Effect of altering dietary calcium and vitamin D in AIN93G diet on bone development in CD-1 male mice

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ABSTRACT

The current levels of vitamin D (vit D) and calcium (Ca) in the AIN93G diet may be higher than required to support healthy bone structure and bone mineral density (BMD) in rodent models. The study objectives were to determine if lower levels of vit D (400 and 100 IU/kg) and Ca (0.35, 0.3, or 0.25%) support healthy bone structure and BMD while maintaining intestinal integrity in weanling male CD-1 mice up to 4 months of age. Lowering the levels of vit D (400 IU/kg) and Ca (0.35%) had no effect on bone structure and BMD, and fecal albumin levels were not different among groups, however, differences were observed in measures of serum LPS among groups. Adjusting the level of vit D to 400 IU/kg and Ca to 0.35% may provide sufficient levels for healthy bone structure and BMD, however further analysis of intestinal integrity is required.

Key Words: AIN-93G, Vitamin D, Calcium, Bone, Intestine
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ABBREVIATIONS

1,25(OH)D: 1,25-hydroxycholecalciferol
25(OH)D: 25-hydroxycholecalciferol
AIN: American Institute of Nutrition
BMC: Bone Mineral Content
BMD: Bone Mineral Density
BV/TV: Percent Bone Volume
Ca: Calcium
Conn. D: Connectivity Density
CS: Cross Sectional Area
Ct. Ar: Cortical Area
Ct. Ar/Tt. Ar: Cortical Area Fraction
Ct. Th: Cortical Thickness
DXA: Dual Energy X-ray Absorptiometry
Ecc.: Eccentricity
Ec. Pm: Endocortical Perimeter
GIT: Gastrointestinal Tract
GM: Gut Microbiota
LPS: Lipopolysaccharides
Ma. Ar: Medullary Area
PBM: Peak Bone Mass
P: Phosphorus
Ps. Pm: Periosteal Perimeter
ROI: Region of Interest
Tb. N: Trabecular Number
Tb. Th: Trabecular Thickness
Tb. Sp: Trabecular Separation
Tt. Ar: Total Bone Area
μCT: Micro Computed Tomography
Chapter 1
INTRODUCTION

Osteoporosis is characterised by low bone mass and structural deterioration resulting in an increased risk of fragility fracture [1]. In Canada, 1 in 3 women and 1 in 5 men will suffer an osteoporotic fracture in their lifetime [2]. All costs associated with osteoporotic fractures have been estimated at 3.9 billion dollars per year in Canada [3]. Individuals who fracture as the result of osteoporosis often endure permanent side effects such as a lower quality of life, chronic pain, loss of mobility, decreased independence, lowered self-esteem, and ultimately a decreased life expectancy [2, 4]. Considering the financial and personal burden of osteoporotic fractures, alongside the lack of ideal treatments, dietary prevention strategies warrant investigation.

Osteoporosis is generally considered a geriatric disease, yet choices made early in life can set a trajectory for either poor or better bone health later in adulthood [5]. Peak bone mass, defined as the maximum amount of bone mass attained, is greatly influenced by nutritional status in early life [6]. Nutritional interventions during early life may provide strategies to achieve or exceed genetically programmed peak bone mass, and thus decrease the risk of an osteoporosis-related fragility fracture later in life. Because of the long duration required to study changes in bone health in humans, rodent models are used and allow researchers to identify how dietary interventions, with whole foods, individual food components or nutrients, affect bone at critical stages of development. The effects of such nutritional interventions can be achieved within a relatively short period of time, often months. Moreover, three-dimensional bone structure can be assessed directly and longitudinally which provides valuable information about how bone structure responds to
a dietary intervention at specific stages of the lifespan, and thus, provides insight into the quality of the bone structure and potentially its ability to withstand fracture.

In the field of Nutritional Sciences, it is essential to use a standardized control diet containing purified components such that studies within or among research groups can be compared. The American Institute of Nutrition (AIN) formed a committee in 1973 to develop the AIN-76 diet, and more recently the AIN-93G diet, to provide a standardized diet for growing laboratory rodent models [7]. While this diet provides sufficient concentrations of nutrients for the growth and development of rodent bones, the evidence supporting the levels of vitamin D (vit D) and calcium (Ca) are based on whole animal growth, bone mineral content (BMC) (measured using an ashing technique, a now primitive method for evaluating bone health) and reducing nephrocalcinosis [7-9]. In fact, vit D and Ca at their current levels (1000 IU/kg and 0.5%, respectively) may be in excess as normal growth, bone structure, and BMD have been observed in rodents at markedly lower levels [10-13]. It is important to identify levels of dietary vit D and Ca that support normal bone development in specific animal models as these higher than required levels may mask potential benefits of novel bone supporting food components used as dietary interventions.

It is now understood that nutritional interventions can influence the health of the gastrointestinal tract (GIT), including the concentration and composition of the microorganisms inhabiting it [14, 15]. The GIT controls the transport of nutrients into the circulation; and acts as a barrier protecting the host from the infiltration of harmful microorganisms and the molecules they synthesize. There is evidence that nutrition can influence the integrity of the intestinal barrier allowing the infiltration of microorganisms
or proinflammatory molecules into circulation and may cause chronic low-grade inflammation which has been linked to the deterioration of bone [15-17].

_This research was designed to determine if a lower level of both dietary vit D (400 or 100 IU/kg) and Ca (0.35, 0.3, or 0.25%) in the AIN-93G diet supports bone health in growing male CD-1 mice as they are commonly used by our research group to study the effects of nutritional interventions on bone outcomes at various stages of the lifespan. This research also investigated if alterations in vit D and Ca influence the integrity of the intestine, measured by serum lipopolysaccharide levels and fecal albumin content, and whether such changes are associated with outcomes of bone health, including BMD and three-dimensional bone structure._
Chapter 2

2 LITERATURE REVIEW

2.1 Bone

The skeletal system has a multitude of functions. The macroscopic structure provides the framework for posture, protects internal organs, acts as a reservoir for minerals to maintain blood mineral homeostasis, and the marrow of bone is responsible for hematopoiesis, red blood cell formation [18]. Bone is also an attachment site for skeletal muscle. When a muscle contracts, it pulls on bone which causes rotation about the joint, creating a lever action. While bone provides a rigid structure, it must also contain a degree of flexibility to absorb forces, as this allows bone to deform reversibly under loads without fracturing. When bone experiences loads greater than its capacity to deform reversibly, the bone enters the “plastic” region and permanent deformation occurs causing microfractures or a fracture of the whole bone [19]. Therefore, bone must be a dynamic organ and be constantly changing its structure in response to the demands from mechanical loading via the processes of modeling and remodeling.

2.1.1 Bone modeling and remodeling

The ability of a bone to adapt, change shape, and alter its composition is governed by the process of modeling and remodeling, and both processes are the coordinated action of osteoblasts, bone forming cells, and osteoclasts, bone resorbing cells [20]. Bone modeling, or formation, causes changes in the bone shape and moves bone surfaces in space to change its thickness, and ultimately to alter the strength of a bone [20]. Remodeling is the process of turning over patches of bone through resorption by osteoclasts cells which is then followed by osteoblasts filling in the excavated site with
new bone. A balance between the activity of osteoclasts and osteoblasts dictates the resultant quantity and strength of the bone. This is controlled by hormonal, mechanical, genetic, and nutritional factors.

2.1.2 Types of bone

The two major types of bone are trabecular (spongy) and cortical (dense) bone. Trabecular bone is found at the epiphysis and metaphysis of long bones, sandwiched between cortical bone in flat bones, and in the vertebral bodies [18]. Consisting of a network of bone struts, providing light-weight support, trabecular bone ‘funnels’ forces toward the dense cortical bone. Because of its high surface area to volume ratio, trabecular bone also provides a site that can be rapidly metabolized during bone resorption [21]. Cortical bone is the dense outer shell of long bones, as well as the inner and outer surfaces of flat bones [18]. The interior surface of cortical bone is referred to as the endocortical surface, and the exterior surface is referred to as the periosteal surface. Over time, resorption occurs on the endocortical surface, and modeling occurs on the periosteal surface resulting in an increased medullary area and wider bone.

Preclinical models are often used to study bone as it allows for more invasive measurements. It is now common practice in bone research to use ex vivo micro-computed tomography (µCT) to assess three-dimensional bone structure that provides a surrogate measure of bone strength. Moreover, in vivo µCT allows for longitudinal assessment of bone structure at multiple time points during the lifespan [22]. Researchers can control and monitor nutritional intake of rodents using standardized control diets such as the AIN-93G diet. Modification of the composition of such diets to investigate the response of bone structure, BMD and growth can also be performed to study the effects
This thesis incorporated the AIN-93G diet, and modified versions containing lower levels of both vit D (400 or 100 IU/kg) and Ca (0.35, 0.3, 0.25%) to investigate the effects on bone. Using µCT, either in vivo (tibia) or ex vivo (spine, mandible), we investigated trabecular and cortical bone in both the appendicular and axial skeleton and mandible, to study structural properties of multiple skeletal sites.

2.2 AIN-93G Diet

Rodent models are commonly used in the fields of Nutritional Sciences, Toxicology, Pathology, Carcinogenesis, and Aging. Scientists recognized that the nutrition they were providing rodents could be influencing their data and sought guidance from nutritional experts [23]. A standardized diet containing adequate nutrition for normal growth and development was needed to control for dietary variables when comparing results within and between research groups. Originally, researchers used cereal based and unrefined non-purified diets commonly referred to as open or closed. Open and closed diets can vary in the nutritional composition creating variability between batches [23]. The nutritional composition of open diets is available to the researchers, and closed formulas refers to diets containing a nutritional composition available only to the manufacturer [24]. Closed cereal based diets proved to be challenging for researchers as they were unable to conclude if nutritional interactions or differences in diet composition could perhaps explain any differences among studies and research groups. It was not until 1972 when the National Institute of Health developed a universal open formula cereal based diet (NIH-07) that scientists had a standardized diet to use in their rodent studies [7].
2.3 Development of the AIN-93G Diet

In 1973, a committee was formed by the AIN consisting of nutritional scientists with the sole purpose of developing standardized diets to allow researchers to more accurately compare results within and among research groups [23]. It was the committee’s opinion that results from biological research may have been influenced by the inconsistency of nutritional composition due to lack of transparency from manufacturers about nutrient content and the use of unrefined ingredients in cereal based diets [23]. To standardize the composition and amount of nutrients used in these research diets, a purified diet named the AIN-76 diet was developed to improve upon and replace the NIH-07 [23]. When comparing the AIN-76 to the NIH-07 diet using a rat model, there was no difference in weight gain over 18 weeks, number of pups born per litter and surviving past weaning [23]. While these outcome measures give limited information to current standards, this gave the committee confidence that the AIN-76 purified diet will provide rodents with a nutritious standardized diet. While the new diet had many improvements, the AIN committee acknowledged that this was a work in progress and that future alterations would be required [23]. Not long after its inception a letter was written to the editor of the Journal of Nutrition voicing concerns over an increased number of hemorrhagic deaths in rodents fed the AIN-76 diet [25]. Sixty of 600 rats died of acute internal hemorrhage into the peritoneal cavity or hemorrhaging from toenail breaks while consuming the AIN-76 diet [25]. Supplementing the AIN-76 diet with 500 µg of vitamin K (a 10-fold increase) ceased deaths within two days and was also shown to drastically decrease signs of hemorrhage [25]. Additional concerns were the shelf life of the AIN-76 diet and how it should be properly stored; below 4°C was later determined to be optimal.
These concerns were addressed in 1980 when the AIN committee created a revised version of the diet, AIN-76A. Vitamin K was increased 10-fold to reduce the risk of haemorrhagic death and an antioxidant was added to stabilize the corn oil. In 1988, the committee held a meeting to discuss various potential modifications to the AIN-76A diet with regards to protein source, carbohydrates, fats, fiber, vitamin and mineral composition [27]. A major issue discussed, which was more prevalent in female rats, was the persistent occurrence of nephrocalcinosis when fed the AIN-76A diet over a 16 week period [28]. To follow up on these findings, the committee developed 2 updated diets in 1993 [7, 8, 27]. An AIN-93G diet, which contains higher protein and fat content, was developed to support pregnancy, lactation and growth, and an AIN-93M was developed as a maintenance diet throughout adulthood. Among many changes from the AIN-76A diet, the Ca to P ratio had been altered to help prevent nephrocalcinosis (Table 1).

### 2.3.1 Evidence for calcium and vitamin D levels in the AIN-93G diet

The current AIN-93G diet, developed in 1993, contains 0.5% Ca, similar to its predecessor the AIN-76 diet, and the recommended level in the Nutritional Requirements for Laboratory Animals, Fourth Edition [29]. A single measurement technique, ashing, was used to determine bone mineral content (BMC) of the tibia and whether the level of Ca was appropriate. Reeves and colleagues investigated the effects of a non-purified diet containing 0.89%, and purified AIN-93G containing 0.33, 0.5, or 0.67% Ca. While tibia BMC was higher in the non-purified diet compared to the AIN-93G diets, there was no difference among the purified diets in both rats and mice [8]. Because there was no difference in tibia BMC among the standardized diets, the AIN committee decided to hold the Ca level at 0.5%. No follow-up study has measured bone structure and strength
to provide further evidence for this level of Ca. However, considering BMC of the tibia was similar to mice fed 0.33% Ca, suggests that 0.5% Ca may be in excess of what is required. This is speculative as we now have the ability to measure BMC, BMD and bone structure. The evidence to support the current level of vit D in the AIN-93G diet for bone development is not based on solid evidence. The National Requirements for Laboratory Animals, Fourth Edition, states “In the absence of additional data, the estimated requirement of 1,000 IU vit D/kg diet is recommended…” [29]. Understanding how levels of Ca and vit D impact bone health when fed at levels lower than that present in AIN-93G are warranted to understand if current levels of these nutrients are in excess of that required for healthy bone development.

When studying bone development, providing a level of vit D and Ca in the diet that supports acquisition of bone mineral and structure without providing excess of these bone-building nutrients is essential to reduce the risk that a novel intervention may be masked by higher than required levels. Furthermore, considering that most people do not meet the nutritional requirements for vit D or Ca, the AIN-93G diet provides an unrealistic diet for extrapolating preclinical results to the human scenario. Using methods such as in vivo and ex vivo µCT to investigate bone structure and BMD, this thesis studied the effects of lower levels of vit D (400 or 100 IU/kg) and Ca (0.35, 0.3, or 0.25%) in the growing CD-1 male mouse, to elucidate lower, yet sufficient, levels to support healthy bone structure and BMD.
Table 1. Composition of common rodent diets.

<table>
<thead>
<tr>
<th>Nutrient</th>
<th>NIH-07</th>
<th>AIN-76A</th>
<th>AIN-93M</th>
<th>AIN-93G</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Gross Energy (Kcal/gm)</strong></td>
<td>4.08</td>
<td>4.34</td>
<td>3.6</td>
<td>3.8</td>
</tr>
<tr>
<td><strong>Amino Acid Profile Percent (%)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Arginine</td>
<td>1.31</td>
<td>0.7</td>
<td>0.45</td>
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<tr>
<td>Lysine</td>
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<td>0.92</td>
<td>1.48</td>
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<td>Methionine</td>
<td>0.48</td>
<td>0.86</td>
<td>0.33</td>
<td>0.56</td>
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<tr>
<td>Cysteine</td>
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<td>0.06</td>
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<td>Vitamin A (IU/g)</td>
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<tr>
<td>Vitamin D3 (IU/g)</td>
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<td>1</td>
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<tr>
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<td>Pantothenic Acid (ppm)</td>
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<td>Pyridoxine (ppm)</td>
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<td>Folic Acid (ppm)</td>
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<td>2</td>
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<tr>
<td>Biotin (ppm)</td>
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<td>25</td>
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<tr>
<td>Vitamin K (ppm)</td>
<td>3.5</td>
<td>0.5</td>
<td>0.86</td>
<td>0.9</td>
</tr>
<tr>
<td><strong>Mineral</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Calcium (%)</td>
<td>1.19</td>
<td>0.52</td>
<td>0.52</td>
<td>0.52</td>
</tr>
<tr>
<td>Phosphorus (%)</td>
<td>0.96</td>
<td>0.44</td>
<td>0.23</td>
<td>0.2</td>
</tr>
<tr>
<td>Potassium (%)</td>
<td>0.83</td>
<td>0.38</td>
<td>0.38</td>
<td>0.38</td>
</tr>
<tr>
<td>Sodium (%)</td>
<td>0.31</td>
<td>0.11</td>
<td>0.11</td>
<td>0.11</td>
</tr>
<tr>
<td>Magnesium (%)</td>
<td>0.19</td>
<td>0.05</td>
<td>0.05</td>
<td>0.05</td>
</tr>
<tr>
<td>Iron (ppm)</td>
<td>288</td>
<td>34.25</td>
<td>34.25</td>
<td>52.02</td>
</tr>
<tr>
<td>Mineral</td>
<td>Value 1</td>
<td>Value 2</td>
<td>Value 3</td>
<td>Value 4</td>
</tr>
<tr>
<td>------------------</td>
<td>---------</td>
<td>---------</td>
<td>---------</td>
<td>---------</td>
</tr>
<tr>
<td>Zinc (ppm)</td>
<td>67</td>
<td>36.76</td>
<td>36.76</td>
<td>36.76</td>
</tr>
<tr>
<td>Manganese (ppm)</td>
<td>103</td>
<td>59.34</td>
<td>11.09</td>
<td>11.33</td>
</tr>
<tr>
<td>Copper (ppm)</td>
<td>16</td>
<td>6.73</td>
<td>6.73</td>
<td>6.73</td>
</tr>
<tr>
<td>Cobalt (ppm)</td>
<td>0.68</td>
<td>0.02</td>
<td>0.02</td>
<td>0.02</td>
</tr>
<tr>
<td>Iodine (ppm)</td>
<td>2</td>
<td>0.21</td>
<td>0.21</td>
<td>0.21</td>
</tr>
</tbody>
</table>

**Guaranteed Analyses Percent (%)**

<table>
<thead>
<tr>
<th>Component</th>
<th>Value 1</th>
<th>Value 2</th>
<th>Value 3</th>
<th>Value 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude Protein (Min.)</td>
<td>22.5</td>
<td>16</td>
<td>12.6</td>
<td>17.9</td>
</tr>
<tr>
<td>Crude Fat (Min.)</td>
<td>5</td>
<td>5</td>
<td>4</td>
<td>7</td>
</tr>
<tr>
<td>Crude Fiber (Max.)</td>
<td>4</td>
<td>5</td>
<td>4.8</td>
<td>4.8</td>
</tr>
<tr>
<td>Ash (Max.)</td>
<td>7.5</td>
<td>4</td>
<td>3.9</td>
<td>4.2</td>
</tr>
<tr>
<td>Moisture (Max.)</td>
<td></td>
<td></td>
<td>7</td>
<td>7</td>
</tr>
</tbody>
</table>
2.4 Calcium and Serum Parathyroid Hormone

Ca is the fifth most abundant element in the body and has several vital functions. Most commonly known for its role in providing stiffness to bone, Ca is deposited as crystalized hydroxyapatite embedded within the extracellular collagen matrix of bone tissue [18]. Approximately 99% of all the Ca in the body is in bone tissue. While this provides rigid bones for locomotion and protection, it also acts as a reservoir of Ca for times of inadequate Ca consumption. Serum Ca is tightly regulated within a tight range, 8.8 to 10.4mg/dl (2.2 to 2.6 mM), in a healthy individual since homeostasis of Ca is essential for life [30]. Because serum Ca levels are so tightly regulated and direct measurements are easily contaminated, parathyroid hormone (PTH) is used as a surrogate measure of serum Ca [31]. This tight regulation of Ca is required to ensure proper electrical gradients are maintained for smooth and cardiac muscle contractions. Ca in the blood is bound to transport proteins such as albumin (40%) or exists as free ionized (51%) and ionic compounds (9%) such as Ca phosphate, Ca carbonate, and Ca oxalate. Ca plays a pivotal role in human health and the consumption, usage, and excretion of Ca affects its homeostasis. Ca is an essential nutrient, meaning it must be consumed in the diet.

*Considering this thesis reduced the level of vit D to 400 and 100 IU/kg and Ca to 0.35, 0.3, or 0.25% in the AIN-93G diet, it is possible that Ca homeostasis would be compromised. To determine if there was stress due to lower levels of these nutrients, serum PTH was measured to determine if Ca homeostasis was altered.*

2.4.1 Sources and recommendations

Ca is naturally found in dark leafy green vegetables, dairy products, and fish (Table 2). While leafy green vegetables contain high amounts of Ca, the bioavailability is reduced
compared to dairy sources [32]. The current dietary recommended intakes (DRI), nutrient reference values for healthy populations that are based off the current scientific knowledge of nutrient requirements, suggests 200 – 260 mg for infants (0-12 months), 700 mg for 1-3 years, 1000 for 4-8 years, and 1300 mg for 9-18 years of age, 1000 mg for both men and women from 19-50 years of age, 1200 mg for women between the ages of 51-70 and 1000 mg for their male counterparts. Men and women over the age of 71 should consume 1200 mg a day, and pregnant and breastfeeding women 1000 mg/day (Table 3) [33]. These DRIs are based on data from studies investigating Ca balance and bone health (BMC, BMD and fracture rate) [34].
Table 2. Common dietary sources of calcium.

<table>
<thead>
<tr>
<th>Food</th>
<th>Serving Size</th>
<th>Calcium (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Fruits and Vegetables</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Spinach, cooked</td>
<td>125 ml</td>
<td>154</td>
</tr>
<tr>
<td>Kale, cooked</td>
<td>125 ml</td>
<td>95</td>
</tr>
<tr>
<td>Orange juice (fortified)</td>
<td>125 ml</td>
<td>155</td>
</tr>
<tr>
<td><strong>Milk and Alternatives</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Milk, chocolate milk</td>
<td>250 ml</td>
<td>291-322</td>
</tr>
<tr>
<td>Cottage cheese</td>
<td>250 ml</td>
<td>146-217</td>
</tr>
<tr>
<td>Yogurt</td>
<td>175 g</td>
<td>292-332</td>
</tr>
<tr>
<td><strong>Fish</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sardines, with bones</td>
<td>75 g</td>
<td>180</td>
</tr>
<tr>
<td>Salmon, canned, with bones</td>
<td>75 g</td>
<td>179-208</td>
</tr>
</tbody>
</table>
Table 3. Dietary reference intakes for calcium.

<table>
<thead>
<tr>
<th>Demographic</th>
<th>Recommended Daily Allowance (mg/day)</th>
<th>Tolerable Upper Level (mg/day)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-3 years of age</td>
<td>700</td>
<td>2500</td>
</tr>
<tr>
<td>4-8 years of age</td>
<td>1000</td>
<td>2500</td>
</tr>
<tr>
<td>9-13 years of age</td>
<td>1300</td>
<td>3000</td>
</tr>
<tr>
<td>14-18 years of age</td>
<td>1300</td>
<td>3000</td>
</tr>
<tr>
<td>Men and Women 19-50 years of age</td>
<td>1000</td>
<td>2500</td>
</tr>
<tr>
<td>Women 51-70 years of age</td>
<td>1200</td>
<td>2000</td>
</tr>
<tr>
<td>Men 51-70 years of age</td>
<td>1000</td>
<td>2000</td>
</tr>
<tr>
<td>Men and Women 71 years of age and older</td>
<td>1200</td>
<td>2000</td>
</tr>
<tr>
<td>Pregnant or Breastfeeding Women 19 years of age</td>
<td>1000</td>
<td>2500</td>
</tr>
</tbody>
</table>
2.4.2 Consumption of calcium and bone growth in humans

During childhood and adolescence, it is important that DRIs of bone supporting essential nutrients are achieved to meet or exceed genetically programmed peak bone mass. In a computer model analysis simulating bone remodeling over 50 years in 30-year-old females, a 10% increase in peak bone mass resulted in a 13-year delay in developing osteoporosis (defined as BMD more than 2.5 SD below the young adult mean). This means that instead of developing osteoporosis at age 60, an individual would develop it at 73 years of age, suggesting peak bone mass is the single most important factor of osteoporosis [35]. While the consumption of Ca rich foods (such as milk) during childhood is associated with higher peak bone mass and reduced fracture risk later in life [36], it is difficult to determine if this bone-supporting effect was due to Ca alone or other nutrients such as magnesium, phosphorus, and protein in milk [37]. Cross-sectional and prospective studies suggest that Ca consumption is positively associated with bone mass and stature [38-40]. Children (3-10 y) who consumed 443 +/-230 mg Ca/day and did not drink milk had a shorter stature and lower BMC compared to age-and sex-matched controls who consumed milk [39]. Similarly, a large study that provided whole or separated (skimmed) milk (354 ml for 6-7 year-olds, and 473 ml for 9-14 year-olds) observed greater height growth in the milk fed group compared to a control group that received an isocaloric biscuit [40]. This is important, as this would equate to a height increase of 0.2 inches over a one-year period. While these studies provide some insight into the importance of Ca during growth, more recent intervention studies in male and female children ranging from the ages of 7 to 18 years of age show consistent increases in BMC and BMD with the addition of dairy or Ca supplementations to the diet for 1
through 1.5 years, with no differences in height between groups [41-45]. Interestingly, a meta-analysis of 19 randomized controlled trials involving 2859 children and Ca supplements ranging from 300 to 1200 mg/day resulted in negligible increases in femoral neck and lumbar spine BMC and BMD and small increases in total body BMC and upper limb BMD. Only the benefit to upper limb BMD persisted after the intervention; and most likely would not convey protection against fractures later in life [46]. The authors suggest that increased consumption of Ca above the current suggested threshold (1400 mg/day) provides little, if any, protective effects, and other nutritional supplementations should be explored, such as vit D. These studies provide insight into the Ca requirements in growing human bones, however, assessment of bone structure in humans is challenging from both a safety and practical perspective. This provides an opportunity to use rodent models to gain more insight into Ca and other nutrients effects on bone structure and BMD. Rodent models allow researchers to more closely manipulate diet and allows for in vivo and ex vivo analysis of multiple skeletal sites.

2.4.3 Effects of dietary calcium on bone structure, strength and BMD in growing rodent models

Using a rodent model, researchers can investigate changes in bone structure, strength and BMD associated with altered dietary Ca consumption. A commonly used rodent model is the Sprague-Dawley rat, and this species has been used extensively to assess the effects of various dietary Ca levels on bone development [10, 13, 47-49]. These studies, and others discussed in this section, used the standard AIN-76 or AIN-93 diets and without manipulating the level of vitamin D. Thus, the level of vit D (1000 IU/kg diet) was
consistent. Most interventions were started at approximately 3 weeks of age, immediately post weaning.

While the Ca level (0.5%) in the current AIN-93G control diet and in its predecessor, the AIN-76 diet, has been used since 1976, researchers continue to investigate how alterations in dietary Ca levels modulate outcomes of bone health while confidently maintaining the level of other nutrients. With the AIN-76A diet, Ca was adjusted to 0.25% until 24 weeks of age in female rats. Decreases in longitudinal tibia bone growth and reduced proximal tibial bone volume of the metaphyseal region compared to 0.5 and 1.0% Ca were observed. Furthermore, increasing Ca to 0.5 or 1.0% at 24 weeks of age until 37 weeks did not substantially improve these outcomes. This suggests a non-reversible deleterious effect of low Ca (0.25%) to bone growth during early development [49]. Similarly, another study fed male and female rats a low (0.25%) or high (1.0%) level of Ca, and BMD was determined using DXA at 4, 13, and 34 weeks of age [50]. Interestingly, in males, the low Ca group had significantly higher BMC at all time points, and there were no long-term differences in BMD despite the low level of dietary Ca. In females, BMC was greater in the low Ca group at 4 weeks of age and BMD was greater in the high Ca group at 13 weeks of age. The inconsistencies of this study may be explained by the fact that the rats on the low Ca diet ate more food, thus gained significantly more weight, than the rats on the high Ca diet [50]. In male rats, Ca restriction (0.25% versus 0.5%) for 10 weeks resulted in a 28% reduction in femur BMD measured by DXA [10]. The methods used in these studies provide no measure of bone quality – how bone is structured and organized.
In recent years, the use of high resolution µCT to assess bone structure *ex vivo* has become routine. µCT provides researchers with the ability to qualitatively and quantitatively measure cortical and trabecular bone structure, with some machines allowing scans to be performed *in vivo*. While µCT is being increasingly used, a limited number of studies to date have used it to assess the effects of altered Ca in growing rodent models. These studies have measured structure *ex vivo* rather than longitudinally in the same rodent. In a growing Sprague-Dawley rat model, altering the AIN-93G dietary Ca level incrementally from 0.7 to 0.1% while holding vitamin D (vit D) constant (1000 IU/kg), no differences in tibia Ca and phosphorus content, breaking force, bending moment, and stress of the femur, and proximal tibia bone volume fraction and trabecular thickness, number and separation were observed when Ca levels were 0.3 through 0.7%. Detrimental effects, while few, to structural properties were observed at 0.2% Ca, and all were compromised with the 0.1% Ca diet [13]. However, a similar study involving male rats found 0.2% Ca was detrimental to femur growth, strength, structure and BMD compared to 0.5 and 1.2% Ca after 4 weeks [48]. Differences in these studies suggest a sex-specific response [51-53]. During adolescence (4-7 weeks of age) female Sprague-Dawley rats showed 7 out of 10 bones (mandible, humerus, sternum, scapula, lumbar-6, thoracic-2, and pelvis) were significantly larger when compared to males [53]. Interestingly, at 24 weeks of age, female rats have increased resorption rates while fed a Ca-restricted (0.02%) diet [51]. Both studies noticed distinct differences in axial bone changes between sexes as 5 out of 6 axial bones were larger in females during growth [53], and a 14% axial bone loss during Ca restriction later in life in females [51]. The female axial skeleton seems to have an increased capacity for deposition and resorption
rates, potentially accommodating for the increased demands of pregnancy and lactation. This may explain the discrepancy between studies investigating dietary Ca levels and bone parameters using different sexes [13, 48]. Considering this, evidence is needed for specific Ca requirements for each sex.

There are discrepancies in the literature regarding the level of dietary Ca for which bone outcomes are compromised and many factors such as length of study and sex makes it difficult to compare results. This thesis research incorporated three levels of Ca (0.35, 0.30, and 0.25%) in combination with lower levels of vit D (100 and 400 IU/kg) to determine if one of these combined lower levels may be identified as a “cut-off point” for supporting bone structure and BMD similarly to the 0.5% Ca and 1000 IU vit D/kg in AIN-93G diet. Considering the above-mentioned studies, there is evidence that 0.25% Ca may be detrimental to bone structure and BMD and therefore was selected as the lowest level. We hypothesized that 0.35% or 0.30% would represent the “cut-off point” for Ca in the context of lower vit D. While this thesis will focus on male CD-1 mice, a parallel project has studied females as there may be a sex-specific response to altering bone supporting nutrients such as Ca and vit D.

2.4.4 Effects on different mouse strains

The previously discussed studies provide an understanding of Ca requirements in the Sprague-Dawley rat model. However, understanding dietary requirements for mouse models is necessary as mice, compared to rats, seem to have improved Ca absorption and bone outcomes when challenged with low dietary Ca [54]. Furthermore, bone development is under strong genetic control, and inbred mouse models have been used extensively to identify genes and loci (location of genes on chromosomes) responsible for
determining bone phenotypes [55-68]. There are a variety of mouse strains used in nutritional research and the trajectory for bone development and adaptability to low dietary Ca can vary [52, 69-71]. In one study, marked differences among 40 strains of mice on voluntary Ca intake, blood Ca, BMC, and BMD were recorded. Interestingly, there was no relationship between Ca consumption, BMC, or BMD, suggesting mineral content is highly heritable. Furthermore, strains of mice that consumed higher amounts of Ca resulted in either significantly low or higher BMD compared to other strains. For females, Ca consumption was significantly higher than males in 11 strains, with males never consuming more Ca, and females had significantly higher BMC and BMD when all strains were combined [52]. A similar study, including a low Ca level (0.25 vs. 0.5%) at 200 IU vit D/kg diet in 11 strains of 4-week old male mice, found variation in femur BMD, bone volume fraction, trabecular thickness, number and separation among strains at 12 weeks. Significant variation in adaptability in BMD, bone volume fraction, and cortical area fraction was also observed among strains when challenged with the low Ca diet [69].

This evidence suggests there are differences in Ca metabolism and bone structure that exists not only between rats and mice, but within mouse strains. Understanding the pattern of bone development, both in terms of mineral accumulation and structural changes, for both rodent models is critical to bone researchers. The aforementioned studies incorporated inbred strains which are more commonly used in mouse research as these models allow for identification of potential genetic influences and decreased variability [72]. Over the past decade, our laboratory has been characterizing the CD-1 mouse model, an outbred stock, to understand how early diet sets a trajectory for
improved bone health at adulthood [73]. We have previously investigated BMC, BMD, and bone strength in CD-1 mice on the AIN-93G diet at monthly intervals over the first 4 months of life [74]. While this provides a baseline measure of expected bone outcomes, there is no literature, to our knowledge, challenging the CD-1 mouse with lower levels of dietary Ca. We hypothesize that effects of past dietary interventions would be even greater in the context of lower dietary vit D (400 or 100 IU/kg) and Ca (0.35, 0.3, or 0.25%), as current levels may mask some of the benefits of these interventions. This hypothesis will be explored in studies beyond this thesis. The current thesis aimed to provide a basis for such studies by elucidating the levels of Ca and vit D that support bone structure and BMD without potentially masking the effects of novel foods or food components. These strategies may help develop preventative approaches against osteoporotic fractures.

2.5 Vitamin D

Vit D is an essential nutrient for Ca homeostasis and normal bone health through direct and indirect effects on bone metabolism. Ensuring efficient intestinal absorption of Ca and P, vit D improves mineral absorption in times of limited and normal consumption [75]. While vit D has been shown to activate 11 genes responsible for bone metabolism, many extra skeletal genes regulating healthy aging, cancer, metabolism, and the immune system are also activated by vit D metabolites [76, 77], elucidating its ubiquitous function in mammalian health.

2.5.1 Sources and recommendations

Vit D is consumed in the diet or endogenously produced in the cutaneous tissue from photolysis of 7-dehydrocholesterol (provitamin D₃) when exposed to ultra-violet beta
radiation (UVB rays) to previtamin D₃ which is then further enzymatically rearranged into functional forms [78]. The amount of vit D that can be produced by the skin is influenced by environmental (latitude, season, time of day, cloud cover) and physiological (age, gender, and ethnicity) factors [79]. Due to concerns regarding skin cancer from exposure to sunlight, dietary sources are recommended [80]. The DRI for vit D is 400 to 800 IU depending on age and pregnancy [33], and these intakes are set with the goal of meeting sufficient circulating levels of serum 25(OH)D₃ (>50 nmol/L). Osteoporosis Canada recommends a higher range for healthy adults at 400-1000 IU of vit D a day, and those over the age of 50 should consume 800 to 2000 IU (Table 4) [2]. Considering that there are a limited number of foods containing substantial amounts of vit D, it can be difficult to meet this DRI. Oily fish is one good source; 1 serving (3.5 ounces) of farmed or wild salmon naturally contain high amounts of vit D (249 and 981 IU, respectively) [81]. However, most people consume fish intermittently, and milk (which undergoes mandatory vit D fortification) may not be commonly consumed while some orange juice and margarine are among other commonly consumed foods fortified with vit D [81]. Milk contains 88 IU/serving of vit D, while orange juice (if fortified) contains a similar amount of vit D (80 IU/cup) [82] (Table 5). However, milk consumption is on the decline with 57% of adolescent Canadians (aged 14-18 years) consuming only 1 cup of milk a day [83], and adult Canadians (aged 19 years and older) consuming <1 cup/day [84].

2.5.2 Serum 25(OH)D₃

Vit D status in humans is assessed by measuring vit D serum 25(OH)D₃. A sufficient serum 25(OH)D₃ level is defined as a level above 50 nmol/L [33], however, some
researchers suggest this level should be increased to a minimum of 75 nmol/L [85]. Vit D deficiency is defined as a serum level of < 30 nmol/L, while inadequacy is within a range of 30 to 50 nmol/L [33]. Only 68% of Canadians have a serum 25(OH)D₃ level above 50 nmol/L [86]. Worldwide, there is an estimated 1 billion people who have deficient or inadequate serum levels of 25(OH)D₃ [79]. Therefore, Osteoporosis Canada now recommends all adult Canadians to take a vit D supplement year round [2].
Table 4. Dietary reference intakes for vitamin D.

<table>
<thead>
<tr>
<th>Age Group</th>
<th>Recommended Daily Allowance (IU/day)</th>
<th>Upper Tolerable Level (IU/day)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Infant (0-6 months)</td>
<td>400</td>
<td>1000</td>
</tr>
<tr>
<td>Infant (7-12 months)</td>
<td>400</td>
<td>1500</td>
</tr>
<tr>
<td>1-3 years</td>
<td>600</td>
<td>2500</td>
</tr>
<tr>
<td>4-8 years</td>
<td>600</td>
<td>3000</td>
</tr>
<tr>
<td>9-70 years</td>
<td>600</td>
<td>4000</td>
</tr>
<tr>
<td>&gt;70 years</td>
<td>800</td>
<td>4000</td>
</tr>
<tr>
<td>Pregnancy &amp; Lactation</td>
<td>600</td>
<td>4000</td>
</tr>
</tbody>
</table>
Table 5. Common dietary sources of vitamin D.

<table>
<thead>
<tr>
<th>Food</th>
<th>Vitamin D (IU)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Salmon (100 g)</td>
<td>272</td>
</tr>
<tr>
<td>Fortified cow’s milk (250 ml)</td>
<td>88</td>
</tr>
<tr>
<td>Fortified orange juice (250 ml)</td>
<td>80</td>
</tr>
<tr>
<td>Egg yolk (1)</td>
<td>25</td>
</tr>
<tr>
<td>Beef (100 g)</td>
<td>25</td>
</tr>
<tr>
<td>Margarine (1 tsp)</td>
<td>25</td>
</tr>
<tr>
<td><strong>Supplements</strong></td>
<td></td>
</tr>
<tr>
<td>Cod liver oil (1 tbsp)</td>
<td>1360</td>
</tr>
<tr>
<td>Infant vitamin D drops (1 ml)</td>
<td>400</td>
</tr>
<tr>
<td>Infant formula (250 ml)</td>
<td>100</td>
</tr>
</tbody>
</table>
2.5.3 Metabolism

To become functional, vit D, whether consumed in the diet or produced in the skin, is transported in the blood to the liver and converted by 25-hydroxylase to 25-hydroxyvitaminD₃ (25(OH)D). 25(OH)D₃ is then transported to the kidneys where it is hydroxylated by 1alpha-hydroxylase into its most biologically active form, 1,25(OH)₂D₃ [87]. 1,25(OH)₂D₃ has the most affinity for the vit D receptor (VDR), and thus, to elicit its effects on target cells, must bind together to form the 1,25(OH)₂D₃ -VDR complex.

2.5.4 1,25(OH)₂D₃ and the vitamin D receptor

The classical role of vit D is to mediate Ca homeostasis by increasing intestinal Ca absorption, Ca reabsorption in the kidneys, and resorption of Ca from bone [88]. To initiate these biological effects, it must bind to the VDR. The VDR is a member of the superfamily, and is a ligand activated DNA-binding transcription factor [88]. The 1,25(OH)₂D₃-VDR complex regulates the expression of genes involved in Ca homeostasis and bone metabolism [76]. The main role of VDR is to maintain high intestinal Ca absorption, and in fact, VDR null mice have decreased Ca absorption causing hypocalcaemia and rickets. Interestingly, these side effects can be treated with a rescue diet containing high amounts of Ca and lactose to improve Ca absorption [88, 89]. 1,25(OH)₂D₃ has been shown to induce osteoclast differentiation and stimulate bone resorption in times of hypocalcemia. This is mediated by the activation of Receptor for the Activation nuclear factor kappa-B (RANK). RANKL, the ligand for RANK, is produced by osteoblast cells, which is regulated by 1,25(OH)₂D₃. Therefore, while 1,25(OH)₂D₃ activity at the intestine indirectly supports bone formation it directly influences bone resorption.
2.5.5 Consumption of vitamin D and bone growth in humans

Severe vit D deficiency is linked to incomplete bone mineralization during growth in children leading to rickets [90]. One study supplemented 38 Hispanic and non-Caucasian infants with 400 IU vit D/day over three months and measured serum 25(OH)D₃, BMC and BMD. The authors found there was no relationship between serum 25(OH)D₃, BMC and BMD at the first week of life or after 3 months of supplementation [91]. Similarly, there were no differences in BMC of the lumbar spine of breast milk-fed versus formula-fed infants [92]. Another study in Caucasian infants, monitored UVB light exposure while investigating the effects of vit D supplementation versus a placebo in 46 infants fed human milk to 6 months of age. While there was a significant increase in serum 25(OH)D₃ in the supplemented group, there was no difference in BMC, serum PTH, or serum 1,25(OH)₂D₃, suggesting that the unsupplemented group had no signs of vit D deficiency [93]. While the above-mentioned studies found significant increases in serum 25(OH)D₃ levels in the supplemented infants, no differences in BMC and BMD were identified [93, 94] up to 12 months of age [94]. However, it is possible that the intervention period was too short to elicit an effect. Furthermore, while it would be interesting to also know if and how bone structure was altered, the invasive nature of computed tomography imaging in humans, and especially in infants and children, means that few studies have information about bone structure. Thus, rodent models can provide some understanding of how bone structure may be affected by different levels of dietary vit D.

Serum PTH and 25(OH)D₃, and BMD of the non-dominant forearm and dominant heel (measured by DXA) was assessed in 12 and 15-year-old boys and girls, while taking into
consideration physical activity, lifestyle and dietary factors. High vit D status (serum 25(OH)D3 ≥ 74.1 nmol/L) in girls resulted in greater forearm BMD and lower PTH [95].

In 100 non-obese children (9.3 +/- 1.9 years), BMC and BMD of the lumbar spine were measured using DXA. 29% were vit D deficient (25(OH)D3 < 20 nmol/L) which correlated with low BMC of the total body and lumbar spine [96]. In 10 to 12-year-old girls with a mean Ca intake of 733 +/- 288 mg/day, 32% of the participants were vit D deficient (25(OH)D3 < or equal to 25 nmol/L) and 46% were insufficient (26-40 nmol/L). Serum PTH was significantly higher in the deficient participants and they had significantly lower cortical volumetric BMD of the distal radius and tibia [97]. Among subjects 12.5 to 17.5 years of age, dietary Ca and vit D, milk intake, 25(OH)D3 status, BMC, and BMD were assessed and there was no relationship between Ca and vit D intakes and bone mass [98]. However, the mean serum level of 25(OH)D3 was 66.3 ± 20 nmol/L, higher than the previously mentioned studies.

The above studies show inconsistencies in the relationship between serum 25(OH)D3 and bone health in children and adolescents. The differences in results may be due to the diverse populations studied, 25(OH)D3 status of the subjects, and their dietary habits. It is difficult to draw conclusions as BMD using DXA is a limited measure of bone as it only measures the amount of mineral present. Moreover, it can be difficult to get accurate scans due to movement of young children [99]. Like studying the skeletal effects of Ca, researchers can opt to use rodent models to investigate specific research questions related to vit D.
2.5.6 Effects of dietary vitamin D on bone structure, BMD and strength in growing rodent models

Strengths of using rodent models to study bone development is that researchers can fully manipulate the composition of the diet as well as conduct analyses that are too invasive in humans, and importantly, provide details about specific effects on bone structure. Some of these outcomes of bone structure are strongly predictive of bone strength [100].

Publications from the early 1980s elucidated the relationship between dietary vit D and bone health in growing rodents [101, 102]. Pups raised by mothers on a vit D deficient diet, then at 23 days-of-age, were given 1 of 3 different vit D metabolites or a vit D deficient diet (Ca level was 0.47%, similar to AIN-93G). The bones of vit D deficient rats were smaller, structurally disorganized, and porous with large vascular channels at 63 days of age [101]. This provided evidence that vit D is important for the normal growth of rodent bones and that various forms of vit D can provide benefits. Rats that had been exposed to vit D deficiency through maternal diet during pregnancy and lactation were then fed, for 10 days, the same diet after weaning and infused with either 15 IU of vit D or a Ca and P solution that had been previously determined to rescue blood Ca and P levels [102]. There was no difference in femur weight, length, ash content or epiphyseal plate width between the vit D deficient group that received Ca and P injections and the vit D infused group. This suggests that vit D supports bone growth and mineralization indirectly by elevating blood Ca and P levels by improving absorption at the gut [102].

41 male Sprague-Dawley rats were fed an AIN diet containing no vit D or the AIN-93G diet with both diets containing 1% Ca until 10 weeks of age, at which point, 10 vit D deficient animals were euthanized and the remainder of the animals were placed on diets
containing 0, 2, 4, 8, 12, or 20 IU vit D/day at 0.4% Ca or a 14 IU vit D/day at 1% Ca for another 3 months [78]. Serum 25(OH)D₃ increased positively with dietary vit D levels. However, an increase in circulating 1,25(OH)₂D₃ was maximized at 8 IU vit D/day, after which, a decrease occurred with increased dietary vit D [78]. While this experiment did not measure bone parameters, it does provide evidence for adequate dietary consumption of vit D to optimize serum 25(OH)D₃ and 1,25(OH)₂D₃ levels in rodent models. Interestingly, they observed no difference in serum Ca or PTH levels among the groups [78]. This suggests that a diet containing 0.4% of Ca provides a sufficient passive gradient for Ca absorption in the absence of vit D. Another study investigated the effects of lowering Ca compared to the current AIN-93G level (0.25% versus 0.5%) at three different levels of vit D (400, 1000, and 10000 IU/kg) in male Sprague-Dawley rats from weaning to 10 weeks of age. Femur BMD, measured by DXA, was reduced in all groups consuming the low Ca diet despite the ten-fold increase in vit D in one of the groups [10]. This suggests the amount of dietary Ca consumed is critical for normal bone growth and cannot be counteracted by simply adding more vit D. Also, this would suggest that the maximal calcitrophic effects of vit D at a Ca level of 0.25% is at or below 400 IU/kg as no increases were seen at 1000 or 10000 IU vit D/kg. Importantly to note, these studies did not measure bone structure, and therefore the authors are unable to conclude if the quality of bone was compromised at these lower levels of vit D.

More recently, our laboratory has used a level of vit D in the CD-1 mouse model that causes a chronic low-grade inflammatory response [12]. Male CD-1 mice were exposed, in utero and during suckling, to the AIN-93G diet with low (25 IU/kg) or high (5000 IU/kg) vit D through the mothers, then randomly assigned to one of the same vit D diets.
until 3 months of age. Serum lipopolysaccharide (LPS) was significantly elevated in mice that received exposure to low vit D from both the maternal environment and diet after weaning. Interestingly, while fed this low level of vit D, no difference in femur and lumbar vertebra BMC, BMD, and strength was observed at 3 months of age [12]. Using the same experimental design and dietary interventions, except in a female inflammatory prone model (IL-10 KO mice), our laboratory found similar no effects to bone in animals fed the 25 IU vit D/kg diet [11]. Considering previous research has observed no effects on bone at half the current level Ca [13], and normal serum Ca and PTH while consuming a diet containing 0.4% Ca and lower levels of vit D [78], 0.5% Ca used in the AIN-93G diet may be compensating for low dietary vit D in these models. Considering this, this thesis project attempted to determine if a lower level of both Ca (0.35, 0.3, or 0.25%) and vit D (400 or 100 IU/kg) in the AIN-93G diet supports healthy bone structure and BMD compared to the current AIN-93G diet.

The studies from our laboratory also provide insight into the role of vit D in modulating the immune system [12]. Vit D plays a role in immune system development [103], and as our research has shown, consuming a diet markedly lower in vit D during growth can lead to a chronic low-grade inflammatory state [12]. This is important for bone researchers to understand, as the immune system influences bone metabolism, and recently, there is evidence emerging linking osteoporosis progression and inflammation [104]. This immunomodulatory effect becomes increasingly complex with the emerging evidence of the gut microbiota bone axis (GM-bone axis) [17]. The GM seems to have similar effects as vit D on the immune system and the influence of vit D on the GM is poorly understood [15].
2.6 Gastrointestinal Tract

GIT functions as a multi-organ system, chemically and mechanically to digest food, to absorb nutrients from the food into circulation, and to excrete the undigested matter. Classically, the influence of GIT on bone was related to the regulation of absorbing bone supporting nutrients, however it is also home to the GM – a potential modulator of bone health [17]. The GIT also acts as a barrier, separating the external environment of the intestinal lumen from the internal environment of the host [16]. It has been observed that dietary factors can compromise this barrier and lead to translocation of pathogenic organisms or the proinflammatory molecules they synthesis into circulation, causing chronic low-grade inflammation [16]. This thesis aimed to determine if lower levels of dietary vit D (400 or 100 IU/kg) and Ca (0.35, 0.3, or 0.25%) influence the intestinal barrier through measures of fecal albumin and a serum proinflammatory marker LPS.

2.6.1 Dietary calcium absorption

The absorption of dietary Ca is dictated by its concentration and electrical gradients across the epithelial wall [105], bioavailability of the dietary source [32], and hormonal activity [88]. Ninety percent of Ca absorption occurs in the small intestine by both passive, paracellular, and active transcellular pathways, mainly 1,25(OH)₂D₃ dependent mechanisms [105]. When concentrations of Ca are high in the lumen of the GIT, paracellular pathways dominate the movement of Ca in the jejunum and ileum of the small intestine [105]; while during low dietary Ca consumption, transcellular pathways actively absorb Ca against its concentration gradient [106]. The presence of a transcellular transport mechanism in the intestine allows Ca to move across the brush border membranes, through the enterocytes and to the basolateral membranes into the
circulation. The transcellular movement of Ca across the luminal wall involves three steps: i. entry into the enterocyte via the TRPV6 channel [107-109]; ii. passive diffusion facilitated by calbindinD9k [105, 109-112], and iii. outward movement of Ca via Ca-ATPase channels on the basolateral surface of the epithelial cells [112].

2.6.2 Gut microbiota

The human gut is colonized by trillions of microorganisms, and the total number of bacteria in the human body is a 1:1 ratio to human cells [113]. While these organisms are genetically different from us, we have evolved in a symbiotic fashion, and the GM can be considered a multicellular organ that communicates with and influences host development and metabolism [114]. Recently, much research has highlighted potential mechanisms of the GM to influence the health of the brain [115], metabolism [116], immune system [117] and even bone [17]. While developmental and overall health benefits of the GM are associated with a healthy multi-colonized gut, a dysbiotic GM can result in the uncontrolled growth of pathogenic organisms leading to or related to disease.

2.7 Gut Microbiota-Bone Axis

2.7.1 Evidence supporting the gut microbiota-bone axis

The GM-bone axis is a new concept with a limited number of studies that have investigated this relationship using rodents [118-123], chickens [124, 125], and zebrafish [126, 127]. One study compared BMD and bone structure in germ free (GF) (raised in a sterile environment resulting in no GM) versus conventionally raised mice (raised in natural environment and develop a normal GM) [118]. GF mice had higher trabecular bone volume to tissue volume, higher trabecular number, less trabecular separation, and higher BMD compared to the conventionally raised mice. GF mice also had increased
rates of bone formation and fewer osteoclasts. Bones of the GF mice also had lower amounts of pro-inflammatory cytokines (IL-6, TNF-alpha). Furthermore, fecal transplant from conventionally-raised mice to GF mice at 3 weeks of age resulted in normalization of bone outcomes (BMD and structure), frequency of T-lymphocytes and number of osteoclastic precursor cells. These findings suggest that the GM-bone axis may be explained through immunomodulatory mechanisms [128].

### 2.7.2 Potential mechanisms of the gut microbiota-bone axis

The intestine contains various specialized endocrine cells that communicate with the GM. Interaction with bacteria can cause these cells to release hormones that enter circulation and communicate with visceral organs, such as bone, afferent nerves, and immune cells [129]. Enterochromaffin cells in the intestine are responsible for much of the serotonin production in the body, and the GM may be responsible for stimulating its release [118]. Bone cells express serotonin receptors and gut derived serotonin has been shown to have negative effects on bone formation in mice [130, 131]. Furthermore, studies have shown inhibition of this mechanism prevented bone loss in rodent models [132-134].

Another potential mechanism of the GM influencing bone is through modulation of the developing immune system during early life. GF mice have under-developed mucosal immune systems, containing hypoplastic Peyer’s patches with fewer germinal centers and reduced number of IgA-producing plasma and T cells [135]. Furthermore, GF mice had twofold lower splenic CD4 T cells compared to conventionally raised mice [136]. These results suggest that aspects of immunity, specifically T cells, are mediated by the GM. Osteoclasts, bone cells responsible for resorbing bone tissue, are derived from myeloid lineage hematopoietic stem cells, and it is the hormones and growth factors in the
microenvironment that determines whether a myeloid precursor cell will differentiate into a macrophage, myeloid dendritic cell, or an osteoclast [137]. In the presence of macrophage colony-stimulating factor (M-CSF) this leads to the upregulation of RANK in osteoclastic precursor cells. RANKL, a ligand for RANK, binds and leads to osteoclastogenesis [138]. M-CSF and RANKL are released by bone marrow stromal cells as well as osteoblasts located in the periosteal and endosteal surfaces to stimulate bone resorption [139]. Altered early development of the immune system by the GM might have long lasting effects on bone metabolism as bone cells are responsive to cytokines released by immune cells. TNF-alpha and IL-1, cytokines released from immune cells, both promote osteoclastogenesis [140].

Lastly, bacteria in the gut can synthesize molecules that translocate into the host circulation and elicit an immune or tissue response [141, 142]. Recently, muramyl dipeptide, an abundant component of the bacteria cell wall, can translocate and be delivered to bone marrow to potentially influence bone metabolism. Administration of 200 µL of muramyl dipeptide at day 0 and 4 in 6-week-old C57Bl6 mice resulted in an increase femur bone volume fraction and trabecular number [141]. The authors concluded that muramyl dipeptide downregulates the ratio of RANKL to OPG, inhibiting osteoclast differentiation. LPS found on the membrane of gram-negative bacteria, can translocate into the host and elicit an immune response. These molecules can activate CD4 T and macrophage cells through their Toll-like receptors and stimulate osteoclastogenesis [142]. Exposure to LPS is associated with chronic low-grade inflammation, a condition that is correlated with low BMD [143]. Researchers have shown moderately elevated C-reactive protein in the blood, a measure of inflammation, is correlated with a low BMD,
elevated rates of bone resorption and increased fracture risk [144-147]. Thus, LPS exposure may have a negative influence on bone via the immune system. Moreover, some species of pathogenic bacteria are invasive and can translocate from the lumen to the host and up-regulate pro-inflammatory genes [148]. This is usually associated with a compromised intestinal barrier caused by dysbiosis or dietary habits [149]. A compromised intestinal barrier allows LPS endotoxins to “leak” into circulation and cause systemic inflammation. Considering the health implications of the GM, understanding factors that influence its health are warranted. Rodent models have been used to study the effects of such nutrients as Ca [16] and vit D [150] on the GM, and both are modifiable factors that can alter the GM, and ultimately bone health.

2.7.3 Calcium and the gut microbiota

There is strong evidence that dietary habits can influence the health of the GM [15, 16]. For example, the ingestion of Ca seems to modulate the GM in a beneficial manner – increasing lactobacilli concentrations [151, 152]. In both rat and human studies, dietary Ca supported resistance against the cytotoxicity of pathogenic bacteria such as salmonella enterotoxigenic Escherichial coli [151-155]. Furthermore, increased dietary Ca intake has been shown to improve intestinal integrity in animal models [16]. When the integrity of the gut is unimpaired, there is a highly selective exchange between the lumen and tissue, and avoidance of pathogenic microorganisms or the molecules they synthesis, such as LPS, entering circulation. In human and animal studies, dietary Ca has been shown to precipitate irritants of the intestinal wall such as bile acids and other surfactants, thus maintaining intestinal integrity and reducing translocation of pathogenic bacteria and LPS [156, 157]. Therefore, increased consumption of Ca may also improve the intestinal
barrier and reduce inflammation; while lower levels may negatively influence the health of the gut microbiota and be detrimental to the intestinal barrier.

2.7.4 Vitamin D and gut microbiota

Serum 1,25(OH)D₃ status is known to modulate the immune system and is linked to autoimmune diseases and intestinal inflammation [158], however, the mechanisms remain elusive. VDR knockout mouse models have shown chronic low grade inflammation [159, 160], increased bacterial burden and mortality when infected with salmonella [161], and in fecal samples, a lower beneficial lactobacillus and higher pathogenic clostridium is observed [162]. While studies using knockout mice provide insight into the roles of vit D, wild type models provide a more realistic scenario of the physiological role of vit D. There are a limited number of vit D deficient studies investigating the GM, however current literature supports the findings from knockout models. A lifelong diet containing low (25 IU) vit D also results in chronic low-grade inflammation [12], and 1,25(OH)D₃ deficiency resulted in an attenuated antimicrobial activity response in the gut [163]. Weaned mice fed a vit D deficient diet and challenged with Citrobacter rodentium showed epithelial barrier dysfunction. Furthermore, vit D deficient animals, infected or not, had altered fecal microbial compositions. These studies provide a framework for the interactions between vit D, the GM and the immune system.

Our group has previously shown that serum LPS and fecal albumin, measures of compromised intestinal barrier function, is higher in a low dietary vit D intervention. Considering the evidence that low vit D intake is associated with chronic inflammation, and low Ca intake is associated with translocation of pathogenic molecules, this research
investigated how lower dietary vit D and Ca influence the intestinal barrier, and if there are relationships to bone health.

2.8 CD-1 Mouse Model

Our group focuses on the ability of food components and nutrients during early life to program bone health, and the CD-1 mouse has been used predominantly in our laboratory [73]. An arguable advantage of using CD-1 mice (an outbred stock) is its genetic heterogeneity which is similar to the human population [164]. Therefore, this model may provide insight into the variable responses that would be seen when an intervention is applied to humans. However, this diversity increases within group variability and the need for increased subjects. This also makes it difficult to determine the genetic background that may be responsible for determining if someone will respond to an intervention. A potential model for overcoming this obstacle is using multiple lines of inbred mice in one study, similar to the design reported by Replogle et al. 2014 [69]. This design potentially allows for genetic diversity while also the ability to control for genetic background. However, an outbred stock may provide a more realistic variance of responses that occur in the human population to an intervention compared to inbred strains [164, 165], and some would argue that the genetic diversity of outbred stocks may make them useful tools for determining genetic basis for variations in responses to interventions such as toxins [164]. Other benefits of CD-1 mice are that they are inexpensive, efficient breeders, respond well to human handling, large litters are obtained and pups can be cross-fostered [73, 165].

Our laboratory has been using the CD-1 mouse for studying the nutritional programming effects on bone, and the findings are promising [73]. Broadly, we have shown that CD-1
mice exposed to soy isoflavones during early life show improved BMD, trabecular interconnectivity of long bones and the lumbar spine at young adulthood when fed the AIN-93G diet [73]. Understanding healthy bone development within this model is critical for interpreting changes to bone outcomes due to nutritional interventions. Our group has previously characterized the biomechanical strength, BMC and BMD of the femur and lumbar vertebrae in male and female CD-1 mice at monthly intervals until 4 months of age [74]. Findings suggested that whole femur and lumbar spine BMC and BMD was maximized at 3 months of age with no differences at 4 months [74]. Interestingly, peak load of the femur midpoint was higher at 2 months of age and did not differ significantly from 3 to 4 months of age. Higher peak load of the femur neck, however, was achieved at 3 months of age. This study suggests peak bone accrual in CD-1 mice is achieved between 3 and 4 months of age. While BMC, BMD, and biomechanical measures are often used as surrogate measures of fracture risk in animal models and provide information regarding the total quantity of bone and its functional strength, respectively, no information regarding the structure of bone is provided. With the use of μCT imaging, structural analysis of trabecular and cortical bone can be completed, and this information can be used to estimate the strength of the bone (or fracture risk) [166]. This is beneficial for bone researches as this technique is non-destructive, allowing for further histomorphological analysis and provides greater details of bone structure. Furthermore, with the use of in vivo μCT, assessment of the same subject at multiple time points can be completed. For example, subjects can be scanned at baseline, then a dietary intervention introduced and more than 1 follow-up scan can be performed during the intervention, as well as post intervention. In such a study design using rodent models, understanding
normal bone development allows for improved interpretation of results as expected
growth and developmental patterns can be taken into consideration. Recently, our lab has
investigated the longitudinal effects of repeated \textit{in vivo} \(\mu \text{CT}\) scanning at 2, 4, and 6
months of age in male and female CD-1 mice. While trabecular bone of the proximal
tibia was higher at 2 months, and declined with age, cortical bone development continued
to 6 months of age [167].

\subsection*{2.9 Rationale}
We hypothesize that the levels of Ca (0.5\%) and vit D (1000 IU/kg) in the AIN-93G diet
may attenuate or mask potential benefits of previously studied dietary intervention.
Considering humans generally do not consume Ca and vit D in excess, providing a diet
with sufficient but not excess Ca and vit D may more closely resemble the human
scenario. To our knowledge, there is limited evidence for vit D and Ca requirements in
the CD-1 mouse. The CD-1 mouse is commonly used in our laboratory to investigate
nutritional programming of bone, and we have previously observed trabecular bone
development to peak at 2 months of age, while cortical bone continues to develop past 4
months of age [73, 167]. Considering previous studies found detrimental effects to BMD
and strength at 0.25\% Ca, we have choosen this level, and 0.3 and 0.35\% to determine if
one of these lower levels supports bone structure and BMD of the tibia. With regards to
vit D, we have choosen to study 100 and 400 IU/kg diet as these levels were previously
shown to produce inadequate and adequate serum 25(OH)D\textsubscript{3} levels in mice [10].
CHAPTER 3

3 RESEARCH QUESTION, OBJECTIVES AND HYPOTHESES

3.1 Research Question

To determine if a lower level of vit D and Ca than is in the AIN-93G diet supports bone development in male CD-1 mice. Given emerging data that vit D and Ca may modulate the intestine, and that a GM-bone axis exists, this project also investigated intestinal integrity.

3.2 Objectives

**Trial 1:** In the context of a diet containing 100 IU vit D/kg diet,

1. to determine if Ca (0.35, 0.3, or 0.25%) supports healthy bone structure and BMD, assessed by µCT at 2 and 4 months of age in male CD-1 mice; and

2. to determine if the health of the intestine, measured by fecal albumin and serum LPS, is altered by lowering Ca (0.35, 0.3, or 0.25%) and thus, potentially, the relationship to bone structure.

**Trial 2:** In the context of a diet containing 400 IU vit D/kg diet,

1. to determine if Ca (0.35, 0.3, or 0.25%) supports healthy bone structure and BMD, assessed by µCT at 2 and 4 months of age in male CD-1 mice; and

2. to determine if the health of the intestine, measured by fecal albumin and serum LPS, is altered by lowering Ca (0.35, 0.3, or 0.25%) and thus, potentially, the relationship to bone structure.
3.3 Hypotheses

1. Tibia, mandible and lumbar vertebra structure and BMD will be compromised in the 0.25% Ca diet group at both 100 and 400 IU vit D/kg compared to the AIN-93G diet.

2. Dietary Ca levels of 0.25% at both levels of vit D (400 and 100 IU/kg) will result in a higher level of serum LPS and fecal albumin compared to the AIN-93G diet.
Bone structure is largely unchanged in growing male CD-1 mice fed lower levels of vitamin D and calcium than in the AIN-93G diet.
4.1 Abstract

**Background:** Nutritional studies often use reference diets to standardize nutritional intake between and within research groups. There is evidence that the level of Ca and/or vit D in the AIN-93G reference diet is higher than required for healthy bone development, and may in fact mask the potential beneficial effect of a dietary intervention. **Objective:** To determine if a lower level of vit D (100 or 400 IU vit D/kg) at a lower level of Ca (0.35, 0.3, or 0.25%) than in the AIN-93G diet supports healthy bone structure and BMD assessed at 2 and 4 months of age. **Methods:** Weanling male CD-1 mice were randomized to modified AIN-93G diets containing either 100 IU vit D/kg diet (Trial 1) or 400 IU vit D/kg diet (Trial 2) within one of three Ca levels (0.35, 0.30, or 0.25% Ca diet) or the AIN-93G diet (REF) (1000 IU/kg vit D and 0.5% Ca) to 4 months of age (n = 13-15/group). At 2 and 4 months of age, BMD and structural properties of the tibia were analysed using *in vivo* µCT. The 4th lumbar vertebrae (L4) and mandible were scanned *ex vivo* at 4 months of age. **Results:** There were no differences in trabecular or cortical bone at the tibia, L4, and mandible between the REF and the 0.35% Ca groups at either vit D level. In Trial 1 (100 IU vit D/kg diet), at the proximal tibia, the 0.25% Ca group had lower trabecular thickness compared to the REF group, and cortical thickness was lower in the 0.25 and 0.3% Ca groups compared to the REF group (p<.05). In Trial 2 (400 IU vit D/kg diet), at tibia midpoint, the 0.25% Ca group had lower (p<0.05) cortical thickness compared to the 0.35% Ca group. **Conclusion:** Dietary Ca can be lowered to 0.35% at either 400 IU vit D/kg without detriment to bone structure or BMD in growing male CD-1 mice.
4.2 Introduction

Osteoporosis is characterized by low BMD and deterioration of structure resulting in an increased risk of fragility fractures [1]. Increasing peak bone mass has been calculated as the single most influential factor for BMD later in life [35], and low BMD during childhood is a strong predictor of low BMD at young adulthood [168]. While osteoporosis is a geriatric disease, nutrition during early life has the ability to set one on a trajectory for better or poor bone health, and nutritional strategies to increase peak bone mass are often studied using preclinical models [73].

Rodents are commonly used as preclinical models in nutritional intervention studies as µCT can be used to obtain detailed information of tissue response to diet interventions. These studies often incorporate standardized reference (REF) diets, such as the AIN-93G, to ensure nutritional consistency between and within research groups using animal models [7, 8, 24, 27]. While the level of Ca in the AIN-93G diet was developed with consideration of whole animal growth, kidney calcification, and tibia Ca content, the current level (0.5%) may be higher than required for bone development as bone structure was unchanged at lower levels of dietary Ca in both rat [13] and mouse [69] models. In weanling female Sprague-Dawley rats, the level of Ca in AIN-93G was studied at levels of 0.7 to 0.1% while holding vit D constant (1000 IU/kg) over a period of 13 weeks. No differences in tibia Ca and P content, breaking force, bending moment, and stress of the femur, and proximal tibia bone volume fraction and trabecular thickness, number and separation were observed among the 0.7 versus 0.3% Ca groups. Detrimental effects to structural properties, while few, were observed at 0.2% Ca, and all were compromised at 0.1% Ca [13]. This suggests dietary Ca can be decreased to less than half the current
level in the AIN-93G diet (0.5%) before Ca content, biomechanical and structural properties are compromised in a rodent model. With regards to vit D, our laboratory has found that lower levels of dietary vit D (25 IU/kg) compared to 5000 IU vit D/kg had no effect on BMD and strength in several different mouse models: growing male CD-1 mice [12], C57BL/6 mice fed a western (high fat) diet [169] and an inflammatory prone mouse model [11]. These studies provide evidence for the need to re-evaluate the current levels of vit D and Ca in the AIN-93G diet for bone development. If these diets contain higher than required levels of vit D and Ca for bone health, this may confound results of nutritional intervention studies targeting bone outcomes such as structure and BMD.

Developing a diet that provides sufficient levels of vit D and Ca that support healthy bone structure and BMD enables researchers to be confident that their dietary interventions aimed at supporting bone health are not being tested in the context of excess vit D and Ca, which might mask a potential benefit of the test diet. This also relates to the human scenario as many North Americans do not consume vit D and Ca at recommended levels [170-172]. Thus, the objective of this study was to determine if lower levels of vit D (100 or 400 IU vit D/kg) and Ca (0.35, 0.3, or 0.25%) support healthy bone structure and BMD assessed using in vivo µCT at 2 and 4 months of age.

4.3 Methods

4.3.1 Animals and diets

The experimental protocol was approved by the Animal Care Committee at Brock University and all experimental procedures complied with the Canadian Council on Animal Care. Female, timed-pregnant CD-1 mice (N = 22) were purchased from Charles River Canada. Upon arrival, mice were caged individually in standard environmental
conditions (12 h light: 12 h dark cycle, room temperature of 23ºC), and acclimatized for one week. LED light was used in the housing room which were confirmed to produce no UVB radiation. All mothers were provided water and the AIN-93G reference diet ad libitum throughout pregnancy and lactation. Litters were randomly assigned to one of two trials consisting of an AIN-93G diet (1000 IU vit D/kg and 0.5% Ca) (Envigo, Madison, Wisconsin) or in Trial 1, 100 IU vit D/kg and either 0.35, 0.30, or 0.25% Ca, or Trial 2, 400 IU vit D/kg and either 0.35, 0.30, or 0.25% Ca. At weaning (21 days of age), male offspring (n = 13-15/group) were randomly assigned a diet within a trial until 4 months of age (Figure 1). Actual dietary levels of vit D and Ca were confirmed by a third-party laboratory (Maxxam Analytics, Mississauga, ON, Canada) (Table 8). All other dietary components were kept constant. Diets were colour coded to allow investigators involved with daily care and analyses to be unaware of dietary intervention assignments. Female offspring were randomized similarly for a separate, parallel study and will be reported in a separate paper. Food intake was measured twice weekly, and body weight was measured once weekly using an electronic scale (Denver Instrument, MXX-5). µCT scans of the right tibia were performed at 2 and 4 months of age. Mice were sacrificed by cervical dislocation at 4 months of age and blood was collected. Lumbar vertebrae 4 (L4), and mandibles were excised, cleaned and stored in saline soaked gauze at -80 ºC until µCT scans were performed ex vivo.
TRIAL 1
Weaning ♀ offspring (PND 21)

TRIAL 2
Weaning ♀ offspring (PND 21)

AIN-93G
0.5% Ca
1000 IU vit D/kg

0.35% Ca 0.30% Ca 0.25% Ca

100 IU vit D/kg diet

AIN-93G
0.5% Ca
1000 IU vit D/kg

0.25% Ca 0.30% Ca 0.35% Ca

400 IU vit D/kg diet

22 timed-pregnant CD-1 mice (11 litters/trial)

Age 2 months
Right tibia: bone structure & BMD (in vivo μCT)

Age 4 months
Right tibia: bone structure & BMD (in vivo μCT)
L4, mandible: bone structure (ex vivo μCT)
Biochemical measures: serum 25(OH)D₃ & serum PTH

Figure 1. Experimental design.
4.3.2  *In vivo* µCT scanning of tibia at 2 and 4 months of age

At 2 and 4 months of age, the right tibia were scanned using a high resolution µCT scanner (SkyScan 1176, Belgium) using methodology previously described [167]. The mice were anaesthetized in an induction chamber using isoflurane inhalant at a rate of 3-5%, transferred to the scanning bed of the µCT, and a nose cone was placed over the face of the mouse to continuously administer isoflurane at a rate of 2.5-3.5% for the duration of the scan. The mouse was positioned supine, with the right leg extended and secured using a customized apparatus and the whole tibia placed in the center of the scan field. To prevent movement and any other structures entering the field of view, the tail and the left leg was secured along the side of the scan bed (*Figure 2*). The following parameters were used to image the trabecular and cortical bone of the tibia: 9µm isotropic pixel size, 40kV, 300µA, rotation step of 0.8 over 180 degrees, 3350 ms exposure time, with a 1.0 mm Al filter, and a scanning duration of 16 minutes.

4.3.3  *Ex vivo* µCT scanning of L4 and mandible

Excised L4 and the right mandibular bone were scanned with a high resolution µCT scanner (SkyScan 1176, Bruker-microCT, Belgium). Samples were removed from storage in saline and then wrapped in parafilm to prevent the samples from losing moisture during scanning. Samples were placed in a polystyrene foam tube and mounted horizontally on the scan bed. The following scanning parameters were used for L4 and right mandibles: bones were wrapped in parafilm wax and secured in a foam tube, 9µm isotropic pixel size, 45kV, 545µA, 0.25mm Al filter, rotation step of 0.2 over 180º, and a scanning duration of 38 minutes.
4.3.4 Image reconstruction

All images were reconstructed using NRecon software (v.1.6.9.10, Bruker-microCT, Belgium). A gaussian filter was applied to the images with smoothing, ring-artifact correction, beam hardening correction, dynamic imaging range, and a defective pixel masking was used and a variable post-alignment compensation calculated by Nrecon was manually adjusted for all images (Table 6).

4.3.5 Image analyses

To obtain the region of interest (ROI) for the proximal tibia trabecular bone, the transaxial slice at which the primary spongiosa of the proximal tibia metaphysis disconnects, and a “bridge” from the low-density cartilage of the growth plate is formed, was located. An offset of 70 slices was calculated based on the average slices required to eliminate the growth plate from the ROI. A height of 75 slices was determined to represent the proximal metaphyseal trabecular bone for analysis. A global threshold of 65 was used to binarize bone tissue from non-bone tissue. The trabecular bone was segmented from the cortical bone manually using a hand-drawn interpolated shape. For cortical bone of the tibia, an ROI was determined by 50 slices distally and proximally from the mid slice as calculated by the half way distance between the medial malleolus and the interchondular eminence (Figure 3). A global threshold of 105 was used to binarize bone tissue from non-bone tissue. Automated processing was used to segment the cortical bone for analysis.

The ROI of the vertebral body was delineated by the lower and upper cartilaginous end plates, which was located and delineated by manual drawing. An adaptive threshold of 77 was set to binarize bone tissue from non-bone tissue.
The ROI of the mandible began 10 slices inferior to the bifurcation of the roof of the roots of the first molar, which was defined as the slice at which the roots became visibly independent structures, and extends 90 slices below this point (Figure 4). An interpolated hand-drawn shape was contoured excluding the dentin ligament surrounding each root, and the medial and lateral boarders were set at the width of the root. Each slice was then visually inspected. An adaptive threshold of 87 was set to binarize bone tissue from non-bone tissue.
Table 6. Reconstruction parameters for tibia, L4 and mandible.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Tibia</th>
<th>L4</th>
<th>Mandible</th>
</tr>
</thead>
<tbody>
<tr>
<td>Smoothing</td>
<td>4</td>
<td>3</td>
<td>5</td>
</tr>
<tr>
<td>Ring artifact</td>
<td>10</td>
<td>8</td>
<td>10</td>
</tr>
<tr>
<td>Beam hardening</td>
<td>35</td>
<td>35</td>
<td>35</td>
</tr>
<tr>
<td>Defective pixel masking</td>
<td>5</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>Dynamic image range</td>
<td>0.000-0.0939</td>
<td>0.000-0.1087</td>
<td>0.0000-0.2041</td>
</tr>
</tbody>
</table>
Figure 2. Depiction of the materials used and the position of the mouse during µCT scanning of the tibia.
A) Mice were positioned supine on the scan bed with the right leg extended and held securely in the plastic holder. The contralateral leg was placed beside the body and secured with tape. B) The right leg was wrapped below the ankle with foam lined with dental wax and tightly secured in the plastic holder to prevent movement during scanning [167].
Figure 3. Representative three-dimensional image of the right tibia. Trabecular region of interest (blue outline) of the proximal tibia, and cortical region of interest at the midpoint (green outline). The length of the tibia was determined as the distance between the medial malleolus (distally) and the intercondylar eminence (proximally).
Figure 4. Representative three-dimensional image of the mandible.
Sagittal view of the mandible and the first molar (A) and transverse (B) and midsagittal (C) view of the inter-radicular region of the first molar (blue).
Trabecular structural measurements that were assessed using 3D analysis included the following: bone mineral density (BMD), percent bone volume (BV/TV), trabecular thickness (Tb.Th), trabecular number (Tb.N), trabecular separation (Tb.Sp), degree anisotropy (DA) and connectivity density (Conn. D). Cortical structural measurements that were analyzed using both 3D and 2D analyses included (Ct.Ar/Tt.Ar), cortical thickness (Ct.Th), periosteal perimeter (Ps.Pm), endocortical perimeter (Ec.Pm), medullary area (Ma.Ar), and mean eccentricity (Ecc.) (Table 7) (CT Analyzer, v. 1.14.4.1 + (64-114 bit), SkyScan, Bruker-microCT, Belgium).
Table 7. Trabecular and cortical bone structural properties [22].

**Trabecular Bone**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Name</th>
<th>Description</th>
<th>Units</th>
</tr>
</thead>
<tbody>
<tr>
<td>BMD</td>
<td>BMD</td>
<td>Volumetric mineral density per unit volume of bone</td>
<td>g/cm³</td>
</tr>
<tr>
<td></td>
<td>Bone Volume</td>
<td></td>
<td></td>
</tr>
<tr>
<td>BV/TV</td>
<td>Fraction</td>
<td>Ratio of the segmented bone volume to the total volume of the region of interest</td>
<td>%</td>
</tr>
<tr>
<td>Tb.N</td>
<td>Trabecular Number</td>
<td>Measure of the average number of trabeculae per unit length</td>
<td>1/mm</td>
</tr>
<tr>
<td>Tb.Th</td>
<td>Trabecular Thickness</td>
<td>Mean thickness of trabeculae, assessed using direct 3D methods</td>
<td>mm</td>
</tr>
<tr>
<td>Tb.Sp</td>
<td>Trabecular Separation</td>
<td>Mean distance between trabeculae, assessed using direct 3D methods</td>
<td>mm</td>
</tr>
<tr>
<td>DA</td>
<td>Degree of Anisotropy</td>
<td>1 = isotropic, &gt;1 = anisotropic</td>
<td>no units</td>
</tr>
<tr>
<td>Conn.D</td>
<td>Connectivity Density</td>
<td>A measure of the degree of connectivity of trabeculae normalized by TV</td>
<td>1/mm³</td>
</tr>
</tbody>
</table>

**Cortical Bone**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Name</th>
<th>Description</th>
<th>Units</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tt.Ar</td>
<td>Total Area</td>
<td>Total cross-sectional area inside the periosteal envelope</td>
<td>mm²</td>
</tr>
<tr>
<td>Ct.Ar</td>
<td>Cortical Bone Area</td>
<td>Cortical bone area = cortical volume ÷ (number of slices x slice thickness)</td>
<td>mm²</td>
</tr>
<tr>
<td>Ct.Ar/Tt.Ar</td>
<td>Cortical Area Fraction</td>
<td>Ratio of cortical bone to total cross-sectional area</td>
<td>%</td>
</tr>
<tr>
<td>Ma.Ar</td>
<td>Medullary Area</td>
<td>Area inside the endosteal envelope</td>
<td>mm²</td>
</tr>
<tr>
<td>Ct.Th</td>
<td>Cortical Thickness</td>
<td>Average cortical thickness</td>
<td>mm</td>
</tr>
<tr>
<td>Ps.Pm</td>
<td>Periosteal Perimeter</td>
<td>Periosteal perimeter</td>
<td>mm</td>
</tr>
<tr>
<td>Ec.Pm</td>
<td>Perimeter</td>
<td>Endocortical perimeter</td>
<td>mm</td>
</tr>
</tbody>
</table>
4.3.6 Biochemical analyses

LC-MS/MS was used to determine serum 25(OH)D₃ concentrations and was performed by the Analytical Facility for Bioactive Molecules of the Centre for the Study of Complex Childhood Diseases, The Hospital for Sick Children (Toronto, ON, Canada). Serum PTH was measured using a mouse PTH 1-84 ELISA kit (REF 60-2305) (Immunotopics Inc., San Clemente, CA, USA) as per manufacturer’s instructions.

4.3.7 Statistical analyses

All statistical analyses were performed using SPSS software. Outliers were defined by 3 interquartile rule and removed before data analysis. Data was assessed for normality by checking skewness and kurtosis. Assumptions of homogeneity, levene’s variance, and sphericity were checked prior to analysis. Body weights and food intake were analysed using a repeated measures ANOVA with 2 factors, age and diet. Tibia BMD and bone structure measures at 2 and 4 months of age were analysed using a mixed ANOVA with 2 factors of age and diet as a between-subject factor. All ex vivo bone structure and serum analyses were analysed using a one-way ANOVA. P < 0.05 was considered as statistical significance.

4.4 Results. Trial 1: AIN-93G or 100 IU vit D/kg and 0.35, 0.30, or 0.25% Ca diets

4.4.1 Food intake

At week 4, the REF and the 0.3% Ca groups were significantly greater than the 0.25% Ca group. At week 5, the REF group ate significantly more food than the 0.35 and 0.3% Ca groups (p < 0.05). At week 6, the 0.35% Ca group ate significantly more food than the 0.3% Ca group. At week 9, the REF group ate significantly more food than the 0.3 and
0.25% Ca group (p < 0.05). The REF group ate significantly more food than the 0.25% Ca group in week 10 and 11. And the 0.35% Ca group ate significantly more food than all other groups in week 14 (p < 0.05) (Figure 5).

4.4.2 Body weight

There were no differences in body weight among groups over the course of the trial, however, there was a significant effect for age as body weight increased with age for all groups (p < 0.001) (Figure 5).

4.4.3 Biochemical analyses

LC-MS/MS revealed serum 25(OH)D₃ was significantly lower for all modified diets compared to the REF diet (p < 0.001). Enzyme linked immunoassays revealed no difference in circulating serum PTH levels among groups (p > 0.05) (Figure 6).
Figure 5. Trial 1: Food intake and body weight.

Food intake (a) and body weight (b). Ear tagging was performed at weaning so body weights prior to weaning (PND 21) were calculated as mean litter weight for each group. Data represented as mean ± SEM. * represents a significant difference among groups at that time (p < 0.05)
Figure 6. Trial 1: Serum 25(OH)D₃ and Serum PTH.
Dietary effects of lower vit D and Ca on serum 25(OH)D₃ (n = 3/group) (a) and serum PTH (n = 8/group. (b) in male CD-1 mice. Mean ± SEM. n = 8/group. * Represents a significant difference from the REF group. P < 0.05
4.4.4  *In vivo* tibia structure

At the proximal tibia, there was a significant main effect for diet where the REF group had greater Tb.Th (mm) compared to the 0.25% Ca group (p < 0.05). There was a main effect for age where bone volume fraction (BV/TV, %) trabecular thickness (Tb.Th, mm), and trabecular separation (Tb.Sp, mm) were greater at 4 months of age, while connectivity density (Conn.D, mm$^{-3}$), trabecular number (Tb.N, mm$^{-1}$), and degree of anisotropy (DA, no unit) were greater at 2 months of age (p < 0.05) (Figure 7).

At the midpoint of the tibia, there was a main effect for diet where the REF group had greater Ct.Th compared to the 0.30 and 0.25% Ca groups (p < 0.05). A main effect for age was observed where tibia length (mm), cortical area fraction (Ct.Ar/Tt.Ar, %), cortical thickness (Ct.Th, mm), periosteal perimeter (Ps.Pm, mm), and eccentricity (Ecc., no units) were greater at 4 months of age, while endocortical perimeter (Ec.Pm, mm) and marrow area (Ma.Ar, mm$^2$) were greater at 2 months of age (p < 0.05) (Figure 8).

4.4.5  *Ex vivo* structure of L4 and mandible

No difference in trabecular or cortical structure among dietary intervention groups at L4 or mandible (p > 0.05) other than the 0.35% Ca group had significantly less Conn. D at the mandible compared to the 0.3% Ca group (p < 0.05) (Figure 10 and Figure 11).
Figure 7. Trial 1: Proximal tibia trabecular structure.
Bone structure of right tibias at 2 months (hatched columns) and 4 months (solid columns) in male CD-1 mice. n = 13-14/group. Data expressed as mean ± SEM. * represents a significant difference from the REF group. P < 0.05
Figure 8. Trial 1: Midpoint tibia cortical structure.
Bone structure of right tibias at 2 months (hatched columns) and 4 months (solid columns) in male CD-1 mice. n = 13-14/group. Data expressed as mean ± SEM. * represents a significant difference from the REF group. P < 0.05
Figure 9. Trial 1: Representative two-dimensional images of the proximal and midpoint tibia. White lines represent 1 mm length. Proximal tibia image was selected as the most bottom slice of the ROI. The middle slice of the ROI is represented for the midpoint tibia.
Figure 10. Trial 1: L4 trabecular and cortical structure.
Trabecular (a-d) and cortical (e-f) bone structure of the L4 scanned ex vivo after 4 months of age in male CD-1 mice. n = 8/group. Data expressed as mean ± SEM. No difference among groups. P > 0.05.
Figure 11. Trial 1: Mandible structure.
Bone structure of the mandible scanned *ex vivo* after 4 months of age in male CD-1 mice. n = 8/group. Data expressed as mean ± SEM. Different letters indicated a significant difference between groups. P < 0.05.
Figure 12. Trial 1: Representative two-dimensional images of the L4 and mandible. The top slice of the ROI was selected to represent the L4 and mandible images. White lines represent 1 mm length for the L4 and 0.1 mm for the mandible.
4.5 Results. Trial 2: AIN-93G or 400 IU Vit D/kg and 0.35 or 0.25% Ca diets

The group fed the 0.30% Ca diet was removed from the study as one cage housing 5 males from this group demonstrated aggressive behaviour causing injury; the sample size was too small to include the group in the final analyses.

4.5.1 Food intake

There was a significant interaction effect where the 0.25% Ca group ate significantly more food compared to the 0.35% Ca group at week 3, and ate significantly more food than the REF group in week 4 (Figure 13).

4.5.2 Body weight

There were no differences in body weight among any of the dietary intervention groups over the course of the trial, however, there was a significant increase in body weight with age (p < 0.001) (Figure 13).

4.5.3 Biochemical analyses

LC-MS/MS revealed no difference in serum 25(OH)D₃ among dietary groups (p > 0.05). Enzyme linked immunoassays revealed no difference in circulating serum PTH levels among groups (p > 0.05) (Figure 14).
Figure 13. Trial 2: Food intake and body weight.
Food intake (a) and body weight (b). Earing tagging was performed at weaning, body weights prior to weaning (PND 21) were calculated as mean litter weight for each group. Data represented as mean ± SEM. * represents a significant difference between groups at that time (p < 0.05)
Figure 14. Trial 2: Serum 25(OH)D₃ and Serum PTH.
Dietary effects of lower vit D and Ca on serum 25(OH)D₃ (n = 3/group) (a) and serum PTH (n = 8/group). (b) in male CD-1 mice. Mean ± SEM. n = 8/group. No difference among groups. P > 0.05.
4.5.4 In vivo tibia structure

There were no differences in trabecular structure of proximal tibia among groups at 2 and 4 months. There was an interaction effect where BV/TV (%) was greater at 2 versus 4 months of age for the 0.35% Ca group, and Tb.Th (mm) was greater at 4 compared to 2 months of age for all dietary groups (p < 0.05). Significant main effect for age revealed Tb.N (mm⁻¹) and Conn. D (mm³) were greater at 2 compared to 4 months, and Tb.Sp (mm) was higher at 4 versus 2 months (p < 0.05) (Figure 15).

At the midpoint of the tibia, the 0.25% Ca group had lower Ct.Th than the 0.35% Ca group (p < 0.05). There was a significant effect for age where Ec.Pm (mm) and Ma.Ar (mm²) were greater at 2 months compared to 4 months, and tibia length (mm), Ps.Pm (mm), and Ct.Th (mm) were greater at 4 months (p < 0.05) (Figure 16).

4.5.5 Ex vivo L4 and mandible structure

There were no differences in trabecular or cortical structure among dietary groups for the L4 and the mandible (p > 0.05) (Figure 18 and Figure 19).
Figure 15. Trial 2: Proximal tibia trabecular structure.
Bone structure of right tibias at 2 months (hatched columns) and 4 months (solid columns) in male CD-1 mice. n = 13-14/group. Mean ± SEM. Different letters represent a significant difference within a dietary group. P < 0.05
Figure 16. Trial 2: Midpoint tibia cortical structure.
Bone structure of right tibias at 2 months (hatched columns) and 4 months (solid columns) in male CD-1 mice. n = 13-14/group. Mean ± SEM. ** represents a significant difference from the 0.35% Ca group. P < 0.05
Figure 17. Trial 2: Representative two-dimensional images of the tibia. White lines represent 1 mm length. Proximal tibia image was selected as the most bottom slice of the ROI. The middle slice of the ROI is represented for the midpoint tibia.
Figure 18. Trial 2: L4 trabecular and cortical structure.
Trabecular (a-d) and cortical (e-f) bone structure of the L4 scanned ex vivo after 4 months of age in male CD-1 mice. n = 8/group. Mean ± SEM. No difference among groups. P > 0.05.
Figure 19. Trial 2: Mandible structure.
Bone structure of the mandible scanned *ex vivo* after 4 months of age. *n* = 8/group. Mean ± SEM. No difference among groups. *P* > 0.05.
Figure 20. Trial 2: Representative two-dimensional images of the L4 and mandible. The top slice of the ROI was selected to represent the L4 and mandible images. White lines represent 1 mm for L4 and 0.1 mm for the mandible.
4.6 Discussion

This study shows that tibia BMD, structure and length as well as structure of L4 and mandible in male CD-1 mice is unchanged when fed the AIN-93G diet containing 100 or 400 IU vit D/kg and 0.35% Ca from weaning to 4 months of age. Moreover, only a few differences in bone structure were observed at lower levels of Ca emphasizing that markedly lower levels of both vit D and Ca than are present in the AIN-93G diet supports healthy bone structure and BMD in male CD-1 mice. Specifically, trabecular thickness in the 0.25% Ca diet group and cortical thickness in the 0.30% and 0.25% Ca groups were decreased compared to the AIN-93G diet group in Trial 1 (100 IU vit D/kg diet). While in Trial 2 (400 IU vit D/kg diet), cortical thickness was significantly less in the 0.25% Ca group compared to the 0.35% Ca group. Our finding that serum PTH concentrations was similar among groups suggests that none of the groups were challenged with low serum Ca homeostasis.

When the Ca levels for the AIN-93G diet were originally tested, the primary goal was to reduce Ca deposits in kidneys of experimental rodent models [8]. Weanling mice and rats were studied for 16 weeks and randomized to one of three diets containing either 0.33%, 0.5%, or 0.67% Ca. No difference in tibia ash content among these modified AIN-93G diets was observed [8]. This research is in agreement with the present studies results which found no difference in tibia BMD and structure between the AIN-93G diet and any of the modified diets containing either 100 or 400 IU vit D/kg and 0.35% Ca. Reeves and colleagues were more concerned with reduce Ca deposits in the kidneys, than setting a sufficient Ca level, that was not in excess for bone health [7]. While understandable, and considering the methods used provided little information regarding
bone health, an excess level of Ca and/or vit D may be problematic for research aimed at elucidating potential dietary interventions to enhance and/or support bone health as excess of either of these nutrients, but particularly Ca, can protect bone when vit D status is low [173].

The findings of the present study suggest that the current level of vit D in the AIN-93G (1000 IU/kg) diet is higher than required for healthy bone structure and BMD in rodent models and is in agreement with previous findings [10]. Low vit D intake (50, 100, 200, or 400 IU/kg) in male mice (C57BL/6) from weaning until 14 weeks of age showed no differences in BMC and BMD of the femur, measured using DXA, and a decrease in these measures were only observed when vit D was fed at 25 IU/kg [10]. Considering that no difference in BMD, structure of the tibia, L4 and mandible was observed at either level of vit D, in combination with a Ca level of 0.35%, provides further evidence for reducing the level of vit D in the AIN-93G diet when using the CD-1 mouse model.

Interestingly, a separate experiment examining the effects of diets containing 0.25% or 0.5% Ca at either 400, 1000, or 10000 IU vit D/kg diet, resulted in a decrease in femur BMD in the 0.25% Ca groups at any level of vit D [10]. While the present study found no changes in BMD and only a few structural outcomes were compromised at this level of Ca (0.25%), the differences between these results may be due to a strain-specific response. The bone phenotype of mice in response to Ca intake differs among inbred strains [52, 69]. For example, eleven genetically diverse inbred strains of male mice were fed a modified AIN-93G diet containing 200 IU vit D/kg and 0.5% or 0.2% Ca from 4 to 12 weeks of age. The authors observed that different mouse strains had specific and differing responses to a low Ca diet in terms of femur BMD, bone volume fraction and
cortical area fraction measured by μCT[69]. This suggests that genetic background significantly influences BMD and structural phenotype, and the ability of each strain to adapt to a low Ca diet. Furthermore, these findings provide justification for investigating the specific vit D and Ca requirements of experimental mouse models. The present study focused on the outbred CD-1 stock which, to our knowledge, has not been extensively compared to other commonly used mouse models. While inbred models provide invaluable information regarding genetic background and trait loci for bone researchers, the CD-1 mouse has been used previously in toxicology [174] and cancer research [175], and relatedly to bone, to study the effects of early life exposure to food components such as soy isoflavones and their ability to influence bone health later in adulthood [73]. These results provide the framework for development of a modified AIN-93G diet containing levels of Ca and vit D that support bone health in growing male CD-1 mice without providing excess that may mask beneficial effects of dietary interventions. Moreover, the positive effects of food components that can program bone health may prove to be greater in the context of a diet not in excess of Ca and vit D.

Previous studies by our group have investigated the programming effects of maternal vit D on BMD and bone structure in male offspring in different mouse models [11, 12, 169]. Mothers consumed either low (25 IU/kg) or high (5000 IU/kg) vit D and male offspring were weaned to a diet containing either the low or high maternal levels of vit D. Interestingly, low dietary vit D consumption in the offspring diet, even when exposed to a low vit D maternal diet, resulted in no effect on BMC and BMD measured by DXA in CD-1 mice [12]. Furthermore, while maternal exposure elicited significant improvements in trabecular number and trabecular separation of femurs in offspring,
exposure to the high vit D diet after weaning did not provide benefits to structure in male C57BL/6 mice using the same experimental design [169]. While these studies were investigating the programming effects of vit D and using a higher level of vit D than the AIN-93G diet, considering multiple studies found no difference in BMD and or bone structure between 25 and 5000 IU vit D/kg, these results call into question the vit D requirements of these models and/or what Ca level would be appropriate. Interestingly, in female C57BL/6 mice, lumbar vertebra BMC and BMD was decreased when consuming the low vit D diet, however no structural differences, may suggest sex differences related to the reliance of vit D [176]. This may be true in the C57BL/6 mouse model, however, a parallel investigation of female siblings of the present study found no difference in BMD, bone structure or serum PTH among dietary groups (unpublished data). This would suggest that the CD-1 mouse, both males and females, have specific dietary requirements for vit D and Ca that need to be further defined.

In summary, findings from the present study suggest that the current level of vit D and Ca is higher than required in the AIN-93G diet to achieve healthy bone structure and BMD in growing male CD-1 mice. This has implications for researchers studying the effects of dietary interventions in rodent studies as use of the AIN-93G may mask or attenuate potential benefits of a dietary intervention. This aspect requires future investigation.
5 STUDY 2

Effects of lower dietary levels of vitamin D and calcium in the AIN-93G diet on intestinal integrity and the relationship to bone health in growing male CD-1 mice
5.1 Abstract

Background: Maintaining a healthy gut barrier is essential for overall health of many tissues including bone. This barrier is influenced by pathogenic organisms, alcohol, fat intake and drugs, and dietary factors such as Ca have been shown to have protective effects. **Objective:** To determine if the health of the intestine, measured by fecal albumin and serum LPS, is altered by lowering the levels of dietary vit D and Ca, and the relationship to bone structure. **Methods:** Weanling male CD-1 mice were randomized to modified AIN-93G diets containing either 100 IU vit D/kg diet (Trial 1) or 400 IU vit D/kg diet (Trial 2) within one of three Ca levels (0.35, 0.30, or 0.25% Ca diet) or the AIN-93G diet (REF) (1000 IU/kg vit D and 0.5% Ca) to 4 months of age (n = 13-15/group). At 4 months of age, fecal pellets were collected and analysed for fecal albumin content. BMD and structural properties of the tibia were analysed using *in vivo* µCT and L4 was scanned *ex vivo*. After which, animals were euthanized and blood was collected and analysed for serum LPS. **Results:** There were no differences in fecal albumin content among dietary groups (p > 0.05) in either Trial. In Trial 2, the group fed modified AIN-93G diet containing 0.35% Ca had significantly higher serum LPS compared to the group fed the REF diet. There was also a significant correlation between fecal albumin content and L4 trabecular thickness (p < 0.05). There were no other significant correlations of fecal albumin and bone outcomes for either the tibia or L4 within either Trial. **Conclusion:** Lowering dietary vit D to 100 IU/kg and Ca to 0.25% diet in the AIN-93G diet did not negatively affect the intestinal integrity of growing male CD-1 mice, and only one correlation between fecal albumin and a bone parameter was observed, suggesting no relationship.
5.2 Introduction

Maintaining a healthy intestinal barrier is essential to control the entry of toxins, pathogenic organisms, and optimization of nutrient absorption. There is evidence that compromised intestinal integrity can be deleterious to bone [15, 17, 169], and nutrition has been shown to both negatively and positively influence the intestinal barrier [177-180]. This creates a complex relation between dietary practices and bone health as the intestine may be a potential target for preventing osteoporosis.

Considering the evidence that increased dietary Ca improves the health of the intestine during times of insult, the purpose of this study was to determine if intestinal integrity is maintained at lower levels of vit D and Ca in the growing male CD-1 mouse model. Rodent models allow researchers to use invasive techniques to study intestinal integrity and the relationship with BMD and bone structure. In an irritable bowel disease rat model, subjects were fed a high fat diet with either low (30 mmol/kg) or high (120 mmol/kg) Ca for 7 weeks. Interestingly, serum LPS was significantly less in the high Ca group suggesting an improved intestinal barrier compared to the low Ca group [179].

Considering that in Study 1 of this thesis, vit D was lowered to one tenth and Ca was lowered to half the current levels in the AIN-93G diet and there were no overt detriments to bone, determining if these levels also maintain intestine health will further support the use of these levels in future diets. The objective of this study was to determine if the health of the intestine, measured by fecal albumin and serum LPS, is altered by lowering the levels of dietary vit D (100 and 400 IU/kg) and Ca (0.25, 0.3, and 0.35%) and the relationship to bone structure.
5.3 Methods

Experimental design and dietary interventions are described in Figure 1. At 4 months of age mouse fecal pellets were collected and mice were subsequently euthanized, and blood was collected in pyrogen free tubes for LPS analysis. Samples were stored at -80ºC until further analysis.

5.3.1 Biochemical analyses

Fecal pellets were resuspended in sterile phosphate buffered saline and albumin concentrations were determined by ELISA following the manufacturer’s instructions (Bethyl Laboratories, Montgomery, TX, USA). Serum LPS was quantified via an enzyme-linked immunosorbent assay (ELISA; Cusabio Biotech, Barksdale, DE, USA).

5.3.2 Statistical analyses

All statistical analysis was performed using SPSS software. Outliers were defined by 3 interquartile rule and removed before data analysis. Data was assessed for normality by checking skewness and kurtosis. Assumptions of homogeneity, and levene’s variance was checked and a one-way ANOVA was used to analyse fecal albumin and serum LPS. P < 0.05 was considered significant. All correlational data was checked for normality and a spearman’s value was used.

5.4 Results

5.4.1 Trial 1: Fecal albumin and serum LPS

There was no difference in fecal albumin content or serum LPS among dietary groups (Figure 21) (p > 0.05).
Figure 21. Trial 1: Fecal albumin and Serum LPS.
Fecal albumin (a) n = 10-12/group, serum LPS (b) n = 2-3/group. P > 0.05. Data expressed as mean ± SEM.
5.4.2 Trial 1: Correlation between fecal albumin and structure at the proximal tibia, tibia midpoint and L4 at 4 months of age

There were no significant correlations of fecal albumin with any of the structural outcomes at the proximal tibia (Figure 22), tibia midpoint (Figure 23) or L4 (Figure 24) (P > 0.05).
Figure 22 Trial 1: Spearman correlations of fecal albumin and proximal tibia structure.
Fecal albumin and trabecular structure outcomes of the proximal tibia. n = 9-11. P > 0.05. REF diet (black), 0.35% Ca diet (red), 0.3% Ca diet (orange), and 0.25% Ca diet (blue).
Figure 23. Trial 1: Spearman correlations of fecal albumin and midpoint tibia structure.

Fecal albumin and cortical structure outcomes of the tibia midpoint. n = 9-11. P > 0.05. REF diet (black), 0.35% Ca diet (red), 0.3% Ca diet (orange), and 0.25% Ca diet (blue).
Figure 24. Trial 1: Spearman correlations of fecal albumin and L4 structure.
Fecal albumin and L4 trabecular (a-d) and cortical (e and f) structure. n = 6-8. P > 0.05. REF diet (black), 0.35% Ca diet (red), 0.3% Ca diet (orange), and 0.25% Ca diet (blue).
5.4.3 Trial 2: Fecal albumin and serum LPS

There was no difference in fecal albumin content and serum LPS among dietary groups other than the 0.35% Ca group had higher serum LPS than the (REF) group (Figure 25) (p < 0.05).
Figure 25. Trial 2: Fecal albumin and serum LPS.
Fecal albumin (a) raw data and SEM plotted. n = 10-11/group. Serum LPS (b) raw data and SEM plotted. n = 4-5/group. P > 0.05.
5.4.4 Trial 2: Correlation between fecal albumin and structure at the proximal tibia, tibia midpoint and L4 at 4 months of age

There was a significant correlation between fecal albumin and Tb.Th (mm) of the L4 (p < 0.05). There were no other significant correlations for fecal albumin with any of the structural outcomes at the proximal tibia (Figure 26), tibia midpoint (Figure 27) or L4 (Figure 28) (P > 0.05).
Figure 26. Trial 2: Spearman correlations fecal albumin and proximal tibia structure.
albumin and trabecular structure outcomes of the proximal tibia n = 9-11/group. P > 0.05. Ref diet (black), 0.35% Ca diet (green), and 0.25% Ca diet (purple).
Figure 27. Trial 2: Spearman correlations of fecal albumin and midpoint tibia structure.
Fecal albumin and cortical structure outcomes of the tibia midpoint. \( n = 9-11/\)group. \( P > 0.05 \). Ref diet (black), 0.35\% Ca diet (green), and 0.25\% Ca diet (purple).
Figure 28. Trial 2: Spearman correlations of fecal albumin and L4 structure. Albumin and L4 trabecular (a-d) and cortical (e-f) structure. n = 7-8. P < 0.05. Ref diet (black), 0.35% Ca diet (green), and 0.25% Ca diet (purple).
5.5 Discussion

This study showed that lowering the level of vit D to 100 IU/kg and Ca to 0.25% in the AIN-93G diet does not increase serum LPS or fecal albumin content in growing male CD-1 mice at 4 months of age. This would suggest that the intestinal integrity of growing male CD-1 mice is not compromised when dietary vit D is lowered to one tenth the level and Ca to half the level in the AIN-93G diet. There was however a significantly higher level of serum LPS in the 400 IU/kg and 0.35% Ca group compared to the REF group, however considering this was not observed at lower levels of vit D and/or Ca suggests this may be by chance. Furthermore, there were no significant correlations with fecal albumin and bone structural outcomes at the tibia, and only Tb.Th (mm) was correlated with fecal albumin at the L4 in Trial 2. The results from this study suggest the intestinal health of growing male CD-1 mice is maintained on an AIN-93G diet that contains lower levels of both dietary vit D and Ca.

To date, while some have studied the effects of increasing dietary Ca, there is little evidence on the effects of lowering dietary Ca on intestinal integrity. There are three proposed mechanisms by which increased dietary Ca improves intestinal barrier function. Firstly, the ingestion of Ca increases gastric acid secretion, decreasing the pH of the stomach. High acidity in the stomach is inhospitable to pathogenic bacteria such as *Salmonella*, and the positive influence of increased dietary Ca has on intestinal integrity may be due to improved microbiota and not changes at the intestinal barrier [16]. Modified AIN-76 diets containing either 0.08, 0.24 or 0.72% Ca were fed to rats who were then orally infected with *S. enteriditis*. Lower dietary Ca resulted in increased *salmonella* count in feces, ileal scraping and lavage, and decreased lactobacilli in all
samples compared to the higher Ca diets. Furthermore, urinary nitric oxide, a measure of bacterial translocation, was reduced in the high Ca diet group suggesting decreased translocation of bacteria [152]. This study suggests the benefits of Ca supplementation are associated with alterations in the microbiota, specifically increased lactobacilli counts and a decrease in the survival of *salmonella*, by creating an environment more resistant to *salmonella* colonization. Creating an environment more favourable for lactobacilli may provide defense (or a more competitive environment) from colonization of potentially infectious pathogens. Secondly, high dietary Ca consumption may increase tight junction expression improving the selectivity of the intestine. Lastly, high Ca concentration in the lumen of the intestine may react with and cause precipitation of bile acids, decreasing their cytotoxicity [16]. A study in male C57BL/6 mice fed an AIN-93G diet or a high fat variation for 7 weeks and the effects on the intestine were investigated. There was increased intestinal permeability, lower tight junction protein expression (ZO-2 and JAM-A) and an increase in primary and secondary bile acid cecal content compared to the AIN-93G diet [177]. This study suggests dietary factors (high fat) increase bile acid concentration within the lumen, which may influence the intestinal barrier through the destruction of intercellular tight junctions. High dietary Ca may provide therapeutic relief [156]. If the intestinal barrier is chronically compromised due to increased bile acid exposure, this suggests a mechanism for bacterial and enterotoxin entry into the circulation leading to chronic low-grade inflammation. These studies provide grounds for the potential use of high Ca diets to combat pathogenic gastrointestinal infections.

While these studies provide evidence for the ability of Ca to aid in improvements in the intestinal barrier function, it is difficult to directly compare these studies to the present
one. Firstly, the above studies incorporated an inflammatory disease or infected model in the study design. This provides a mechanism for insult to the intestinal barrier, while the present study only adjusted the dietary vit D and Ca levels. This adjustment may not be enough stress to deteriorate the intestinal barrier, therefore no differences among the groups would be observed. Secondly, many studies incorporated a high fat diet to mimic a Western diet, and this is known to decrease intestinal integrity as measured by FD-4 permeation which is absorbed via the paracellular route [177]. High fat diets also cause an increase in the release of bile acids. While the main function of bile acids is to aid in the digestion of dietary fat, there is evidence that they also play a role as signalling molecules for intestinal epithelial cells for transporting nutrients and barrier function [181]. Increased levels of bile acids in the lumen of the intestine is cytotoxic, and have been shown to increase intestinal permeability to large molecules, and this loss of intestinal barrier was associated with compromised intercellular tight junction complexes [177].

Recently, our lab studied male C57BL/6 mice fed either high (5000 IU/kg) or low (25 IU/kg) vit D in utero and weaning from the maternal diet, then weaned to an obesogenic diet containing either the high or low vit D to 7 months of age [169]. While there was a significant main effect for maternal diet and offspring diet where serum LPS was higher in the groups that had experienced lower dietary vit D, there was also a main effect where fecal albumin was also higher for the dam diet. This study showed that maternal dietary vit D has a beneficial programming effect on trabecular bone at the distal femur, and fecal albumin was positively correlated with vertebral and femur trabecular separation, and negatively for vertebral trabecular number. This would suggest that the health of the
intestinal barrier may influence bone health, however, by which mechanism this occurs was not assessed. It is difficult to directly compare results of the present study and Villa et al, 2016, as the present study did not alter maternal diet. Furthermore, Villa et al. 2016 used a high fat diet which is known to negatively impact the intestinal barrier. Considering the present study did not find differences in fecal albumin or serum LPS among dietary groups, this would suggest that lower levels of dietary vit D, in combination with lower Ca, in the AIN-93G diet does not affect the intestinal integrity of growing male CD-1 mice.

In summary, this study suggests the intestinal health of growing male CD-1 mice is unchanged when dietary Ca and vit D are reduced in the AIN-93G diet. This provides further evidence, in conjunction with the first study of this thesis, that bone structure and BMD is also largely unaffected.
This thesis research has shown that lowering the level of vit D to 400 or 100 IU/kg and Ca to 0.35% does not negatively impact bone structure and BMD in growing male CD-1 mice. Only a few differences in bone structure were observed with diets containing either 0.3 or 0.25% of Ca in both trials of vit D. Specifically, within Trial 1 (100 IU vit D/kg diet), trabecular thickness in the 0.25% Ca diet group and cortical thickness in the 0.30% and 0.25% Ca groups were lower compared to the AIN-93G diet. While in Trial 2 (400 IU vit D/kg diet), cortical thickness was significantly less in the 0.25% Ca group compared to the 0.35% Ca diet. Interestingly, all modified diets containing 100 IU vit D/kg had significantly lower serum 25(OH)D$_3$ compared to the AIN-93G REF diet. However, there were no differences in serum PTH levels among groups, indicating that Ca homeostasis was maintained. Surrogate measures of intestinal integrity (fecal albumin, and serum LPS) demonstrated that intestinal integrity was maintained at both levels of vit D (400 and 100 IU/kg) when Ca is 0.25% diet. However, considering the 400 IU vit D/kg and 0.35% Ca group had significantly elevated serum LPS, and no analysis of intestinal tissue was completed, this aspect requires further study. Lastly, there were no correlations between fecal albumin and bone structure, other than for trabecular thickness at the L4, suggesting there was no relationship between intestinal integrity and bone outcomes.

The current level of vit D (1000 IU/kg) was added to the AIN-93G as recommended by the National Research Council, Committee on Animal Nutrition, and was based on a personal opinion [29]. Considering one of the goals of the AIN committee when developing the AIN-93G diet was to ensure levels of nutrition are not in excess, the
findings of the present study align with the aim of the AIN committee when developing the AIN-93G diet [8]. Previously, C57BL/6 mice fed the AIN-93G diet containing 0.25% Ca at either 400, 1000, or 1000 IU vit D/kg resulted in lower femur BMD at any level of vit D compared to diets containing 0.5% Ca [10]. This would suggest the bone supporting effects of vit D are maximized at or below 400 IU vit D/kg in a diet containing 0.25% Ca. While there was no effect on BMD of the tibia in the present thesis research, differences in bone structure (trabecular thickness and cortical) was lower in the modified diet containing 100 IU vit D/kg and 0.25% Ca, while the 0.25% Ca diet containing 400 IU vit D/kg had significantly lower cortical thickness compared to the 0.35% diet group. While this is only a few parameters, this suggests there may be a threshold near dietary levels of 0.25% Ca for bone health. Similarly, in female Sprague-Dawley rats, the level of Ca in AIN-93G was studied at levels of 0.7 to 0.1% while holding vit D constant (1000 IU/kg) over a period of 13 weeks, from weaning through 4 months of age. No differences in tibia Ca and P content, breaking force, bending moment, and stress of the femur, and proximal tibia bone volume fraction and trabecular thickness, number and separation were observed among the 0.7 versus 0.3% Ca groups. Detrimental effects to structural properties, while few, were observed at 0.2% Ca, and all were compromised at 0.1% Ca in the tibia [13]. This evidence suggests a Ca level of 0.25% in the AIN-93G diet may have deleterious effects on bone structure, strength and/or BMD in rodent models. Furthermore, results from Hunt et al., 2008 and this thesis research suggests a Ca level of approximately 0.35%, at a vit D level of 100, 400 or a 1000 IU/kg, is sufficient to achieve healthy bone structure and BMD in growing rodent models.
Ensuring nutritional composition supports the health of tissues other than bone is necessary to confirm the safety of lowering the levels of nutrients such as vit D and Ca. Considering there is evidence that both these nutrients support the health of the intestine, investigating if the modified diets containing lower vit D (100 or 400 IU/kg) combined with lower Ca (0.35, 0.3, or 0.25%) incorporated in this thesis was warranted. This thesis observed that these levels had no effect on the intestinal barrier as measured by fecal albumin, however serum LPS was significantly higher in the modified diet containing 400 IU vit D/kg and 0.35% Ca compared to the AIN-93G diet. Considering the small sample size for serum LPS analysis and that higher serum LPS was not observed at lower levels of vit D (100 IU/kg) and/or Ca (0.3 or 0.25%) suggests this finding may have been due to a type 2 error. This would suggest that lowering vit D to 100 or 400 IU/kg and Ca (0.35, 0.3, or 0.25%) alone does not cause insult to the intestine. The effect of lower levels of Ca and vit D on intestinal health requires further analysis.

While previously our group has observed correlations between fecal albumin and bone structure this thesis research project did not find similar relationships. It is difficult to directly compare the results of these studies as many factors were different between studies (vit D levels, dietary fat levels, mouse model, and exposure during in utero life).
7 STRENGTHS AND LIMITATIONS

Strengths of this study include the use of *in vivo* µCT which provides not only an analysis of BMD but also the structure of bone. Considering trabecular and cortical bone structure have specific growth patterns from 2 to 4 months of age, imaging structure within each mouse at these points decreases variability within groups and provides a better understanding of BMD and bone structural changes [167]. This provides an opportunity to use a within subject measures statistical design to test tibia outcomes at 2 and 4 months of age and therefore, increasing the power of the statistical model. Additionally, measuring bone structure using µCT provides detailed information regarding the structure in a non-destructive manner. Considering the interdependent physiological effects of Ca and vit D, reducing the levels of both nutrients provides evidence for a combined reduction of these nutrients in CD-1 mice for future studies. Lastly, the assessment of multiple skeletal sites, including the appendicular and axial skeleton as well as mandible, provides a broader understanding of how the skeletal system will respond to these dietary levels.

A limitation of this study may be the relatively short duration of the intervention. Considering osteoporosis occurs later in life, studying mice into older age (18-24 months) when BMD and structure decline, vit D and Ca at these lower levels may alter bone outcomes. Considering the findings of Replogle et al. 2014, that there is variation in BMD among strains of inbred mice, the findings of this study may be limited to the CD-1 mouse, as other mouse models may respond differently. While the CD-1 outbred stock display a relatively high degree of genetic heterogeneity, creating within group variability, outbred models may better reflect the variability of responses that would be
seen in a human population [164]. Another limitation to this study includes some factors related to the analysis of fecal albumin. Through visual observation, the moisture content of the fecal pellets was different among samples. This may have affected the total fecal mass analysis skewing the corrected sample results to the measured fecal weight. Additionally, Fluorescein isothiocyanate-conjugated dextran is the gold standard technique for measuring intestinal integrity, however we used fecal albumin and serum LPS [182]. While a secondary objective of this study was to determine if the gut of growing male CD-1 mice remained healthy at these lower levels of Ca and vit D, this data may only be relevant for this mouse model. However, the original study by Reeves et al., 1993 only included one rat and mouse model and these results were then used for diets for all mouse and rat models [8].
Trial 1: In the context of a diet containing 100 IU vit D/kg diet, there was a main effect where the 0.25% Ca group was less than the AIN-93G diet group, and cortical thickness was less for the 0.3 and 0.25% Ca groups compared to the AIN-93G group. No differences in intestinal integrity was observed. Therefore:

1. Bone structure and BMD measured by µCT were supported at 2 and 4 months of age when growing male CD-1 mice consumed 0.35% Ca.

2. Intestinal integrity measured by fecal albumin and serum LPS was maintained at 4 months of age when growing male CD-1 mice were fed 0.25% Ca.

Trial 2: In the context of a diet containing 400 IU vit D/kg diet, there was a main effect where the 0.25% Ca group had lower cortical thickness compared to the 0.35% Ca group. The 0.35% Ca group had higher serum LPS, and there was a significant correlation between fecal albumin and L4 tabecular thickness. Therefore:

1. Bone structure and BMD measured by µCT were supported at 2 and 4 months of age when growing male CD-1 mice consumed 0.35% Ca.

2. Intestinal integrity measured by fecal albumin and serum LPS was maintained at 4 months of age when growing male CD-1 mice were fed 0.25% Ca.
9 FUTURE DIRECTIONS

Developing a diet that will provide adequate, yet not in excess, nutrition is ideal for studying the effectiveness of dietary strategies to promote bone development. The findings from this study provide evidence to reduce the levels of vit D and Ca at or below 400 IU/kg and 0.35%, respectively, while introducing a novel food component or nutrient that may support bone health. For example, our laboratory has previously found that CD-1 mice exposed to isoflavones during early life have increased BMD and trabecular interconnectivity [73]. Considering this, it would be interesting to see if lowering vit D and Ca to the levels stated above combined with administration of isoflavones has a more pronounced programming effect [73]. Considering North Americans generally consume less Ca and vit D than recommended, performing the same experiment when Ca and vit D are provided at insufficient levels (causing slight deterioration to bone outcomes) may provide a better context for the human scenario. Investigating how the diversity and concentration of the microbiota is altered in the CD-1 mouse when fed at 400 IU vit D/kg and 0.35% Ca in the AIN-93G diet would elucidate the effects on the gut microbiota. Additionally, investigating if there is a relationship between bone and the composition of the gut microbiota with this diet is warranted. Considering the importance of an appropriate Ca:P ratio for reducing nephrocalcinosis in rodent models, determining the effects of 400 IU vit D/kg and 0.35% Ca and maintaining a Ca:P ratio of 1.3 (current ratio of the AIN-93G diet) is warranted. Lastly, investigating what mechanisms improve absorption (intestine) and reabsorption (kidneys) of bone supporting nutrients such as Ca at such low dietary levels is also warranted to further our understanding the physiological mechanisms controlling Ca homeostasis.
10 REFERENCES


167. Sacco, S.M., et al., Repeated irradiation from micro-computed tomography scanning at 2, 4 and 6 months of age does not induce damage to tibial bone microstructure in male and female CD-1 mice. BoneKEy Rep, 2017. 6(855).


Date: April 11, 2016

Dear Dr. Ward,

Your “Animal Use Protocol (AUP)” entitled:

Determining the level of dietary calcium and vitamin D that supports bone development without providing ‘excess’ bone-supporting nutrients has been approved by the Animal Care Committee.

This approval expires in one year on the last day of the month.
The number for this project is AUP # 16-03-02.
This number must be indicated when ordering animals for this project.

ANIMALS APPROVED: 28 timed-pregnant females CD-1 mice

Fiona Hunter, Chair of ACC

THIS PROTOCOL IS IN EFFECT FOR A PERIOD OF ONE YEAR ONLY AND IS SUBJECT TO POST APPROVAL MONITORING.

ALL UNEXPECTED MORTALITIES MUST BE REPORTED TO ANIMAL CARE SERVICES STAFF IMMEDIATELY.
Table 8. The level of vitamin D and calcium in each diet as determined by an independent laboratory.*

<table>
<thead>
<tr>
<th></th>
<th>Intervention Diets</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td><strong>Trial 1</strong></td>
</tr>
<tr>
<td></td>
<td></td>
<td>100 IU vit D/kg Diet</td>
</tr>
<tr>
<td>Diet vit D, IU</td>
<td>REF</td>
<td>.35% Ca</td>
</tr>
<tr>
<td></td>
<td>880</td>
<td>ND</td>
</tr>
<tr>
<td>Diet Ca, %</td>
<td>0.44</td>
<td>0.31</td>
</tr>
<tr>
<td>Envigo, TD Code</td>
<td>94045</td>
<td>160266</td>
</tr>
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</table>

*Testing was completed by Maxxam Analytics, Mississauga Ontario, Canada. The reportable detection limit for vitamin D is 200 IU. ND=not detectable
Table 9. Trial 1: Tibia trabecular and cortical structure.

<table>
<thead>
<tr>
<th>Diets</th>
<th>Age (month)</th>
<th>Mixed ANOVA, P-values</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Diet</td>
</tr>
<tr>
<td>AIN-93G n = 14</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.35% Ca n = 14</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.30% Ca n = 14</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.25% Ca n = 13</td>
<td></td>
<td></td>
</tr>
<tr>
<td>100 IU vit D/kg</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Tibia Length, mm</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>18.22 ± 0.11</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>19.08 ± 0.15</td>
<td></td>
</tr>
<tr>
<td><strong>Tibia (Trabecular) BMD, g/cm²</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>0.25 ± 0.01</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>0.23 ± 0.016</td>
<td></td>
</tr>
<tr>
<td><strong>BV/TV, %</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>20.70 ± 1.73</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>19.62 ± 2.66</td>
<td></td>
</tr>
<tr>
<td><strong>Tb.Th., mm</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>0.08 ± 0.00</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>0.09 ± 0.00</td>
<td></td>
</tr>
<tr>
<td><strong>Tb.Sp., mm</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>0.21 ± 0.01</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>0.30 ± 0.03</td>
<td></td>
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<tr>
<td><strong>Tb.N, mm⁻¹</strong></td>
<td></td>
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<tr>
<td>2</td>
<td>2.71 ± 0.17</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>2.12 ± 0.27</td>
<td></td>
</tr>
<tr>
<td><strong>DA, no units</strong></td>
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</tr>
<tr>
<td>2</td>
<td>2.00 ± 0.07</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>1.84 ± 0.05</td>
<td></td>
</tr>
<tr>
<td><strong>Conn.Dn, mm⁻³</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>147.50 ± 10.40</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>107.77 ± 13.24</td>
<td></td>
</tr>
<tr>
<td><strong>Tibia (Cortical) Cl.Ar/Tt.Ar, %</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>63.64 ± 1.14</td>
<td></td>
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<tr>
<td>4</td>
<td>68.16 ± 0.96</td>
<td></td>
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<tr>
<td><strong>Cl.Th, mm</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>0.25 ± 0.01</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>0.28 ± 0.01</td>
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<td><strong>Ps.Pm, mm</strong></td>
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<tr>
<td>2</td>
<td>5.22 ± 0.07</td>
<td></td>
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<tr>
<td>4</td>
<td>5.40 ± 0.06</td>
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<tr>
<td><strong>Ec.Pm, mm</strong></td>
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<td></td>
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<tr>
<td>2</td>
<td>3.28 ± 0.08</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>3.08 ± 0.07</td>
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<tr>
<td><strong>Ma.Ar, mm²</strong></td>
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<tr>
<td>2</td>
<td>0.55 ± 0.06</td>
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</tr>
<tr>
<td>4</td>
<td>0.55 ± 0.02</td>
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</tr>
<tr>
<td><strong>Ecc, no units</strong></td>
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<td></td>
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<tr>
<td>2</td>
<td>0.73 ± 0.01</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>0.74 ± 0.01</td>
<td></td>
</tr>
</tbody>
</table>

1 Values are mean ± standard error of the mean (SEM), NS = not significant
Table 10. Trial 1: L4 and mandible structure.

<table>
<thead>
<tr>
<th>Diet</th>
<th>100 IU vit D/kg</th>
<th>One way - ANOVA P-value</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>REF n = 8</td>
<td>0.35% Ca n = 8</td>
</tr>
<tr>
<td>L4 (Trabecular)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>BV/TV, %</td>
<td>27.65 ± 1.48</td>
<td>28.17 ± 1.15</td>
</tr>
<tr>
<td>Tb.Th., mm</td>
<td>0.07 ± 0.00</td>
<td>0.07 ± 0.00</td>
</tr>
<tr>
<td>Tb.Sp., mm</td>
<td>0.21 ± 0.01</td>
<td>0.21 ± 0.01</td>
</tr>
<tr>
<td>Tb.N, mm⁻¹</td>
<td>3.87 ± 0.21</td>
<td>3.88 ± 0.14</td>
</tr>
<tr>
<td>DA, no units</td>
<td>1.85 ± 0.04</td>
<td>1.82 ± 0.07</td>
</tr>
<tr>
<td>Conn.Dn, mm⁻³</td>
<td>188.53 ± 21.61</td>
<td>164.42 ± 10.47</td>
</tr>
<tr>
<td>L4 (Cortical)</td>
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<tr>
<td>Ct.Ar, (mm²)</td>
<td>0.46 ± 0.01</td>
<td>0.45 ± 0.01</td>
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<tr>
<td>Ct.Th, mm</td>
<td>0.09 ± 0.00</td>
<td>0.09 ± 0.00</td>
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<tr>
<td>Mandible</td>
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<td></td>
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<tr>
<td>BV/TV, %</td>
<td>77.59 ± 2.00</td>
<td>76.54 ± 2.88</td>
</tr>
</tbody>
</table>

Bone structure of the L4 and mandible scanned *ex vivo* after 4 months of age in male CD-1 mice. n = 8/group.
Data expressed as mean ± SEM. P < 0.05. NS = not significant.
Table 11. Trial 2: Tibia trabecular and cortical structure.

<table>
<thead>
<tr>
<th>Age</th>
<th>REF n = 14</th>
<th>0.35% Ca n = 14</th>
<th>0.25% Ca n = 13</th>
<th>Diet</th>
<th>Age</th>
<th>Diet x Age</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><strong>Tibia Length, mm</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>18.00 ± 0.10</td>
<td>17.93 ± 0.10</td>
<td>17.86 ± 0.10</td>
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¹ Values are mean ± standard error of the mean (SEM), NS = not significant.
Table 12. Trial 2: L4 and mandible structure.

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<th>One way - ANOVA</th>
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<td>0.35% Ca n = 8</td>
<td>0.25% Ca n = 8</td>
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<td>BV/TV, %</td>
<td>28.26 ± 1.7</td>
<td>29.49 ± 2.37</td>
<td>27.36 ± 0.95</td>
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<td>Tb.Th., mm</td>
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<td>0.07 ± 0.00</td>
<td>0.07 ± 0.00</td>
</tr>
<tr>
<td>Tb.Sp., mm</td>
<td>0.21 ± 0.01</td>
<td>0.21 ± 0.01</td>
<td>0.22 ± 0.01</td>
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<td>1.91 ± 0.07</td>
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<td>170.83 ± 16.47</td>
<td>195.56 ± 19.21</td>
<td>160.16 ± 9.49</td>
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<td>L4 (Cortical)</td>
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<td>73.90 ± 5.90</td>
<td>77.14 ± 2.61</td>
<td>71.50 ± 3.79</td>
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</table>

¹ Values are mean ± standard error of the mean (SEM), NS = not significant