Yaqoob *et al.*, 1999). The technique used in this study determines cytokine concentrations based on a known volume of blood; the number of cytokine-producing cells is neither known nor controlled. Therefore measurement of effect of IL-6 and IL-10 levels in this study may have been confounded by the large inter-individual variation that occurs, and the fact that no baseline samples, or cell count, were taken to

allow a point of reference. Hence, the observed differences may be due to an individual-variation, rather than the effect of a dietary component, and may explain the large SEMs observed within certain diet groups.

4.5. Bone Mineral Density

There was no significant effect of pre- and / or pro-biotic supplementation on bone density (BMD) or bone mineral content (BMC) assessed by *ex vivo* DEXA. However, as there was no benefit in mineral balance after eight weeks of supplementation, this is as may be expected. The apparent improvement of certain dietary manipulations observed during the first balance study may have been followed by a period of reduced mineral absorption, allowing other diets to "catch-up" any advances in BMD and BMC gained by these groups. Peak bone mass is thought to be achieved in rats at about three months of age (Sengupta *et al.*, 2005); DEXAs in this study would have therefore been carried out on animals that would have reached this stage. *In vivo* DEXAs may have shown an apparent benefit of probiotic and vitamin K supplementation after two weeks, as calcium, magnesium and zinc fractional and / or absolute absorption was improved at this point. However, this is purely speculation, and without *in vivo* DEXAs having been carried out then, it is impossible to confirm this. DEXAs cannot be carried out on animals under 150g (Brown, 2001), however, and therefore *in vivo* DEXAs were not possible at this point.

The findings of this study confirm the results of Darragh *et al.* (2005), who fed DR20™ to neonatal piglets for four weeks. It is also in agreement with the findings of Ohta *et al.* (2002) who fed six-week old sham-operated mice with control or 5% FOS diets for six weeks. They found total, bone density in the femur to be the same between control and FOS animals; these findings were echoed when bone density was measured

in proximal, central and distal areas of the femur. Kruger *et al.* (2003b) found four weeks of feeding inulin (degree of polymerisation, DP>23) to seven-week old male rats increased femur BMD compared to control animals; no significant difference was found between control animals and those fed short-chain FOS (DP2-8) or a mixture of inulin and short-chain FOS. The Kruger *et al.* (2003b) trial was shorter than Ohta *et al.* (2002) trial and the present one, and unlike the present study, neither trial supplied both probiotics and prebiotics.

Narva *et al.* (2004) studied the effect of 14 weeks of *Lactobacillus helveticus* supplementation to growing rats on BMD. At the end of the study, animal body weights differed significantly between groups. Using raw data, there was no significant difference in BMD between control and *L. helveticus* groups; results became significant when BMD was divided by body weight for each animal, and then analysed. It may not be ideal to compare this with results from the current study, as they will have been analysed differently (body weight was used as a covariate in this study).

Kruger *et al.* (2003a) fed OVX rats DR20™ for three months, and observed a significant increase in BMC and BMD in femur and lumbar spine compared to OVX control animals. Animals received a diet with calcium at a level of 0.5%. OVX rats fed 0.5% calcium diet have reduced calcium absorption compared to sham-operated animals (Kalu and Orhii, 1999). Therefore, it is possible that although DR20™ did not show a significant effect in improving bone quality in actively growing animals in this study, probiotics may prove beneficial to animals who are already compromised in terms of bone metabolism (such as ovariectomised rats), or may prove beneficial when not combined with prebiotics.

4.6. Biomechanical properties of bone

Bone biomechanics were not significantly affected by supplementation with a prebiotic. In fact, the only significant difference observed in the parameters measured was in measurements of bone stiffness (elasticity) between the two diets containing vitamin K; additional supplementation with the probiotic (in the HCMPK + diet) resulted in lower stiffness compared to its exclusion (HCMPK –). HCMPK + and HCMPK – were not statistically different from HCMP –, HCMP + or FOS, suggesting that addition of vitamin K and DR20™ had an additive effect; supplementation of vitamin K alone did not affect bone stiffness.

Vitamin K mediates the post-translational modification of osteocalcin, which in turn promotes calcium binding and bone mineralisation. If the probiotic is utilising vitamin K for its own ends, there may be a reduction in its availability for the host organism, and hence bone mineralisation would occur less or be less effective, than if the probiotic were not supplied. Mineralisation provides mechanical rigidity (Lian *et al.*, 1999); supplementation of the probiotic and vitamin K could cause a reduction in bone stiffness. Roy *et al.* (2001) demonstrated an inverse relationship between osteocalcin content and bone elasticity. Several studies have suggested a link between vitamin K status and fracture risk in humans (reviewed by Weber (2001)), and it is frequently used as a predictor of bone fracture risk (Suttie, 2000). Rats are thought to achieve peak bone mass, an important indicator of bone strength, at three months of age (Sengupta *et al.*, 2005), and humans at 20-years of age (Heaney *et al.*, 2000). Improving bone quality before this age can reduce the likelihood of fractures (summarised in Figure 35).

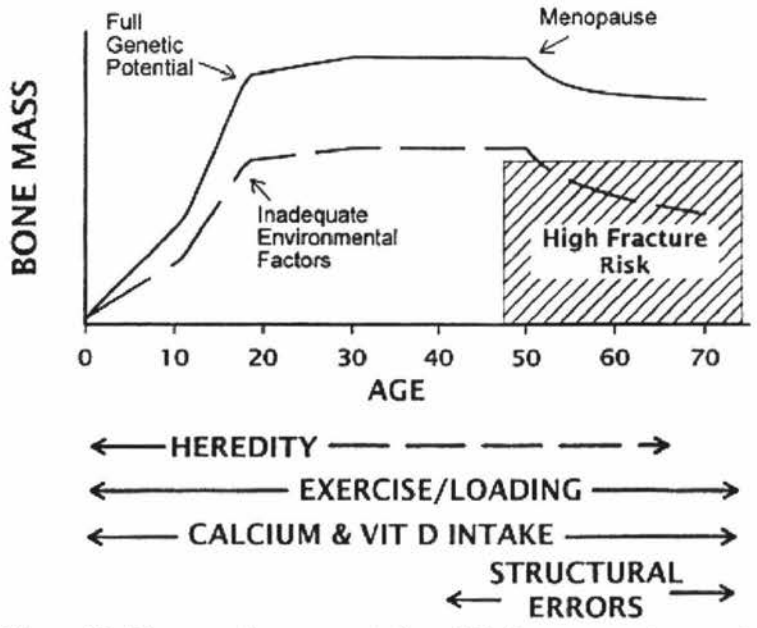

Figure 35: Diagramatic representation of the bone-mass changes during the life-time of an individual who achieves their full genetic potential, and one who does not (magnitude of differences between these two is not to scale). If both individuals experience the same drop in bone mass later in life (as a result of menopause etc), the person with lower bone mass will reach a level of high fracture risk sooner. Underneath the graph are written several of the factors known to be of particular importance in determining bone mass. Taken from Heaney (2000).

The observation that vitamin K administration is more effective at increasing bone stiffness than the provision of the probiotic and vitamin K to growing animals is important. Vitamin K has the potential to reduce the risk of osteoporotic fractures later in life (Kalkwarf *et al.*, 2004). Osteoporosis was once considered an inevitable consequence of aging (World Health Organisation, 2003b). It has been increasingly recognised that actions can be taken to prevent osteoporosis, minimising its financial and physical debilitating effects, or even death. Attempts to decrease the likelihood of fractures using vitamin K, should be wary of including probiotics as this may result in a reduction of bone stiffness, therefore potentially increasing in likelihood of fractures.

Other bone biomechanical parameters did not appear to be affected, suggesting that although bone mineralisation may be reduced in probiotic diets supplemented with vitamin K, the stress it is able to withstand may not differ. In addition, bone stiffness did not differ between diets that were or were not supplemented with vitamin K; perhaps suggesting the observed differences were fallible.

4.7. Bone Ash Content

Bone ash content, non-mineral bone matter and the ratio of the two did not appear to be affected by probiotic and / or prebiotic supplementation. Again, it is possible that long-term supplementation may result in down-regulation allowing mineral absorption and deposition in bone to occur at a greater rate in diets that lagged at the beginning.

The results of this study confirm those of Ohta *et al.* (2002), who observed no difference in bone ash content in six-week old sham-operated or OVX mice after receiving a control or FOS diet for six weeks (although there was a difference between control-diet fed sham-operated and OVX rats). Narva *et al.* (2004) found femur ash content to be lower in *Lactobacillus helveticus* supplemented rats compared to controls after 14 weeks. However, control diets differed from *L. helveticus* diets in all the parameters listed in diet composition, such as calcium, energy, protein, fat and carbohydrate. It is unclear whether this was taken into account during the analysis.

4.8. Bone Mineral Content

Pro- and / or pre-biotic supplementation did not have any apparent effect on bone contents of calcium, magnesium or zinc, which may be due to down-regulation of mineral absorption as a result of long-term supplementation. Ohta *et al.* (2002) observed no difference between six-week old control and FOS-fed sham-operated and OVX mice in bone content of calcium and magnesium after six weeks (although there was a difference between control-fed sham and OVX animals). Raschka and Daniel (2005) found 15 days of feeding 10% inulin to growing rats resulted in a significant increase in calcium, magnesium and zinc content in femurs. Narva *et al.* (2004) found no difference in calcium content between growing rats receiving a *L. helveticus* or control diet; differences in calcium intake and body weight appear to have been taken into account

during analysis. It is possible, therefore, that long-term supplementation of prebiotics and / or administration of probiotics to growing animals may not increase the mineral content of bone, which providing prebiotics alone may do. Any advances which may have been obtained by short-term supplementation may have not have been so pronounced by the end of the trial.

4.9. Gut Bacteriology

Lactobacillus spp. colonies were cultured and counted from caecal samples in order to determine whether diets had successfully manipulated gut flora. There is, at present, no probe available to directly quantify DR20™. The method chosen was specific for *Lactobacillus* spp.; this is the easiest method for analysing the potential functional significance of diets (whilst there are other methods available, time and financial constraints prevented their use in this study). In addition, although DR20™ was chosen as the probiotic supplied, the aim of the study was to alter the balance of gut flora in favour of probiotics, as these are the bacteria most likely to benefit the host, and potentially (as this study aimed to determine) affect mineral absorption and bone quality.

Analysis of caecal counts of *Lactobacillus* spp. suggested that there was no significant difference introduced by the administration of DR20™ or vitamin K. Supplementation of FOS caused a significantly different caecal count compared to HCMP + and HCMPK –, but not other diets. It should be noted, however, that the analysis used quantifies all species of *Lactobacillus*. Therefore, the fact that certain diets caused higher numbers of *Lactobacillus* is not conclusive evidence of its effect on DR20™, but rather provides a possible reflection of DR20™ abundance. FOS caecal count is, therefore also confounded by the fact that it is a prebiotic, and would, therefore

be expected to increase several other species of *Lactobacillus* (as well as other probiotics) in addition to DR20™. However, be that as it may, an increase in *Lactobacillus* spp. is still beneficial to the host. Having said that, if all that was intended was to alter the populations of probiotics in the gut, it appears that supplying DR20™ alone may not be as effective as supplementation of FOS. Whether FOS is as effective when provided alone or in combination with DR20™ has not been determined, although several studies have shown its effectiveness when used alone (reviewed in Chapter 1). Indeed, these studies also found changes in calcium, magnesium and zinc absorption and retention, and bone quality, which were not found in this study. However, this may be due to the length of the study, and the possible adaptation to mineral absorption and retention (i.e. a down-regulation of active transport), which occurs following a lengthy period of high mineral availability.

4.10. Context

Although the study aimed to provide animals with 1 x 10^9 cfu per day, in several cases, animals did not always eat all food provided. However, since this was not limited to one dietary group (analysis of results at the end of the trial showed total and weekly intakes of food to be similar between diet groups), this may not be a confounding factor. Due to computer malfunction and data loss, it was also not possible to determine whether bacterial counts in diets differed significantly from the required dosage of 1 x 10^9 cfu per day, or whether bacterial counts differed significantly between diets, therefore hindering data interpretation. In addition, the effect of leaving food out in rat cages (i.e. exposing bacteria to oxygen) on bacterial numbers was not determined. However, being that *Lactobacillus rhamnosus* is a facultative anaerobe (Sutter *et al.*, 1985), it should not have been killed by such exposure. The trial was also carried out for ten weeks, during which period two balance studies were carried out. This is

particularly useful as it provides some indication of the effect that long-term supplementation may have on mineral balance and bone quality of consumers, who may supplement their diets for a similar period.

The rat is a coprophaging animal, and therefore may have limited use as a model of human mineral absorption, since this is not a practice adopted by or necessary for mineral absorption in humans. This may be a particular problem in experiments such as this one, which rely on NDO-induced changes occurring in the large intestine, which affect mineral absorption. In the balance studies, for example, coprophagy was prevented since faeces fell through the wire floor into collection pots. Ohta *et al.* (1996) found that FOS increases mineral absorption in rats regardless of whether or not coprophagy was prevented.

SUMMARY

This study aimed to test the hypothesis that probiotic supplementation could improve calcium balance and increase bone density in growing animals. The study also hypothesised that joint supplementation with a prebiotic would have a synergistic effect.

Short-term (three-weeks) probiotic supplementation increased the absolute absorption of calcium and magnesium; long-term (nine-weeks) supplementation did not affect calcium, magnesium or zinc fractional or absolute absorption. The mechanism for the short-term increase of absolute absorption of these minerals in response to probiotic supplementation is unclear. Long-term supplementation may result in down-regulation of active and / or passive pathways of mineral absorption, due to their increased availability in the large intestine. Alternatively, the minerals may have been provided in a form that aided absorption (i.e. no phytic or oxalic acids present, which are known to

decrease bioavailability) and therefore reduced the presence of these minerals in the large intestine.

Short-term vitamin K supplementation increased calcium and magnesium absolute absorption, but not fractional absorption; long-term supplementation did not affect absolute or fractional absorption of any of the minerals. Bone stiffness, was improved in animals supplemented with vitamin K (HCMPK –) compared to those receiving vitamin K and probiotic (HCMPK +). Vitamin K is required for the post-translational modification of osteocalcin, which in turn promotes bone mineralisation. Improving bone quality in animals that are actively growing has the potential to reduce the likelihood of osteoporotic fractures later in life. This finding bears importance for any nutritional interventions, which aim to improve bone quality as a result of probiotic supplementation.

Prebiotic supplementation in combination with probiotic and vitamin K supplementation did not affect absolute or fractional absorption of any of the minerals either long- or short-term. This is in contrast to the findings of several (but not all) previous studies. Differences may have been observed because a different prebiotic was supplied, or at a different level. Most other studies have also not provided a probiotic in addition to the prebiotic. It is possible that this may increase growth of probiotic(s) in the large intestine and therefore cause a consequential increase in nutritional demand. Faecal weight was increased in the short-term by prebiotic supplementation, but not long-term. Increased faecal bulking as a result of NDOs is a well-accepted phenomenon; therefore why this did not occur later on is unclear. Prebiotic supplementation appears to have been the most effective method for increasing *Lactobacillus* species levels in the caecum. Whilst the method used to quantify the caecal content of bacteria analysed that of all *Lactobacillus* species, rather than DR20™

specifically, it provides a more useful idea of the practical significance of dietary interventions. As *Lactobacillus* species are probiotics, improving the gut bacterial balance in their favour can only benefit the host organism.

Bone resorption and formation did not appear to have been changed as a result of pre-, pro-biotic or vitamin K supplementation, as shown by CTx, IL-6 and IL-10 results. There also appears to have been no functional significance of dietary interventions on BMD, BMC, bone calcium, magnesium and zinc contents, bone ash content, as well as most of the bone biomechanical parameters measured.

This study continues the work already carried out into investigating the effect of pre- and pro-biotics on mineral absorption and bone quality. The current study has suggested that pre- and pro-biotic supplementation may prove more useful in younger rats, which are close to peak-absorption than older rats. Alternatively, the absence of an effect may have been a result of long-term interventions. Further studies, could, therefore, be carried out to determine whether age and / or length of administration is / are responsible for the observed lack of effect on mineral absorption and bone quality towards the end of the trial, as well as improving understanding of the mechanisms behind any possible effects observed. Should these studies prove fruitful, human studies could be carried out in their wake. Whilst there has been insufficient research carried out in humans, it may be that, as is the case with rats, aging results in a decrease in mineral absorption. Pre- and pro-biotics may, therefore, prove useful for the older generation, who may experience compromised absorption, and post-menopausal women, whose reduced levels of oestrogen cause bone loss, and, potentially osteoporosis. Further research is required to determine whether this is the case.

There is an ever-growing realisation of the importance of nutrition in evading preventable illnesses and disease. This study has suggested that pre- and pro-biotics

may indeed have a place in improvement of mineral absorption, and potentially their use in promoting bone health, providing another source of ammunition in the fight against osteoporosis and other bone-diseases. To be truly effective, however, more work is needed to be able to determine the circumstances required for optimal results.

References

Abrams, S. A., Yergey, A. L. and Heaney, R. P. (1994). "Relationship between Balance and Dual Tracer Isotopic Measurements of Calcium-Absorption and Excretion." Journal of Clinical Endocrinology and Metabolism 79(4): 965-969.

Abu-Amer, Y., Abbas, S. and Hirayama, T. (2004). "TNF receptor type 1 regulates RANK ligand expression by stromal cells and modulates osteoclastogenesis." Journal of Cellular Biochemistry 93(5): 980-989.

Adachi, J. D. (1996). "The correlation of bone mineral density and biochemical markers to fracture risk." Calcified Tissue International 59: S16-S19.

Ahdjoudj, S., Lasmoles, F., Oyajobi, B. O., Lomri, A., Delannoy, P. and Marie, P. J. (2001). "Reciprocal control of osteoblast/chondroblast and osteoblast/adipocyte differentiation of multipotential clonal human marrow stromal F/STRO-1(+) cells." Journal of Cellular Biochemistry 81(1): 23-38.

Armbrecht, H. J. and Wasserman, R. H. (1976). "Enhancement of Ca++ Uptake by Lactose in Rat Small-Intestine." Journal of Nutrition 106(9): 1265-1271.

Armbrecht, H. J., Zenser, T. V., Bruns, M. E. H. and Davis, B. B. (1979). "Effect of Age on Intestinal calcium absorption and adaptation to dietary calcium." American Journal of Physiology 236(6): E769-74.

Aubin, J. E. and Bonnelye, E. (2000). "Osteoprotegerin and its ligand: A new paradigm for regulation of osteoclastogenesis and bone resorption." Osteoporosis International 11(11): 905-913.

Baba, S., Ohta, A., Ohtsuki, M., Takizawa, T., Adachi, T. and Hara, H. (1996). "Fructooligosaccharides stimulate the absorption of magnesium from the hindgut in rats." Nutrition Research 16(4): 657-666.

Baron, R. (1999). Anatomy and Ultrastructure of Bone. Primer on Metabolic Bone Diseases and Disorders of Mineral Metabolism. M. J. Favus. Philadelphia, Lippincott, Williams and Wilkins: 3-10.

Bassett, J. H. D. and Williams, G. R. (2003). "The molecular actions of thyroid hormone in bone." Trends in Endocrinology and Metabolism 14(8): 356-364.

Benway, D. A. and Weaver, C. M. (1993). "Assessing Chemical Form of Calcium in Wheat, Spinach, and Kale." Journal of Food Science 58(3): 605-608.

Beynen, A. C., Baas, J. C., Hoekemeijer, P. E., Kappert, H. J., Bakker, M. H., Koopman, J. P. and Lemmens, A. G. (2002). "Faecal bacterial profile, nitrogen excretion and mineral absorption in healthy dogs fed supplemental oligofructose." Journal of Animal Physiology and Animal Nutrition 86(9-10): 298-305.

Blair, H. C., Zaidi, M. and Schlesinger, P. H. (2002). "Mechanisms balancing skeletal matrix synthesis and degradation." Biochemical Journal 364(2): 329-41.

Bossard, M. J., Tomaszek, T. A., Thompson, S. K., Amegadzie, B. Y., Hanning, C. R., Jones, C., Kurdyla, J. T., McNulty, D. E., Drake, F. H., Gowen, M. and Levy, M. A. (1996). "Proteolytic Activity of Human Osteoclast Cathepsin K." J. Biol. Chem. 271(21): 12517-12524.

Brandaoneto, J., Stefan, V., Mendonca, B. B., Bloise, W. and Castro, A. V. B. (1995). "The Essential Role of Zinc in Growth." Nutrition Research 15(3): 335-358.

Brink, E. J., Dekker, P. R., Vanberesteijn, E. C. H. and Beynen, A. C. (1991). "Inhibitory Effect of Dietary Soybean Protein Vs Casein on Magnesium Absorption in Rats." Journal of Nutrition 121(9): 1374-1381.

Brommage, R., Binacua, C., Antille, S. and Carrie, A. L. (1993). "Intestinal Calcium-Absorption in Rats Is Stimulated by Dietary Lactulose and Other Resistant Sugars." Journal of Nutrition 123(12): 2186-2194.

Bronner, F. (1998). "Calcium absorption - A paradigm for mineral absorption." Journal of Nutrition 128(5): 917-920.

Bronner, F. (2003a). "Mechanisms of intestinal calcium absorption." Journal of Cellular Biochemistry 88(2): 387-393.

Bronner, F. (2003b). "Mechanisms and functional aspects of intestinal calcium absorption." Journal of Experimental Zoology Part a-Comparative Experimental Biology 300A(1): 47-52.

Bronner, F. and Pansu, D. (1999). "Nutritional Aspects of Calcium Absorption." J. Nutr. 129(1): 9-12.

Bronner, F., Pansu, D. and Stein, W. D. (1986). "An Analysis of Intestinal Calcium-Transport across the Rat Intestine." American Journal of Physiology 250(5): G561-G569.

Brown, A. J., Krits, I. and Armbrecht, H. J. (2005). "Effect of age, vitamin D, and calcium on the regulation of rat intestinal epithelial calcium channels." Archives of Biochemistry and Biophysics 437(1): 51-58.

Brown, I. L. (2004). "Applications and uses of resistant starch." Journal of Aoac International 87(3): 727-732.

Brown, J. P. and Josse, R. G. (2002). "2002 clinical practice guidelines for the diagnosis and management of osteoporosis in Canada." CMAJ 167(90100): 1S-34.

Brown, K. (2001). Measurement of Bone Quality in growing male rats using DEXA and bone ash content. IFNHH. Palmerston North, Massey: 88.

Buckley, M. and Bronner, F. (1980). "Calcium-Binding Protein-Biosynthesis in the Rat - Regulation by Calcium and 1,25-Dihydroxyvitamin-D3." Archives of Biochemistry and Biophysics 202(1): 235-241.

Burger, E. H. and Klein-Nulend, J. (1999). "Mechanotransduction in bone - role of the lacuno-canalicular network." Faseb Journal 13: S101-S112.

Cashman, K. D. and Flynn, A. (1996). "Effect of dietary calcium intake and meal calcium content on calcium absorption in the rat." British Journal of Nutrition 76(3): 463-470.

Caverzasio, J., Montessuit, C. and Bonjour, J. P. (1996). "Functional Roles of Pi Transport in Osteogenic Cells." News in physiological sciences 11: 119-25.

Cenci, S., Weitzmann, N. M., Roggia, C., Namba, N., Novack, D., Woodring, J. and Pacifici, R. (2000). "Estogen deficiency induces bone loss by enhancing T-cell production of TNF-α." Journal of Clinical Investigation 106(10): 1229-1237.

Chambers, T. J. (2000). "Regulation of the differentiation and function of osteoclasts." Journal of Pathology 192(1): 4-13.

Champ, M. M. J. (2004). "Physiological aspects of resistant starch and in vivo measurements." Journal of Aoac International 87(3): 749-755.

Chen, D., Zhao, M. and Mundy, G. R. (2004). "Bone morphogenetic proteins." Growth Factors 22(4): 233-241.

Chonan, O., Takahashi, R. and Watanuki, M. (2001). "Role of activity of gastrointestinal microflora in absorption of calcium and magnesium in rats fed beta 1-4 linked galactooligosaccharides." Bioscience Biotechnology and Biochemistry 65(8): 1872-1875.

Christenson, R. H. (1997). "Biochemical markers of bone metabolism: An overview." Clinical Biochemistry 30(8): 573-593.

Coudray, C., Bellanger, J., CastigliaDelavaud, C., Remesy, C., Vermorel, M. and Rayssignuier, Y. (1997). "Effect of soluble or partly soluble dietary fibres supplementation on absorption and balance of calcium, magnesium, iron and zinc in healthy young men." European Journal of Clinical Nutrition 51(6): 375-380.

Coudray, C., Tressol, J. C., Gueux, E. and Rayssiguier, Y. (2003). "Effects of inulin-type fructans of different chain length and type of branching on intestinal absorption and balance of calcium and magnesium in rats." European Journal of Nutrition 42(2): 91-98.

Creedon, A., Flynn, A. and Cashman, K. (1999). "The effect of moderately and severely restricted dietary magnesium intakes on bone composition and bone metabolism in the rat." British Journal of Nutrition 82(1): 63-71.

Cui, L., Takagi, Y., Nezu, R., Iiboshi, Y., Yoshida, H., Sando, K. and Okada, A. (1995). "Prolonged zinc-deficient diet alters alkaline phosphatase and disaccharidase activities and induces morphological changes in the intestine of rats." Journal of Trace Elements in Experimental Medicine 8(4): 249-261.

Daftarian, P. M., Kumar, A., Kryworuchko, M. and DiazMitoma, F. (1996). "IL-10 production is enhanced in human T cells by IL-12 and IL-6 and in monocytes by tumor necrosis factor-alpha." Journal of Immunology 157(1): 12-20.

Dahl, W. J., Whiting, S. J., Isaac, T. M., Weeks, S. J. and Arnold, C. J. (2005). "Effects of thickened beverages fortified with inulin on beverage acceptance, gastrointestinal function, and bone resorption in institutionalized adults." Nutrition 21(3): 308-311.

Darragh, A., Kruger, M., Wolber, F., Molan, A., Cross, F., Booth, C., Dey, D., Fray, L., Wylie, K. and Broomfield, A. (2005). Effect of pre- and / or pro-biotic dietary supplementation on nutrient uptake in a neonatal piglet model. Confidential Report. Palmerston North, Institute of Food, Nutrition & Human Health, Massey University: 62.

Davis, S. R., McMahon, R. J. and Cousins, R. J. (1998). "Metallothionein knockout and transgenic mice exhibit altered intestinal processing of zinc with uniform zinc-dependent zinc transporter-1 expression." Journal of Nutrition 128(5): 825-831.

Delzenne, N., Aertssens, J., Verplaetse, H., Roccaro, M. and Roberfroid, M. (1995). "Effect of Fermentable Fructo-Oligosaccharides on Mineral, Nitrogen and Energy Digestive Balance in the Rat." Life Sciences 57(17): 1579-1587.

Dobnig, H. and Turner, R. T. (1995). "Evidence That Intermittent Treatment with Parathyroid-Hormone Increases Bone-Formation in Adult-Rats by Activation of Bone Lining Cells." Endocrinology 136(8): 3632-3638.

Ducy, P. and Karsenty, G. (1998). "Genetic control of cell differentiation in the skeleton." Current Opinion in Cell Biology 10(5): 614-619.

Ducy, P., Zhang, R., Geoffroy, V., Ridall, A. L. and Karsenty, G. (1997). "Osf2/Cbfa1: A Transcriptional Activator of Osteoblast Differentiation." Cell 89: 747–754.

Ellis, K. J. (2000). "Human body composition: In vivo methods." Physiological Reviews 80(2): 649-680.

Fleet, J. C. (2000). Zinc, Copper and Manganese. Biochemical and Physiological Aspects of Human Nutrition. M. H. Stipanuk. Philadelphia, W.B. Saunders: 741-760.

Ford, D. (2004). "Intestinal and placental zinc transport pathways." Proceedings of the Nutrition Society 63(1): 21-29.

Franck, A. (1998). "Prebiotics stimulate calcium absorption: a review." Milchwissenschaft-Milk Science International 53(8): 427-9.

Freund, T. and Bronner, F. (1975a). "Stimulation Invitro by 1,25-Dihydroxy-Vitamin-D3 of Intestinal Cell Calcium-Uptake and Calcium-Binding Protein." Science 190(4221): 1300-1302.

Freund, T. and Bronner, F. (1975b). "Regulation of Intestinal Calcium-Binding Protein by Calcium Intake in Rat." American Journal of Physiology 228(3): 861-869.

Gabbitas, B. and Canalis, E. (1997). "Retinoic acid regulates the expression of insulin-like growth factors I and II in osteoblasts." Journal of Cellular Physiology 172(2): 253-264.

Gertner, J. M. (1999). Childhood and Adolescence. Primer on the Metabolic Bone Diseases and Disorders of Mineral Metabolism. M. J. Favus. Philadelphia, Lippincott Williams and Wilkins: 45-49.

Ghosh-Choudhury, N., Abboud, S. L., Nishimura, R., Celeste, A., Mahimainathan, L. and Choudhury, G. G. (2002). "Requirement of BMP-2-induced phosphatidylinositol 3-kinase and Akt serine/threonine kinase in osteoblast differentiation and Smad-dependent BMP-2 gene transcription." Journal of Biological Chemistry 277(36): 33361-33368.

Gibson, G. R. and Roberfroid, M. B. (1994). "D."

Goytain, A. and Quamme, G. A. (2005). "Identification and characterization of a novel mammalian Mg2+ transporter with channel-like properties." Bmc Genomics 6.

Greenfield, E. M., Bi, Y. M. and Miyauchi, A. (1999). "Regulation of osteoclast activity." Life Sciences 65(11): 1087-1102.

Griffin, I. J., Davila, P. M. and Abrams, S. A. (2002). "Non-digestible oligosaccharides and calcium absorption in girls with adequate calcium intakes." British Journal of Nutrition 87: S187-S191.

Griffin, I. J., Hicks, P. M. D., Heaney, R. P. and Abrams, S. A. (2003). "Enriched chicory inulin increases calcium absorption mainly in girls with lower calcium absorption." Nutrition Research 23(7): 901-909.

Grynpas, M. D., Chachra, D. and Lundon, K. (2000). "Bone Quality In Animal Models of Osteoporosis." Drug Development Research 49: 146-158.

Gudiel-Urbano, M. and Goni, I. (2002). "Effect of fructooligosaccharide on nutritional parameters and mineral bioavailability in rats." Journal of the Science of Food and Agriculture 82(8): 913-917.

Halleen, J. M., Raisanen, S., Salo, J. J., Reddy, S. V., Roodman, G. D., Hentunen, T. A., Lehenkari, P. P., Kaija, H., Vihko, P. and Vaananen, H. K. (1999). "Intracellular Fragmentation of Bone Resorption Products by Reactive Oxygen Species Generated by Osteoclastic Tartrate-resistant Acid Phosphatase." J. Biol. Chem. 274(33): 22907-22910.

Heaney, R. P., Abrams, S., Dawson-Hughes, B., Looker, A., Marcus, R., Matkovic, V. and Weaver, C. (2000). "Peak bone mass." Osteoporosis International 11(12): 985-1009.

Hoenderop, J. G. J., van der Kemp, A. W. C. M., Hartog, A., van de Graaf, S. F. J., van Os, C. H., Willems, P. H. G. M. and Bindels, R. J. M. (1999). "Molecular Identification of the Apical Ca2+ Channel in 1,25-Dihydroxyvitamin D3-responsive Epithelia." J. Biol. Chem. 274(13): 8375-8378.

Hofbauer, L. C., Gori, F., Riggs, B. L., Lacey, D. L., Dunstan, C. R., Spelsberg, T. C. and Khosla, S. (1999). "Stimulation of osteoprotegerin ligand and inhibition of osteoprotegerin production by glucocorticoids in human osteoblastic lineage cells: Potential paracrine mechanisms of glucocorticoid-induced osteoporosis." Endocrinology 140(10): 4382-4389.

Holick, M. F. (2000). Vitamin D. Biochemical and Physiological Aspects of Human Nutrition. M. H. Stipanuk. Philadelphia, W.B. Saunders: 624-39.

Hong, M. H., Williams, H., Jin, C. H. and Pike, J. W. (2000). "The inhibitory effect of interleukin-10 on mouse osteoclast formation involves novel tyrosine-phosphorylated proteins." Journal of Bone and Mineral Research 15(5): 911-918.

Ichikawa, H. and Sakata, T. (1998). "Stimulation of epithelial cell proliferation of isolated distal colon of rats by continuous colonic infusion of ammonia or short-chain fatty acids is nonadditive." Journal of Nutrition 128(5): 843-847.

Idriss, H. T. and Naismith, J. H. (2000). "TNF alpha and the TNF receptor superfamily: Structure-function relationship(s)." Microscopy Research and Technique 50(3): 184-195.

Jiang, C. S., Giger, M. L., Kwak, S. M., Chinander, M. R., Martell, J. M. and Favus, M. J. (2000). "Normalized BMD as a predictor of bone strength." Academic Radiology 7(1): 33-39.

Jilka, R. L., Weinstein, R. S., Bellido, T., Roberson, P., Parfitt, A. M. and Manolagas, S. C. (1999). "Increased bone formation by prevention of osteoblast apoptosis with parathyroid hormone." Journal of Clinical Investigation 104(4): 439-446.

Kalkwarf, H. J., Khoury, J. C., Bean, J. and Elliot, J. G. (2004). "Vitamin K, bone turnover, and bone mass in girls." American Journal of Clinical Nutrition 80(4): 1075-1080.

Kalu, D. N. and Orhii, P. B. (1999). "Calcium absorption and bone loss in ovariectomized rats fed varying levels of dietary calcium." Calcified Tissue International 65(1): 73-77.

Kamchan, A., Puwastien, P., Sirichakwal, P. P. P. P. and Kongkachuichai, R. (2004). "In vitro calcium bioavailability of vegetables, legumes and seeds." Journal of Food Composition and Analysis 17(3-4): 311-320.

King, J. C., Shames, D. M. and Woodhouse, L. R. (2000). "Zinc Homeostasis in Humans." J. Nutr. 130(5): 1360S-1366.

Kipp, D. E., McElvain, M., Kimmel, D. B., Akhter, M. P., Robinson, R. G. and Lukert, B. P. (1996). "Scurvy results in decreased collagen synthesis and bone density in the guinea pig animal model." Bone 18(3): 281-288.

Kishi, S. and Yamaguchi, M. (1997). "Characterization of zinc effect to inhibit osteoclast-like cell formation in mouse marrow culture: Interaction with dexamethasone." Molecular and Cellular Biochemistry 166(1-2): 145-151.

Kohrt, W. M., Bloomfield, S. A., Little, K. D., Nelson, M. E. and Yingling, V. R. (2004). "Physical activity and bone health." Medicine and Science in Sports and Exercise 36(11): 1985-1996.

Komori, T., Yagi, H., Nomura, S., Yamaguchi, A., Sasaki, K., Deguchi, K., Shimizu, Y., Bronson, R. T., Gao, Y. H., Inada, M., Sato, M., Okamoto, R., Kitamura, Y., Yoshiki, S. and Kishimoto, T. (1997). "Targeted Disruption of Cbfa1 Results in a Complete Lack of Bone Formation owing to Maturational Arrest of Osteoblasts." Cell 89: 755–764.

Kong, Y. Y., Yoshida, H., Sarosi, I., Tan, H. L., Timms, E., Capparelli, C., Morony, S., Oliveira-dos-Santos, A. J., Van, G., Itie, A., Khoo, W., Wakeham, A., Dunstan, C. R., Lacey, D. L., Mak, T. W., Boyle, W. J. and Penninger, J. M. (1999). "OPGL is a key regulator of osteoclastogenesis, lymphocyte development and lymph-node organogenesis." Nature 397(6717): 315-323.

Konrad, M., Schlingmann, K. P. and Gudermann, T. (2004). "Insights into the molecular nature of magnesium homeostasis." American Journal of Physiology-Renal Physiology 286(4): F599-F605.

Krebs, N. F. (2000). "Overview of Zinc Absorption and Excretion in the Human Gastrointestinal Tract." J. Nutr. 130(5): 1374S-1377.

Kruger, M. C., Brown, K. E., Collett, G., Layton, L. and Schollum, L. M. (2003b). "The effect of fructooligosaccharides with various degrees of polymerization on calcium bioavailability in the growing rat." Experimental Biology and Medicine 228(6): 683-688.

Kruger, M. C., Gallaher, B. W. and Schollum, L. M. (2003a). The Effect Of DR10™ And DR20™ On Bone Density And Biochemical Markers Of Bone Turnover In The Ovariectomised Female Rat. Confidential Report. Palmerston North, IFNHH, Massey University: 26.

Kuhn, R., Lohler, J., Rennick, D., Rajewsky, K. and Muller, W. (1993). "Interleukin-10-Deficient Mice Develop Chronic Enterocolitis." Cell 75(2): 263-274.

Langdahl, B. L., Kassem, M., Moller, M. K. and Eriksen, E. F. (1998). "The effects of IGF-I and IGF-II on proliferation and differentiation of human osteoblasts and interactions with growth hormone." European Journal of Clinical Investigation 28(3): 176-183.

Le Blay, G., Michel, C., Blottiere, H. M. and Cherbut, C. (1999). "Prolonged intake of fructo-oligosaccharides induces a short-term elevation of lactic acid-producing bacteria and a persistent increase in cecal butyrate in rats." Journal of Nutrition 129(12): 2231-2235.

Lian, J. B., Stein, G. S., Canalis, E., Robey, P. G. and Boskey, A. L. (1999). Bone Formation: Osteoblast Lineage Cells, Growth Factors, Matrix Proteins, and the Mineralisation Process. Primer on the Metabolic Bone Diseases and Disorders of Mineral Metabolism. M. J. Favus. Philadelphia, Lippincott Williams & Wilkins: 14-29.

Liuzzi, J. P., Blanchard, R. K. and Cousins, R. J. (2001). "Differential regulation of zinc transporter 1, 2, and 4 mRNA expression by dietary zinc in rats." Journal of Nutrition 131(1): 46-52.

Liuzzi, J. P. and Cousins, R. J. (2004). "Mammalian zinc transporters." Annual Review of Nutrition 24: 151-172.

Lomaga, M. A., Yeh, W. C., Sarosi, I., Duncan, G. S., Furlonger, C., Ho, A., Morony, S., Capparelli, C., Van, G., Kaufman, S., van der Heiden, A., Itie, A., Wakeham, A., Khoo, W., Sasaki, T., Cao, Z. D., Penninger, J. M., Paige, C. J., Lacey, D. L., Dunstan, C. R., Boyle, W. J., Goeddel, D. V. and Mak, T. W. (1999). "TRAF6 deficiency results in osteopetrosis and defective interleukin-1, CD40, and LPS signaling." Genes & Development 13(8): 1015-1024.

Lonnerdal, B. (2000). "Dietary factors influencing zinc absorption." Journal of Nutrition 130(5): 1378S-1383S.

Lopez, H. W., Coudray, C., Bellanger, J., Younes, H., Demigne, C. and Remesy, C. (1998). "Intestinal fermentation lessens the inhibitory effects of phytic acid on mineral utilization in rats." Journal of Nutrition 128(7): 1192-1198.

Lopez, H. W., Coudray, C., Levrat-Verny, M. A., Feillet-Coudray, C., Demigne, C. and Remesy, C. (2000). "Fructooligosaccharides enhance mineral apparent absorption and counteract the deleterious effects of phytic acid on mineral homeostasis in rats." Journal of Nutritional Biochemistry 11(10): 500-508.

Lopez, H. W., Leenhardt, F., Coudray, C. and Remesy, C. (2002). "Minerals and phytic acid interactions: is it a real problem for human nutrition?" International Journal of Food Science and Technology 37(7): 727-739.

Lopez, H. W., Levrat-Verny, M. A., Coudray, C., Besson, C., Krespine, V., Messager, A., Demigne, C. and Remesy, C. (2001). "Class 2 resistant starches lower plasma and liver lipids and improve mineral retention in rats." Journal of Nutrition 131(4): 1283-1289.

Loty, C., Sautier, J. M., Tan, M. T., Oboeuf, M., Jallot, E., Boulekbache, H., Greenspan, D. and Forest, N. (2001). "Bioactive glass stimulates in vitro osteoblast differentiation and creates a favorable template for bone tissue formation." Journal of Bone and Mineral Research 16(2): 231-239.

Manolagas, S. C. (2000). "Birth and death of bone cells: Basic regulatory mechanisms and implications for the pathogenesis and treatment of osteoporosis." Endocrine Reviews 21(2): 115-137.

Marcus, R. (1999). Physical Activity and Regulation of Bone Mass. Primer on the Metabolic Bone Diseases and Disorders of Mineral Metabolism. M. J. Favus. Philadelphia, Lippincott Williams and Wilkins: 262 - 264.

Mariadason, J. M., Catto-Smith, A. and Gibson, P. R. (1999). "Modulation of distal colonic epithelial barrier function by dietary fibre in normal rats." Gut 44(3): 394-399.

Martin, T. J. and Sims, N. A. (2005). "Osteoclast-derived activity in the coupling of bone formation to resorption." Trends in Molecular Medicine 11(2): 76-81.

Mason, A. C., Weaver, C. M., Kimmel, S. and Brown, R. K. (1993). "Effect of Soybean Phytate Content on Calcium Bioavailability in Mature and Immature Rats." Journal of Agricultural and Food Chemistry 41(2): 246-249.

McMahon, R. J. and Cousins, R. J. (1998). "Regulation of the zinc transporter ZnT-1 by dietary zinc." Proceedings of the National Academy of Sciences of the United States of America 95(9): 4841-4846.

Minamida, K., Sujaya, I. N., Tamura, A., Shigematsu, N., Sone, T., Yokota, A., Asano, K., Benno, Y. and Tomita, F. (2004). "The effects of di-D-fructofuranose-1,2 ': 2,3 '-dianhydride (DFA III) administration on human intestinal microbiota." Journal of Bioscience and Bioengineering 98(4): 244-250.

Mineo, H., Hara, H., Kikuchi, H., Sakurai, H. and Tomita, F. (2001). "Various indigestible saccharides enhance net calcium transport from the epithelium of the small and large intestine of rats in vitro." Journal of Nutrition 131(12): 3243-3246.

Morishita, T., Tamura, N., Makino, T. and Kudo, S. (1999). "Production of Menaquinones by Lactic Acid Bacteria." J. Dairy Sci. 82(9): 1897-1903.

Morohashi, T., Sano, T., Ohta, A. and Yamada, S. (1998). "True calcium absorption in the intestine is enhanced by fructooligosaccharide feeding in rats." Journal of Nutrition 128(10): 1815-1818.

Mosekilde, L. (1995). "Assessing bone quality -- Animal models in preclinical osteoporosis research." Bone 17(4, Supplement 1): S343-S352.

Mundy, G. R. (1999). Bone Remodeling. Primer on Metabolic Bone Diseases and Disorders of Mineral Metabolism. M. J. Favus. Philadelphia, Lippincott, Williams and Wilkins: 30-38.

Naber, T. H. J., Baadenhuysen, H., Jansen, J., vandenHamer, C. J. A. and vandenBroek, W. (1996). "Serum alkaline phosphatase activity during zinc deficiency and long-term inflammatory stress." Clinica Chimica Acta 249(1-2): 109-127.

Nagy, T. R., Prince, C. W. and Li, J. (2001). "Validation of Peripheral Dual-Energy X-ray Absorptiometry for the Measurement of Bone Mineral in Intact and Excised Long Bones of Rats." Journal of Bone and Mineral Research 16(9): 1682-1687.

Nakashima, T., Kobayashi, Y., Yamasaki, S., Kawakami, A., Eguchi, K., Sasaki, H. and Sakai, H. (2000). "Protein expression and functional difference of membrane-bound and soluble receptor activator of NF-kappa B ligand: Modulation of the expression by osteotropic factors and cytokines." Biochemical and Biophysical Research Communications 275(3): 768-775.

Nanes, M. S. (2003). "Tumor necrosis factor-alpha: molecular and cellular mechanisms in skeletal pathology." Gene 321: 1-15.

Narva, M., Collin, M., Lamberg-Allardt, C., Karkkainen, M., Poussa, T., Vapaatalo, H. and Korpela, R. (2004). "Effects of long-term intervention with Lactobacillus helveticus-fermented milk on bone

mineral density and bone mineral content in growing rats." Annals of Nutrition and Metabolism 48(4): 228-234.

Nesbitt, S. A. and Horton, M. A. (1997). "Trafficking of matrix collagens through bone-resorbing osteoclasts." Science 276(5310): 266-69.

Ohta, A., Baba, S., Ohtsuki, M., Taguchi, A., Adachi, T. and Hara, H. (1996). "Prevention of coprophagy modifies magnesium absorption in rats fed with fructooligosaccharides." British Journal of Nutrition 75(775-784).

Ohta, A., Motohashi, Y., Ohtsuki, M., Hirayama, M., Adachi, T. and Sakuma, K. (1998b). "Dietary fructooligosaccharides change the concentration of calbindin-D9k differently in the mucosa of the small and large intestine of rats." Journal of Nutrition 128(6): 934-939.

Ohta, A., Ohtsuki, M., Baba, S., Hirayama, M. and Adachi, T. (1998a). "Comparison of the nutritional effects of fructo-oligosaccharides of different sugar chain length in rats." Nutrition Research 18(1): 109-120.

Ohta, A., Ohtsuki, M., Baba, S., Takashi, A., Sakata, T. and Sakaguchi, E. (1994). "Calcium and Magnesium Absorption from the colon and rectum are increased in rats fed frutooligosaccharides." Journal of Nutrition 125: 2417 - 2424.

Ohta, A., Uehara, M., Sakai, K., Takasaki, M., Adlercreutz, H., Morohashi, T. and Ishimi, Y. (2002). "A combination of dietary fructooligosaccharides and isoflavone conjugates increases femoral bone mineral density and equol production in ovariectomized mice." Journal of Nutrition 132(7): 2048-2054.

Olsen, B. R., Reginato, A. M. and Wang, W. F. (2000). "Bone development." Annual Review of Cell and Developmental Biology 16: 191-220.

Otto, F., Thornell, A. P., Crompton, T., Denzel, A., Gilmour, K. C., Rosewell, I. R., Stamp, G. W. H., Beddington, R. S. P., Mundlos, S., Olsen, B. R., Selby, P. B. and Owen, M. J. (1997). "Cbfa1, a Candidate Gene for Cleidocranial Dysplasia Syndrome, Is Essential for Osteoblast Differentiation and Bone Development." Cell 89: 765–77.

Ovesen, J., Moller-Madsen, B., Thomsen, J. S., Danscher, G. and Mosekilde, L. (2001). "The positive effects of zinc on skeletal strength in growing rats." Bone 29(6): 565-570.

Oxford Reference Online (2002). Osteopetrosis. Concise Medical Dictionary. O. U. Press. Oxford, Oxford University Press. 2005.

Oxford Reference Online (2004). Ossification. A Dictionary of Biology. O. U. Press. Oxford, Oxford University Press. 2005.

Oxford University Press (2003). Bone. A Dictionary of Nursing. Oxford Reference Online. Oxford, Oxford University Press. 2005.

Pansu, D., Bellaton, C. and Bronner, F. (1983b). "Developmental-Changes in the Mechanisms of Duodenal Calcium-Transport in the Rat." American Journal of Physiology 244(1): G20-G26.

Pansu, D., Bellaton, C., Roche, C. and Bronner, F. (1983a). "Duodenal and Ileal Calcium-Absorption in the Rat and Effects of Vitamin-D." American Journal of Physiology 244(6): G695-G700.

Parker, P., Jones, G. and Smith, S. (2003). "Mixed cultures of food-grade probiotic bacteria and enteric bacteria demonstrate both synergism and inhibition of menaquinone production." Journal of Food Science 68(7): 2325-2330.

Peacock, M. (1991). "Calcium-Absorption Efficiency and Calcium Requirements in Children and Adolescents." American Journal of Clinical Nutrition 54(1): S261-S265.

Peng, J. B., Chen, X. Z., Berger, U. V., Vassilev, P. M., Tsukaguchi, H., Brown, E. M. and Hediger, M. A. (1999). "Molecular cloning and characterization of a channel-like transporter mediating intestinal calcium absorption." Journal of Biological Chemistry 274(32): 22739-22746.

Perez-Llamas, F., Garaulet, M., Martinez, J. A., Marin, J. F., Larque, E. and Zamora, S. (2001). "Influence of dietary protein type and iron source on the absorption of amino acids and minerals." Journal of Physiology and Biochemistry 57(4): 321-328.

Pestka, S., Krause, C. D., Sarkar, D., Walter, M. R., Shi, Y. F. and Fisher, P. B. (2004). "Interleukin-10 and related cytokines and receptors." Annual Review of Immunology 22: 929-979.

Plebani, M., Bernardi, D., Zaninotto, M., DePaoli, M., Secchiero, S. and Sciacovelli, L. (1996). "New and traditional serum markers of bone metabolism in the detection of skeletal metastases." Clinical Biochemistry 29(1): 67-72.

Pocock, G. and Richards, C. D. (2004). The Physiology of Bone. Human Physiology: The Basis of Medicine. Oxford, Oxford University Press: 554-556.

Raschka, L. and Daniel, H. (2005). "Diet composition and age determine the effects of inulin-type fructans on intestinal calcium absorption in rat." European Journal of Nutrition 44(6): 360-364.

Rideout, T. C. and Fan, M. Z. (2004). "Nutrient utilisation in response to dietary supplementation of chicory inulin in growing pigs." Journal of the Science of Food and Agriculture 84(9): 1005-1012.

Riggs, B. L. (2000). "The mechanisms of estrogen regulation of bone resorption." Journal of Clinical Investigation 106(10): 1203-1204.

Roberfroid, M. and Slavin, J. (2000b). "Nondigestible oligosaccharides." Critical Reviews in Food Science and Nutrition 40(6): 461-480.

Roberfroid, M. B. (2000a). "Chicory fructooligosaccharides and the gastrointestinal tract." Nutrition 16(7-8): 677-679.

Roberfroid, M. B. (2005). "Introducing inulin-type fructans." British Journal of Nutrition 93(S1): S13-S25.

Roller, M., Rechkemmer, G. and Watzl, B. (2004). "Prebiotic inulin enriched with oligofructose in combination with the probiotics Lactobacillus rhamnosus and Bifidobacterium lactis modulates intestinal immune functions in rats." Journal of Nutrition 134(1): 153-156.

Rousselle, A. V. and Heymann, D. (2002). "Osteoclastic acidification pathways during bone resorption." Bone 30(4): 533-540.

Roy, M. E., Nishimoto, S. K., Rho, J. Y., Bhattacharya, S. K., Lin, J. S. and Pharr, G. M. (2001). "Correlations between osteocalcin content, degree of mineralization, and mechanical properties of C-Carpio rib bone." Journal of Biomedical Materials Research 54(4): 547-553.

Rude, R. K. (2000). Magnesium. Biochemical and Physiological Aspects of Human Nutrition. M. H. Stipanuk. Philadelphia, W.B. Saunders: 671 - 685.

Rude, R. K., Gruber, H. E., Norton, H. J., Wei, L. Y., Frausto, A. and Kilburn, J. (2005). "Dietary magnesium reduction to 25% of nutrient requirement disrupts bone and mineral metabolism in the rat." Bone 37(2): 211-219.

Rude, R. K., Gruber, H. E., Wei, L. Y., Frausto, A. and Mills, B. G. (2003). "Magnesium deficiency: Effect on bone and mineral metabolism in the mouse." Calcified Tissue International 72(1): 32-41.

Rude, R. K., Kirchen, M. E., Gruber, H. E., Stasky, A. A. and Meyer, M. H. (1998). "Magnesium deficiency induces bone loss in the rat." Mineral and Electrolyte Metabolism 24(5): 314-320.

Rude, R. K., Oldham, S. B. and Singer, F. R. (1976). "Functional Hypoparathyroidism and Parathyroid-Hormone End-Organ Resistance in Human Magnesium-Deficiency." Clinical Endocrinology 5(3): 209-224.

Sainsbury, R. and Richards, R. (1997). Prevention of Osteoporosis in Older Populations: A report to the National Health Committee. Wellington: 50.

Sako, T., Matsumoto, K. and Tanaka, R. (1999). "Recent progress on research and applications of non-digestible galacto-oligosaccharides." International Dairy Journal 9(1): 69-80.

Sakuma, K. (2002). "Molecular Mechanism of the Effect of Fructoologosaccharides on Calcium Absorption." Bioscience Microflora 21(1): 13 - 20.

Salo, J. and Lehenkari, P. (1997). "Removal of osteoclast bone resorption products by transcytosis." Science 276(5310): 270-3.

Schlingmann, K. P., Weber, S., Peters, M., Nejsum, L. N., Vitzthum, H., Klingel, K., Kratz, M., Haddad, E., Ristoff, E., Dinour, D., Syrrou, M., Nielsen, S., Sassen, M., Waldegger, S., Seyberth, H. W. and Konrad, M. (2002). "Hypomagnesemia with secondary hypocalcemia is caused by mutations in TRPM6, a new member of the TRPM gene family." Nature Genetics 31(2): 166-170.

Scholz-Ahrens, K. E., Acil, Y. and Schrezenmeir, J. (2002). "Effect of oligofructose or dietary calcium on repeated calcium and phosphorus balances, bone mineralization and trabecular structure in ovariectomized rats." British Journal of Nutrition 88(4): 365-377.

Schweigel, M. and Martens, H. (2000). "Magnesium transport in the gastrointestinal tract." Frontiers in Bioscience 5: D666-D677.

Sengupta, S., Arshad, M., Sharma, S., Dubey, M. and Singh, M. M. (2005). "Attainment of peak bone mass and bone turnover rate in relation to estrous cycle, pregnancy and lactation in colony-bred Sprague-Dawley rats: Suitability for studies on pathophysiology of bone and therapeutic measures for its management." The Journal of Steroid Biochemistry and Molecular Biology 94(5): 421-429.

Shalhoub, V., Faust, J., Boyle, W. J., Dunstan, C. R., Kelley, M., Kaufman, S., Scully, S., Van, G. and Lacey, D. L. (1999). "Osteoprotegerin and osteoprotegerin ligand effects on osteoclast formation from human peripheral blood mononuclear cell precursors." Journal of Cellular Biochemistry 72(2): 251-261.

Simpson, R. J., Hammacher, A., Smith, D. K., Matthews, J. M. and Ward, L. D. (1997). "Interleukin-6: Structure-function relationships." Protein Science 6(5): 929-955.

Slepchenko, B. M. and Bronner, F. (2001). "Modeling of transcellular Ca transport in rat duodenum points to coexistence of two mechanisms of apical entry." American Journal of Physiology-Cell Physiology 281(1): C270-C281.

Song, H. M., Nacamuli, R. P., Xia, W., Bari, A. S., Shi, Y. Y., Fang, T. D. and Longaker, M. T. (2005). "High-dose retinoic acid modulates rat calvarial osteoblast biology." Journal of Cellular Physiology 202(1): 255-262.

Steeve, K. T., Marc, P., Sandrine, T., Dominique, H. and Yannick, F. (2004). "IL-6, RANKL, TNF-alpha/IL-1: interrelations in bone resorption pathophysiology." Cytokine & Growth Factor Reviews 15(1): 49-60.

Stendig-Lindberg, G., Koeller, W., Bauer, A. and Rob, P. M. (2004). "Experimentally induced prolonged magnesium deficiency causes osteoporosis in the rat." European Journal of Internal Medicine 15: 97-107.

Stenvinkel, P., Ketteler, M., Johnson, R. J., Lindholm, B., Pecoits-Filho, R., Riella, M., Heimburger, O., Cederholm, T. and Girndt, M. (2005). "IL-10, IL-6, and TNF-alpha: Central factors in the altered

cytokine network of uremia - The good, the bad, and the ugly." Kidney International 67(4): 1216-1233.

Subcommittee on Laboratory Animal Nutrition, Committee on Animal Nutrition, Board on Agriculture and National Research Council (1995). Nutrient requirements of laboratory animals. Washington, National Academy of Sciences.

Sutter, V. L., Citron, D. M., Edelstein, M. A. C. and Finegold, S. M. (1985). Wadsworth Anaerobic Bacteriology Manual. California, Star Publishing Company.

Suttie, J. K. (2000). Vitamin K. Biochemical and Physiological Aspects of Human Nutrition. M. H. Stipanuk. Philadelphia, W.B. Saunders: 568 - 583.

Swarthout, J. T., D'Alonzo, R. C., Selvamurugan, N. and Partridge, N. C. (2002). "Parathyroid hormone-dependent signaling pathways regulating genes in bone cells." Gene 282(1-2): 1-17.

Tahiri, M., Tressol, J. C., Arnaud, J., Bornet, F., Bouteloup-Demange, C., Feillet-Coudray, C., Ducros, V., Pepin, D., Brouns, F., Roussel, A. M., Rayssignuier, Y. and Coudray, C. (2001). "Five-Week Intake of Short-Chain Fructo-oligosaccharides Increases Intestinal Absorption and status of magnesium in post-menopausal women." Journal of Bone and Mineral Research 16(11): 2152 - 2160.

Tahiri, M., Tressol, J. C., Arnaud, Y., Bornet, F. R. J., Bouteloup-Demange, C., Feillet-Coudray, C., Brandolini, M., Ducros, V., Pepin, D., Brouns, F., Roussel, A. M., Rayssiguier, Y. and Coudray, C. (2003). "Effect of short-chain fructooligosaccharides on intestinal calcium absorption and calcium status in postmenopausal women: a stable-isotope study." American Journal of Clinical Nutrition 77(2): 449-457.

Takahara, S., Morohashi, T., Sano, T., Ohta, A., Yamada, S. and Sasa, R. (2000). "Fructooligosaccharide consumption enhances femoral bone volume and mineral concentrations in rats." Journal of Nutrition 130(7): 1792-1795.

Takamizawa, S., Maehata, Y., Imai, K., Senoo, H., Sato, S. and Hata, R. I. (2004). "Effects of ascorbic acid and ascorbic acid 2-phosphate, a long-acting vitamin C derivative, on the proliferation and differentiation of human osteoblast-like cells." Cell Biology International 28(4): 255-265.

Takeuchi, Y., Suzawa, M., Fukumoto, S. and Fujita, T. (2000). "Vitamin K-2 inhibits adipogenesis, osteoclastogenesis, and ODF/RANK ligand expression in murine bone marrow cell cultures." Bone 27(6): 769-776.

Tate, M. L. K. (2003). ""Whither flows the fluid in bone?" An osteocyte's perspective." Journal of Biomechanics 36(10): 1409-1424.

Thomas, D. M., Udagawa, N., Hards, D. K., Quinn, J. M. W., Moseley, J. M., Findlay, D. M. and Best, J. D. (1998). " Insulin Receptor Expression in Primary and Cultured Osteoclast-like Cells." Bone 23(3): 181-186.

Tondravi, M. M., McKercher, S. R., Anderson, K., Erdmann, J. M., Quiroz, M., Maki, R. and Teitelbaum, S. L. (1997). "Osteopetrosis in mice lacking haematopoietic transcription factor PU.1." Nature 386(6620): 81-84.

Tsuchiya, H. and Bates, C. J. (2003). "Comparison of vitamin C deficiency with food restriction on collagen cross-link ratios in bone, urine and skin of weanling guinea-pigs." British Journal of Nutrition 89(3): 303-310.

Tungland, B. C. (2003). Fructooligosaccharides and other fructans: Structures and Occurrence, Production, Regulatory Aspects, and Nutritional Health Significance. Oligosaccharides in Food and Agriculture. G. Eggleston and G. L. Cote. Washington, American Chemical Society. 849: 135-152.

Turner, C. H. (2002). "Biomechanics of Bone: Determinants of Skeletal Fragility and Bone Quality." Osteoporosis International 13: 97-104.

Udagawa, N., Takahashi, N., Yasuda, H., Mizuno, A., Itoh, K., Ueno, Y., Sjinki, T., Gillespie, M. T., Martin, T. J., Higashio, K. and Suda, T. (1999). "Osteoprotegerin Produced by Osteoblasts is an Important Regulator in Osteoclast Development and Function." Endocrinology 141(9): 3478 - 3484.

Vaananen, H. K., Zhao, H., Mulari, M. and Halleen, J. M. (2000). "The cell biology of osteoclast function." Journal of Cell Science 113(3): 377-381.

van den Heuvel, E., Muys, T., van Dokkum, W. and Schaafsma, G. (1999). "Oligofructose stimulates calcium absorption in adolescents." American Journal of Clinical Nutrition 69(3): 544-548.

van den Heuvel, E., Schaafsma, G., Muys, T. and van Dokkum, W. (1998). "Nondigestible oligosaccharides do not interfere with calcium and nonheme-iron absorption in young, healthy men." American Journal of Clinical Nutrition 67(3): 445-451.

Van Loo, J. (2004). "The specificity of the interaction with intestinal bacterial fermentation by prebiotics determines their physiological efficacy." Nutrition Research Reviews 17(1): 89-98.

Van Loo, J., Cummings, J., Delzenne, N., Englyst, H., Franck, A., Hopkins, M., Kok, N., Macfarlane, G., Newton, D., Quigley, M., Roberfroid, M., van Vliet, T. and van den Heuvel, E. (1999). "Functional food properties of non-digestible oligosaccharides: a consensus report from the ENDO project (DGXII AIRII-CT94-1095)." British Journal of Nutrition 81: 121-132.

Voets, T., Nilius, B., Hoefs, S., van der Kemp, A., Droogmans, G., Bindels, R. J. M. and Hoenderop, J. G. J. (2004). "TRPM6 forms the Mg2+ influx channel involved in intestinal and renal Mg2+ absorption." Journal of Biological Chemistry 279(1): 19-25.

Wagner, E. F. and Karsenty, G. (2001). "Genetic control of skeletal development." Current Opinion in Genetics & Development 11(5): 527-532.

Walder, R. Y., Landau, D., Meyer, P., Shalev, H., Tsolia, M., Borochowitz, Z., Boettger, M. B., Beck, G. E., Englehardt, R. K., Carmi, R. and Sheffield, V. C. (2002). "Mutation of TRPM6 causes familial hypomagnesemia with secondary hypocalcemia." Nature Genetics 31(2): 171-174.

Wang, K., Zhou, B., Kuo, Y. M., Zemansky, J. and Gitschier, J. (2002). "A novel member of a zinc transporter family is defective in acrodermatitis enteropathica." American Journal of Human Genetics 71(1): 66-73.

Wang, L. X., Walia, B., Evans, J., Gewirtz, A. T., Merlin, D. and Sitaraman, S. V. (2003). "IL-6 induces NF-kappa B activation in the intestinal epithelia." Journal of Immunology 171(6): 3194-3201.

Wasserman, R. H. and Fullmer, C. S. (1995). "Vitamin-D and Intestinal Calcium-Transport - Facts, Speculations and Hypotheses." Journal of Nutrition 125(7): S1971-S1979.

Watkins, B. A., Li, Y., Lippman, H. E. and Seifert, M. F. (2001). "Omega-3 polyunsaturated fatty acids and skeletal health." Experimental Biology and Medicine 226(6): 485-497.

Weaver, C. M., Heaney, R. P., Nickel, K. P. and Packard, P. I. (1997). "Calcium bioavailability from high oxalate vegetables: Chinese vegetables, sweet potatoes and rhubarb." Journal of Food Science 62(3): 524-525.

Weber, P. (2001). "Vitamin K and bone health." Nutrition 17(10): 880-887.

Wolf, R. L., Cauley, J. A., Baker, C. E., Ferrell, R. E., Charron, M., Caggiula, A. W., Salamone, L. M., Heaney, R. P. and Kuller, L. H. (2000). "Factors associated with calcium absorption efficiency in pre- and perimenopausal women." American Journal of Clinical Nutrition 72(2): 466-471.

Wood, R. J. (2000). Calcium and Phosphorous. Biochemical and Physiological Aspects of Human Nutrition. M. H. Stipanuk. Philadelphia, W.B. Saunders: 643-670.

Wood, R. J. and Serfaty-Lacrosniere, M. D. (1992). "Gastric Acidity, Atrophic Gastritis, and Calcium Absorption." Nutrition Reviews 50(2): 33-40.

World Health Organisation (2003a). Epidemiology and Risk Factors. Prevention and Management of Osteoporosis. World Health Organisation Scientific Group. Geneva. 921: 44.

World Health Organisation (2003b). Introduction. Prevention and Management of Osteoporosis. Geneva, World Health Organisation: 1-9.

Xing, L. P. and Boyce, B. F. (2005). "Regulation of apoptosis in osteoclasts and osteoblastic cells." Biochemical and Biophysical Research Communications 328(3): 709-720.

Yagami, K., Suh, J. Y., Enomoto-Iwamoto, M., Koyama, E., Abrams, W. R., Shapiro, I. M., Pacifici, M. and Iwamoto, M. (1999). "Matrix GLA protein is a developmental regulator of chondrocyte mineralization and, when constitutively expressed, blocks endochondral and intramembranous ossification in the limb." Journal of Cell Biology 147(5): 1097-1108.

Yamaguchi, M. and Kishi, S. (1995). "Differential-Effects of Transforming Growth-Factor-Beta on Osteoclast-Like Cell-Formation in Mouse Marrow Culture - Relation to the Effect of Zinc-Chelating Dipeptides." Peptides 16(8): 1483-1488.

Yamaguchi, M. and Kishi, S. (1996). "Zinc compounds inhibit osteoclast-like cell formation at the earlier stage of rat marrow culture but not osteoclast function." Molecular and Cellular Biochemistry 158(2): 171-177.

Yaqoob, P., Newsholme, E. A. and Calder, P. C. (1994). "The effect of dietary lipid manipulation on rat lymphocyte subsets and proliferation." Immunology 82: 603–610.

Yaqoob, P., Newsholme, E. A. and Calder, P. C. (1999). "Comparison of cytokine production in cultures of whole human blood and purified mononuclear cells." Cytokine 11(8): 600-605.

Younes, H., Coudray, C., Bellanger, J., Demigne, C., Rayssiguier, Y. and Remesy, C. (2001). "Effects of two fermentable carbohydrates (inulin and resistant starch) and their combination on calcium and magnesium balance in rats." British Journal of Nutrition 86(4): 479-485.

Younes, H., Demigne, C. and Remesy, C. (1996). "Acidic fermentation in the caecum increases absorption of calcium and magnesium in the large intestine of the rat." British Journal of Nutrition 75(2): 301-314.

Yu, X., Scholler, J. and Foged, N. T. (1996). "Interaction between effects of parathyroid hormone and bisphosphonate on regulation of osteoclast activity by the osteoblast-like cell line UMR-106." Bone 19(4): 339-345.

Zafar, T. A., Weaver, C. M., Zhao, Y. D., Martin, B. R. and Wastney, M. E. (2004). "Nondigestible oligosaccharides increase calcium absorption and suppress bone resorption in ovariectomized rats." Journal of Nutrition 134(2): 399-402.

Zaidi, M., Iqbal, J., Sun, L. and Blair, H. C. (2005). "Molecules, mechanisms, and momentum in bone and cartilage research." Biochemical and Biophysical Research Communications 328(3): 647-650.

Zornitze, A. e. and Bronner, F. (1971). "In-Situ Studies of Calcium Absorption in Rats." American Journal of Physiology 220(5): 1261-&.