PREBIOTIC ACTIVITY OF TART CHERRY COMPARED TO FRUCTOOLIGOSACCHARIDES ON THE GUT-BONE AXIS

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Abstract:

Objectives: Prebiotics are known to have beneficial effects on bone, but their mechanisms of action remain uncertain. Recently, probiotics were reported to modulate the gut-bone axis via the short chain fatty acid (SCFA), butyrate's effects on regulatory T (Treg) cells. This study investigated how two different prebiotics (i.e. tart cherry [TC] and fructooligosaccharide [FOS]) affect bone cells and whether the response is mediated by Treg cells within the gut-bone axis.

Methods: Eight-week-old C57BL/6 female mice (n=96) were assigned to treatments in 2x3 factorial design with diet (0, 10% TC or 10% FOS) and CD-25 (+CD25 antibody or –CD25 control) as factors. After 8 weeks of treatment, bone structural parameters, biochemical markers, Treg cells in the ileum and bone marrow, fecal SCFAs, and key regulators of bone cell differentiation and activity were assessed. Normally distributed data were analyzed using a 2-way ANOVA; otherwise, Friedman's test was run. For post-hoc analysis Fischer's least square means was used and alpha was 0.05.

Results: As anticipated, the relative abundance of Treg cells in the ileum and bone marrow was suppressed in the groups receiving the CD25 antibody. FOS treatment increased bone mineral content *($P < 0.01$)* and density *($P < 0.05$)*, trabecular bone volume in the vertebra and proximal tibia *($P < 0.01$)*, and length of the tibia *($P < 0.01$)* compared to the control and TC groups. No effect of FOS or TC treatments was observed on the cortical bone. A similar skeletal response was observed in the FOS groups, irrespective of CD25. After 8 wks of treatment, TC increased serum c-terminal end of the telopeptide of type I collagen 1 (CTX-1) compared to control and there was no effect of either prebiotic on procollagen-1 N-terminal peptide (P1NP). FOS increased fecal SCFA *($P < 0.01$)* to a greater extent than TC. The relative abundance of mRNA for regulators of osteoblast differentiation (i.e., bone morphogenetic protein 2 [*Bmp2*] and *Osterix*), and osteoblast activity (i.e., type 1 collagen [*Col1α1*]) in the hard tissue of bone were increased with the FOS diet. Likewise, osteocytes were activated as indicated by increased phosphate regulating endopeptidase x-linked (*Phex*), dentin matrix acidic phosphoprotein 1 (*Dmp-1*), matrix extracellular phosphoglycoprotein (*Mepe*) and sclerostin (*SOST)* gene expression in response to the FOS, but not the TC diets. No changes were noted in osteoclastic genes with either prebiotic. In the ileum, the inflammatory cytokine interleukin-17 (*Il17*) and T cell trafficking molecules (i.e., C-C motif chemokine receptor 7 [*CCR7*], C-X-C chemokine receptor type 4 [*CXCR4*], C-X-C motif chemokine ligand 10 [*CXCL10*], C-X-C motif chemokine ligand 12 [*CXCL12*], vascular cell adhesion protein 1 [*Vcam1*]) decreased in the presence of FOS. All findings support that FOS promotes bone formation.

Conclusion: The findings of this study indicate FOS, but not TC supplementation exerted beneficial effects on bone. This response did not require Treg cells to be present, suggesting that the mechanism through which the prebiotic such as FOS affects bone differs from that of probiotics.

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CHAPTER I

INTRODUCTION

Osteoporosis is a growing public health issue [1, 2]. It is a condition of low bone mass and microstructural defect affecting the skeletal system due to an imbalance in bone formation and bone resorption. Though modern science has increased life expectancy, maintaining one's health in older age is difficult because of the high fracture risk [3]. Fractures are associated with pain, functional limitations, and deformity, as well as a dependent lifestyle that can result in depression, low self-esteem, mood disorders, and social isolation. Worldwide, approximately 200 million people suffer from osteoporosis and more than 9 million fractures occur annually [4]. Worldwide a person suffers a fracture every 3 seconds [4, 5]. Taking into consideration the current accelerated fracture rate, it is expected that the prevalence of hip fracture will exceed 21 million per year by 2050 [6, 7]. Each year, $19 billion is expended on osteoporosis treatment and by 2025, these expenditures are expected to increase to $25.3 billion annually [8].

Focusing on the gender disparities, 1 in 3 women and 1 in 5 men aged over 50 years will suffer an osteoporotic fracture in their remaining life [9]. The lumbar spine, proximal femur, and distal forearm are the most common sites of fracture [10]. Mainly, postmenopausal women are susceptible to this condition due to estrogen deficiency that occurs with menopause [11]. Estrogen suppresses pro-inflammatory cytokines that cause bone destruction via accelerated osteoclast differentiation and enhances osteoblast differentiation, thus promoting bone

turnover [12]. The elderly are reluctant to accept available treatment options for osteoporosis because of concern with the cost of long-term use of medication and possible side effects [13, 14]. As a result, researchers continue to search for safe, cost-effective, alternative strategies for osteoporosis treatment and prevention.

Osteoporosis can be divided into primary and secondary types [4]. Primary osteoporosis is further divided into senile and postmenopausal sub-groups. Usually, senile osteoporosis is related to old age and occurs in both sexes. Inadequate sunlight exposure, low dietary intake of vitamin D, age-related decreasing organ function (e.g., renal failure), and malabsorption of calcium can contribute to a negative calcium balance causing stimulation of parathyroid hormone ultimately resulting in both trabecular and cortical bone loss [15, 16]. Postmenopausal osteoporosis occurs in two phases. The early phase is rapid and characterized by increased osteoclast activity or bone resorption, resulting in the loss of trabecular and cortical bone. It is estimated that postmenopausal women lose bone up to 2-3% per year and this continues for 4-8 years [17-20]. In the subsequent slow phase of postmenopausal bone loss, the decreasing activity of osteoblasts (bone-forming cells) prolongs and exacerbates the loss [21]. In contrast, secondary osteoporosis is involved with diseases (e.g., inflammatory bowel disease [IBD] and celiac disease), smoking, medications (e.g., glucocorticoids), and lifestyle factors (e.g., sedentary lifestyle and low calcium intake) which can negatively affect skeletal health.

An important indicator of fracture risk is the concept of bone mass, which is clinically expressed as bone mineral density (BMD). Bone tissue is accrued as a part of prenatal development and reaches its peak in the decade following puberty [22, 23]. In the third-decade bone mass plateaus for a period of time prior to the beginning of age-related bone loss [24]. One standard deviation increase in peak bone mass decreases 50% of the fracture risk [25]. According to the World Health Organization (WHO), a person is osteoporotic if their BMD is 2.5 standard

deviation or more (i.e., T-score ≤ - 2.5 SD) below the average value for young healthy women [3].

Two important determinants of bone mass are bone modeling and bone remodeling. Bone modeling is the process where-by bone formation by osteoblasts exceeds bone resorption by osteoclasts, resulting in the accrual of bone tissue or mass. Osteoclasts and osteoblasts work independently at different surfaces to reshape the bone or change to adapt to mechanical loading. Bone modeling is usually prominent during skeletal development and growth occurring in periosteal, endocortical, or trabecular bone surfaces [26]. In contrast, with bone remodeling the osteoclasts first resorb bone to remove old, or damaged bone, and then osteoblasts form new bone at the same site. This process aims to repair the micro-fracture and renew the bony structure [27]. In osteoporosis, bone remodeling plays a vital role. There are hormonal factors such as parathyroid hormone, calcitonin, calcitriol (1,25 dihydroxycholecalciferol), growth factors (e.g., transforming growth factor [TGF-β] and insulin-like growth factor-1 [IGF-1], and other factors such as cytokines (e.g., interleukin-[IL-1β] and tumor necrosis factor-[TNF]-α) as well as biomechanical factors that contribute to bone remodeling. Among the cytokines, IL-1β, TNF-α, IL-6, and macrophage colony-stimulating factor (M-CSF) simultaneously induce osteoclast proliferation, differentiation, maturation, and activation contributing to bone destruction [28, 29]. During the reversal phase of bone remodeling, TGF-β and IGF-1 stimulate the production of bone matrix proteins such as bone sialoprotein (BSP) and osteopontin (OPN) that form the extracellular matrix of bone and give the tissue its elastic properties. Bones are continuously exposed to different mechanical loads by a variety of stress and strains that can affect bone remodeling.

The gut-bone axis is a term used to describe the interconnection among gut microorganisms, immune cells, and bone cells [30, 31]. Around 100 trillion bacteria live in the lumen of the gut [32]. Some populations can ferment non-digestible carbohydrates and produce

metabolites such as short-chain fatty acids (SCFA), which have a variety of health benefits, including promoting the gut barrier function, providing energy for intestinal epithelial cells, and improving bone health [33, 34]. Among the SCFAs, acetate, propionate, and butyrate are predominant in the gut [30, 35]. The microbiota also produce other compounds such as indole derivatives and IGF-1 that influences gut and bone cell activity [34, 36-38]. Gut epithelial cells line the mucosa and perform many functions including absorption of nutrients [39]. The mucosal layer act as a physical barrier to the lumen and the underlying connective tissue known as lamina propria. In lamina propria, immune cell populations such as macrophages and dendritic cells reside. Dendritic cells, recognized as key antigen presenting cells, can present antigens to naïve $CD4^+$ T cells, initiating their differentiation into subsets as indicated by their expression of forkhead box protein 3 ($CD25^+$ $FOXP3^+$ Tregs) Treg cells and IL-17 in the case of Th 17 cells [40, 41]. Differentiated T cells can enter the circulation and traffic to tissues, including the bone marrow where they can alter osteoblast, osteoclast, and osteocyte function [42, 43]. Another T-cell population, cytotoxic $CD8^+$ T-cells that are produced in the bone marrow has been reported to regulate Wnt/β catenin signaling, which is an important pathway in the formation of osteoblasts [44]. Paneth cells embedded with intestinal epithelial cells secrete antimicrobial peptides, play a role in neutrophil migration and halt infection in the gut, and interacts with the immune cells performing immunomodulatory functions [45]. As SCFA can modulate the activity of the gut and bone, altering the gut microbiota and regulating their metabolite such as SCFA using probiotics and prebiotics may have beneficial effects on bone remodeling.

It has been shown that prebiotics and probiotics can potentiate SCFA production by targeting the gut microbiome, and thus contribute to increasing bone formation and suppressing bone resorption. Accumulating evidence indicates that probiotics (e.g., *Lactobacillus rhamnosus* GG) alter the gut commensal community, increase SCFA (i.e., butyrate, propionate, and acetate) producing microbial populations, and has positive effects on bone via immunomodulatory

mechanisms as well direct effects of gut-derived metabolites [46-48]. However, it remains unclear whether the prebiotics act through these same mechanisms.

Prebiotics have been classically defined as the non-digestible component of food that induce the growth of beneficial microorganisms in the gut and have health benefits in the gut and at distal sites. More recently other bioactive components in food have been shown to have prebiotic activity [49]. Prebiotics such as fructooligosaccharides (FOS), inulin, and phenolic acids have a positive effect on BMD and skeletal strength in animal models [50-53]. These observations have coincided with a shift in gut microbiota composition, increase SCFA production, lowered luminal pH, and increase calcium absorption [50, 54]. Whereas SCFAs exert immunomodulatory function by downregulating the pro-inflammatory cytokines TNF-α and upregulating anti-inflammatory cytokine production IL-10, further studies are needed to establish the mechanisms of how prebiotics directly and/or indirectly affect bone cells at distal sites.

Bone homeostasis may be maintained by FOS either by host metabolic system or host immune system. It has been hypothesized that the mechanism of action of FOS is via the increase in SCFA [55]. Another possible mechanism of FOS may be directly increasing Treg cell population or indirectly upregulating Treg production by SCFA. Treg cells can suppress osteoclast differentiation. In addition, FOS-induced Treg cells in conjunction with $CD8^+$ T cells may activate Wnt signaling pathway or TGF-β dependent Smad signaling pathway for bone formation [46]. It is still not explored how FOS increases bone mass via gut bone axis from a specific target standpoint.

Fruits, such as tart cherry, that are rich in prebiotic compounds, might also exert beneficial effects on bone via the gut-bone axis. Montmorency tart cherry ***(Prunus cerasus),*** is a species of ***Prunus*** from the Rosaceae family [56, 57]. Dietary consumption of tart cherries has a number of health benefits, including anti-inflammatory, antioxidant, and bone protective

functions [58, 59]. Previously, our lab has investigated that tart cherry not only prevented bone loss, but also exhibited anabolic effects (increased BMD) in spine and long bone tibia [58]. Furthermore, we showed that postmenopausal women consuming tart cherry juice twice daily exhibited a decrease in the bone resorption marker, tartrate-resistant acid phosphatase type 5b (TRAP5b) [60]. Tart cherry is a good source of anthocyanin, phenolic acids, and FOS [61]. It is known that both phenolic acids and FOS have prebiotic activity [62-64]. Phenolic acids reduce inflammation by inhibiting receptor activator of nuclear factor κβ (RANKL) and promoting osteoblast differentiation via TGF-β mediated Wnt signaling in both bone and gut in a Treg cell differentiation-dependent manner [65, 66]. However, these phenolic acids are relatively poorly absorbed, which raises the possibility that their effects are mediated through the gut [53, 67].

With increasing recognition that the gut may serve as a target for preventing and treating osteoporosis, renewed interest in foods with prebiotic activity has emerged. Previously, most reports focused on the ability of a single prebiotics (i.e., non-digestible carbohydrates) to improve BMD and bone strength [51, 55, 68]. With growing research, multiple prebiotics containing food like tart cherries have been identified and it is expected they may have additional advantages. Thus, it is important understand how simple and more complex prebiotics compare in their effects on the bone and to understand whether this response is mediated through the gut-bone axis.

The **purpose** of this project is to compare how tart cherry and FOS alter bone metabolism and structure, and whether their effects are mediated via Treg cells. This will be accomplished by performing a study using young adult, C57BL6 female mice. Mice will be randomly assigned to diets supplemented with tart cherry or FOS and will receive a CD25 antibody or isotype control antibody. CD25 antibody suppresses Treg cell function [46]. The **hypothesis** that is being tested is incorporating tart cherry and FOS into the diet will enhance bone structural and metabolic parameters, and this response will be inhibited in mice receiving the CD25 antibody. The hypothesis will be tested by accomplishing the following specific aims for the experiment.

Aim 1: To characterize the effect of tart cherry and FOS on bone quality (i.e., bone mass and microarchitecture) and to determine whether Tregs are required for this response.

Working Hypothesis: Tart cherry and FOS will improve bone mass and trabecular and cortical bone microarchitecture, but this response will be inhibited in the mice receiving the CD25 antibody.

Aim 2: To determine the effect of tart cherry or FOS supplementation on systemic markers of bone metabolism (e.g., cross-linked C-telopeptide of type I collagen (CTX) an indicator of bone resorption and N-terminal propeptide of type I procollagen (PINP), an indicator of bone formation).

Working Hypothesis: Incorporating tart cherry and FOS alone into the diet will reduce serum CTX, whereas serum PINP will be increased. The CD25-treated mice will show no alteration in systemic bone markers in response to dietary treatments.

Aim 3: To evaluate the abundance of Treg and Th17 cells in the gut and bone marrow utilizing fluorescence-assisted cell sorting in response to the tart cherry and the FOS supplemented diets.

Working Hypothesis: In comparison to mice who received CD25 antibody injections, animals fed tart cherry and FOS will have more Treg and fewer Th17 cells in the gut and bone marrow.

Aim 4: To determine how tart cherry or FOS alters fecal SCFAs and whether or not this is affected by CD25.

Working Hypothesis: Tart cherry or FOS supplementation will stimulate the production of SCFAs in the gut whereas animals who receive CD25 antibody will have no effect on the response to diet will have lower production of SCFAs.

Aim 5: To determine the effect of tart cherry or FOS on gut inflammatory mediators at the gene expression level.

Working Hypothesis: Tart cherry or FOS supplementation will downregulate pro-inflammatory cytokines (e.g., TNF-α, IL-1β, IL-6, IL-17) and upregulate anti-inflammatory cytokines in the gut (e.g., TGF-β, IL-10). On the contrary, the CD25 antibody will ablate this response.

Aim 6: To characterize the alterations in gene expressions of key regulators of osteoclast and osteoblast differentiation, indicators of the osteoclast and osteoblast activity and osteocyte activation in response to tart cherry or FOS.

Working Hypothesis: Tart cherry or FOS will suppress gene expression involved in osteoclastogenesis and enhance gene encoding for osteoblastogenesis pathways in the bone marrow and CD25 antibody-treated mice will not be able to demonstrate such responses. These alterations in bone cell activity will occur in conjunction with alterations in osteocyte signaling.

Limitations and Potential Pitfalls:

There are some limitations to our study. First, we have only focused on $CD4^+CD25^+$ Treg cells. We do not know whether the result of dietary supplementation will be similar in all subtypes of Treg cells. Second, our study was designed to examine the response to treatment at a single time point of 8 weeks. It is conceivable that we may have learned more about the response to FOS with an earlier time point or the effects of tart cherry with a longer duration treatment. Third, we have focused the work in this project on the response of young female adult (naïve) mice. Further studies are needed to determine if the response differs based on the age and gender of the animals. Last of all, this study was intended to provide new insights into prebiotics'

mechanisms of action. Because of this, we have used animal models. Additional studies would be required in humans to determine if they have a similar response to the prebiotics.

CHAPTER II

LITERATURE REVIEW

2.1 Introduction to Osteoporosis

According to the World Health Organization (WHO) osteoporosis is defined as a "progressive systemic skeletal disease characterized by low bone mass and microarchitectural deterioration of bone tissue, with a consequent increase in bone fragility and susceptibility to fracture" [69]. In the U.S. alone, more than 10 million people suffer from osteoporosis [4, 70]. According to data from the Centers for Disease Control and Prevention, the incidence of osteoporosis is as high as 27% among women as they age compared to 6% in men [71]. It is expected that the incidence of osteoporotic hip fractures will increase to 6.3 million annually by 2050 [72, 73]. This rising incidence of osteoporosis and its related fractures among the world's aging population is a major public health concern.

Osteoporotic fractures have been linked to increased morbidity, death, and a lower quality of life [74, 75]. In the U.S., the annual incidence of osteoporotic fractures surpasses 1.5 million, and hip fractures have the most severe impact on health. Unfortunately, one out of every five patients who experience a hip fracture dies within the first year following their fracture, and one-third of those who survive require assistance [74, 76]. Only a small percentage of those who survive return to their earlier level of physical activity. With the aging demographic worldwide,

it is essential to identify better prevention and treatment strategies for osteoporosis beyond the existing pharmacological treatment options that have side effects, poor compliance, and high cost [77, 78].

The World Health Organization (WHO) has established guidelines for diagnosing osteoporosis based on measures of BMD from dual-energy x-ray absorptiometry (DXA) scans [79]. T-scores, which are defined by the number of standard deviations from a young, healthy adult of the same sex is used as the criteria (Table 1).

Table 1. World Health Organization Diagnostic Criteria for Osteoporosis [13, 75]

T-score	Classification
> -1.0	Normal
-1.0 to -2.5	Osteopenic
< -2.5	Osteoporotic

Although using T-score criteria to diagnose hip and lumber spine fracture risk in elderly adults is appropriate, they are not appropriate for children and individuals under the age of 50. For such population groups, using the Z-score is appropriate, which compares an individual's BMD to that of a reference group that is similar in age, gender, and ethnicity. As osteoporosis remains “silent” until a fracture occurs, identifying the higher-risk population group would be beneficial.

Major risk factors for osteoporosis include both genetic and lifestyle factors. Peak bone mass, gender, and family history are the main genetic factors [80-84]. Low bone mass is the leading risk factor for osteoporotic fracture [85-87]. Females tend to have a lower peak bone mass and experience a rapid phase of bone loss in the 5-10 years following menopause and women's

smaller skeletal or frame size. As a result, women are four times more likely to develop osteoporosis and two times more likely to develop osteopenia or low bone mass [83]. Though men are at a lower risk of osteoporosis, there is evidence that men who fracture, experience more complications [88]. Apart from these genetic factors, modifiable lifestyle factors include diet, physical activity, and medications [13, 89, 90]. Nutrients such as calcium, vitamin D, protein, zinc, phosphorus, magnesium, and vitamin K are important in the context of bone health. In addition to these traditional nutrients, probiotics and prebiotics have also been shown to affect osteoporotic risk by modulating the gut microbiota and SCFA production [91, 92]. Physical activity is another modifiable lifestyle factor that significantly affects fracture risk. For example, a sedentary lifestyle increases fracture risk [93]. In contrast, weight-bearing activity stimulates bone formation [94]. Finally, drugs such as glucocorticoids, anti-convulsants, loop diuretics, and anti-depressants are also known to increase osteoporotic fracture risk [90]. Taken together it is evident that diet, physical activity, and maintaining good overall health provide important means through which the risk for osteoporosis can be reduced.

2.2 Anatomy and Physiology of Bone:

Bone is a connective tissue that is constantly undergoing change. The adult human skeleton, which is made up of 206 bones, serves a variety of functions including movement, hematopoiesis, and protection for internal organs [28, 95]. Bone material can be divided into organic and inorganic components. The organic portion is comprised of 90% collagenous and 10% glycoprotein and proteoglycans. Cross-linking of the triple helix structure of the collagen provides bone flexibility and durability, allowing it to withstand torsion and bending forces [96]. In contrast, the inorganic portion is made up of the mineral salts of hydroxyapatite comprised primarily of calcium and phosphate [97]. This gives bone rigidity and makes it more resistant to compression forces. In combination with hormonal signaling (e.g., parathyroid hormone, calcitonin, calcitriol, sex hormones), bone regulates these minerals and maintains skeletal

remodeling in adults. Bone marrow is the home of hematopoietic stem cells (HSCs) and mesenchymal stem cells (MSCs). HSCs can differentiate into myeloid or lymphoid progenitor cells with myeloid cells serving as the precursors for osteoclasts as shown in **Figure 1** and lymphoid cells having the capacity to differentiate into T cell subsets. T-lymphocytes populations (e.g., T regulatory and Th17 cells) are key players in bone remodeling. To understand the pathophysiology of osteoporosis, an understanding of bone anatomy is important.

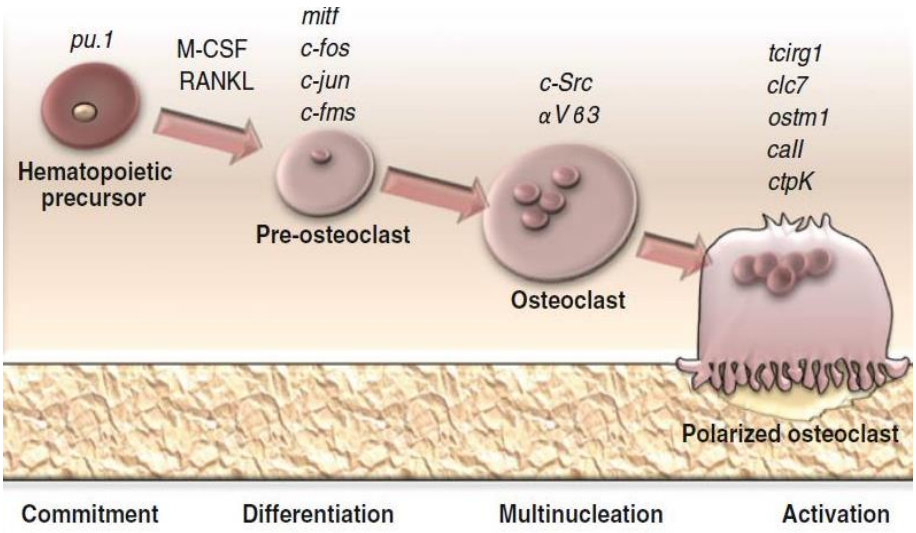


Figure 1.

Different stages of osteoclast differentiation with associated genes for bone resorption. Peruzzi et al. 2012 Clinical Reviews in Bone and Mineral Metabolism

Overall, eighty percent of the adult human skeleton is made up of dense cortical bone and twenty percent is made up of trabecular (i.e., woven or lamellar) bone [98]. Cortical bone has multiple concentric rings of lamellae with a central canal referred to as the "Haversian System" which contains blood and lymphatic vessels, as well as nerves. Within the cortical bone, osteocytes are located within the lacunae and are responsible for mechanosensing and coordination of the osteoclast and osteoblast [99]. Osteocytes' radial cytoplasmic extensions remain in tiny canals called canaliculi and these extensions are joined by gap junctions allowing for cell-to-cell communication. The bone is protected by a fibrous connective tissue known as the periosteum, which covers the outer cortical surface, and the endosteum, a membranous tissue that covers the inner surface of both cortical and trabecular bone.

Bone modeling and bone remodeling are essential for skeletal growth and maintenance. Defective physiological processing can cause osteoporosis and subsequently fracture [28, 95]. Bone modeling is the dominant physiological process during growth [100]. This process is characterized by asynchronous bone deposition and bone resorption. In bone modeling, bones modify their shape while increasing their size to overcome physical stimuli or mechanical stress as described by Wolff's Law [98]. In the adult skeleton, bone modeling is less pronounced, though in some pathological conditions (e.g., hypoparathyroidism, renal osteodystrophy, treatment with anabolic agents) this modeling persists to correct deformities [101].

In contrast, bone remodeling is the primary physiological process occurring in the adult skeleton where osteoclasts remove bone followed by osteoblasts initiating bone formation within a bone multicellular unit (BMU). These processes with the BMU occur in a cyclical and synchronized series of events namely, resorption, reversal, and formation [29].

In the resorption phase, bone is removed by osteoclasts. Osteoclast precursor cells migrate to the damaged site of the bone. In response to osteocyte's apoptotic death, bone damage sensed by osteocytes, or other potential stimuli that remain to be defined, RANKL is released by osteoblasts and stromal cells and binds with its receptor (i.e., RANK) located at pre-osteoclasts [102]. An adaptor protein, TNF receptor-associated factor 6 (TRAF 6) causes the translocation c-Jun, c-fos, and the master regulator of osteoclast differentiation factor known as "nuclear factor of activated T cells, cytoplasmic1" (NFATc1) to the nucleus. In response, calcium is released from the calcium/calmodulin-dependent protein calcineurin to support the self-augmented effect of NFATc1 coupled with other transcription factors to stimulate NF-κB for osteoclast differentiation [103, 104]. Under influence of RANKL, chemokines are released and cell fusion occurs resulting in the formation of osteoclasts which are characterized as giant polarized multinucleated cells with ruffled borders. The multinucleated mature osteoclasts attach their ruffled borders at the damaged bone site and form a sealed zone where they release hydrochloric

acid and proteases (e.g., cathepsin K, matrix metalloproteinase 9, metalloproteinase 13) that degrade and solubilize the minerals and form a cavity known as “Howship’s lacunae” [105, 106]. To regulate the osteoclastic activity, osteoblasts express osteoprotegerin (OPG) and prevent RANKL-RANK binding and the subsequent responses [107]. This resorption phase lasts for two to four weeks in humans. At the end of the resorption phase, osteoclasts undergo apoptosis.

In the reversal phase of bone remodeling, the remaining undigested demineralized collagen matrix is replaced by a single layer of undetermined lineage cells. It has been suggested that these bone lining cells are osteoblast lineage-derived cells [108]. Proteins are released during the digestion of the matrix and these proteins TGF-β, IGF-1, and bone morphogenetic proteins (BMPs) attract the osteoblasts [109-111]. Basically, the reversal phase represents a transition from bone resorption to bone formation.

Lastly, the bone formation stage of bone remodeling takes place with subsequent protein synthesis and extracellular matrix deposition. TGF-β, BMPs, and IGF-1 signaling molecules initiate the Wnt/β-catenin signaling pathway of osteoblastogenesis by promoting the expression of Runt-related transcription factor 2 (Runx2) by MSC-derived osteoblast progenitors. The downstream target of Runx2 is osterix (Osx), which promotes the maturation of pre-osteoblasts into osteoblasts [112, 113]. Mature osteoblasts secrete collagen type 1 alpha 1 chain (Col1α1), non-collagenase proteins like bone osteocalcin (Ocn), osteopontin (Opn), and bone sialoprotein (Bsp) and alkaline phosphatase (Alp) [114-117]. Opn and Bsp have a strong affinity for calcium and regulate bone mineralization by increasing hydroxyapatite formation [118-120]. Some osteoblasts undergo apoptosis, some become bone lining cells, and others transform into osteocytes that are entombed within the bone. The bone formation and mineralization phase take up to 4 months in humans [29]. The outcome of each bone remodeling cycle is a new osteoid formation. The whole process of bone remodeling in humans takes 4 to 6 months whereas, in

mice, it takes only 2 weeks [121]. The adult skeleton continues to remodel to maintain skeletal homeostasis, with about 15% of the mature skeleton being replaced every year in humans [23].

Osteocytes plays a major role in bone remodeling maintaining a harmony between osteoclastic activity and osteoblastic activity. At the site of microdamage, dying osteocytes express RANKL and signal osteoclasts for osteoclastogenesis [122]. Osteocytes can also regulate osteoblasts activity for bone formation by controlling the protein sclerostin which is encoded by *SOST* gene [123]. Downregulation of the *SOST* gene is involved in the upregulation of BMP proteins and Wnt signaling. Osteocyte secretes phosphate regulating neutral endopeptidase on the chromosome X (Phex), dentin matrix Protein 1 (Dmp1) and matrix extracellular phosphoglycoprotein (Mepe) which are involved in mineralization and phosphate metabolism [124-126]. Dietary component may influence osteoblastic activity and their proliferation and differentiation to osteocytes [127].

2.3 Gut Mucosal Anatomy and Immunity:

The gastrointestinal tract is made up of the small intestine (i.e., duodenum, jejunum, and ileum), which are the primary sites of digestion and absorption of nutrients, and the large intestine (i.e., ascending, transverse, and descending colon) that is responsible for reabsorption of water and the mineral ions, sodium and chloride. Bacterial fermentation of indigestible materials occurs mainly in the colon, but to some degree in the distal end of the small intestine and cecum as well. The wall of the intestine is comprised of the serosa, muscularis externa, submucosa, and mucosa (**Figure 2**). The mucosal layer consists of epithelial cells, lamina propria, and muscularis mucosa. The epithelial lining with tight junctions (e.g. occludin, zonula occludens, or ZO), claudin, junctional adhesion molecules, or JAM) acts as a barrier that allows selective permeability of substances from the lumen to enter into mucosal layer [128]. Beneath the epithelial layer, the

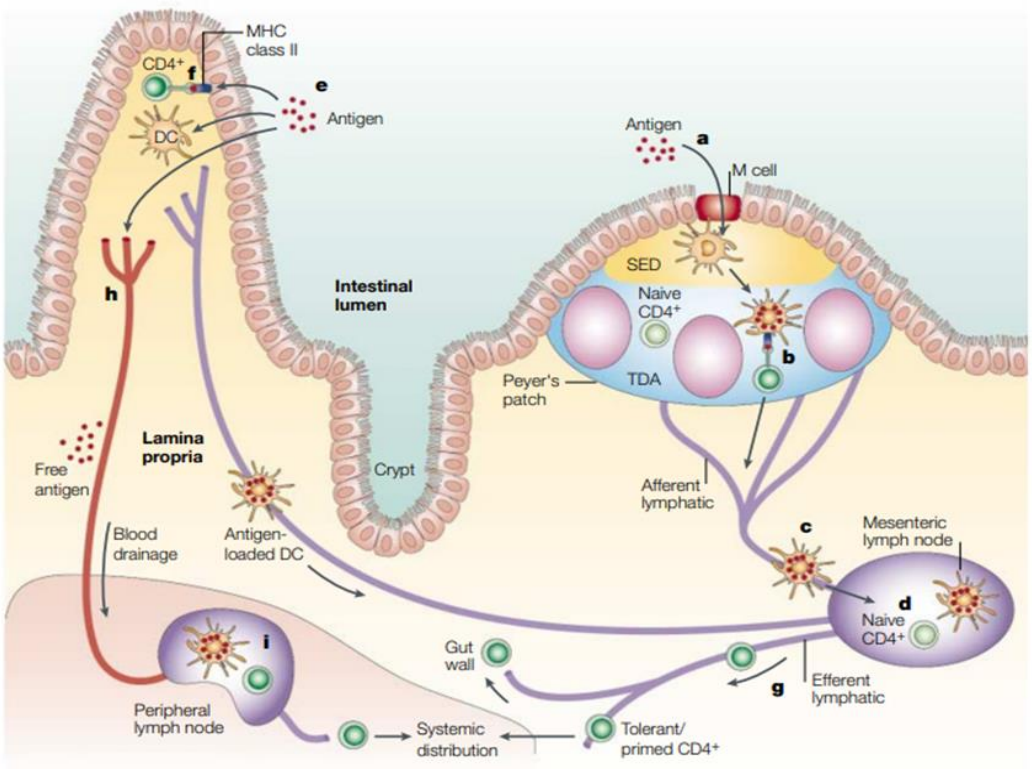


Figure-2: Gut mucosa with immune components. Mowat et al. 2003 Nature Reviews Immunology [129]

lamina propria is comprised of connective tissue containing lymphatic and blood vessels, immune cells (e.g., T cells, dendritic cells, B-cells, macrophages), and nerve plexus. $CD4^+$ T cells with subtypes of T helper (Th1), Th2, Th17, forkhead box $P3^+$ (Foxp3) regulatory T cells (Treg), and Th17 cells can be found within the lamina propria of the gut.

Although Th1 and Th2 cell numbers are relatively unchanged throughout the small and large intestine, Treg cells are most abundant in the colon and Th17 cells decrease in number from the small intestine to the large intestine [31, 130]. The macrophages release interleukin IL-10, which is important for the survival of the immunosuppressive $Foxp3^+$ Treg cells [131]. Other important components of the gut-associated lymphoid tissue (GALT) are the mesenteric lymph nodes (MLNs) and the Peyer's patches that are primarily found in the distal end of the small

intestine. Peyer's patches are primarily located along the small intestine, increasing in number and size from the jejunum to the terminal ileum. The follicle-associated epithelium cells of Peyer's patches have specialized cells (i.e., M cells) that sample antigens. Also residing within the Peyer's patches are B-cells, naïve T-cells, and resident dendritic cells.

The primary role of intraepithelial cells is to ensure the integrity of the intestinal epithelium and maintain local immune protection [132]. The gut has additional protective cells called paneth cells, goblet cells, and intraepithelial lymphocytes. Small intestinal crypts anchor the paneth cells at the bottom that produces antimicrobial peptides (AMPs) like α-defensin and β-defensin, lysozymes, secretory phospholipase A2, cathelicidins that are stored in secretory granules. The goblet cells synthesize mucus that does not only act as a physical barrier, but is also rich in antimicrobial peptides, IgA, and glycol proteins that kill harmful microbes [133, 134]. Research has shown that the SCFA, butyrate, can upregulate the MUC2 gene expression in vitro which affects intestinal barrier function [135].

In the Peyer's patches, antigens from microbes and the diet are presented to naïve $CD4^+$ T-cells by resident dendritic cells (DC) **(Figure 2).** Cytokines TGF-β, IL-6, and IL-21 stimulate naïve $CD4^+$ cells to develop into RORγt and RORα transcription factor induced Th-17 cells [41, 136]. Differentiated Th-17 cells express IL-23 receptors (IL-23R) ensuring the survival of Th-17 cells [137]. Together IL-23 and IL-6 stimulate pathogenic Th-17 cell conversion. Dendritic cells containing Toll-like receptor 5 assist in this conversion in an MHC-II dependent manner in the lamina propria of the small intestine [138]. Bacterial genera such as *Clostridium, Ruminococcus, Veillonella, Butyricimonas,* and *Prevotella* adhere to the human ileum mucosa and are associated with activating intestinal Th-17 cells [139]. High fat, as well as high salt diets, can also induce differentiation of Th-17 cells [140]. Recent research studies reported, segmented filamentous bacteria (SFB) promotes Th-17 cell differentiation in the small intestinal lamina propria and is linked to subsequent Th-17-dependent inflammatory response in mice [141, 142]. The effector T-

cells can migrate from the MLN back to the lamina propria of the mucosa or enter into systemic circulation where they traffic to other tissues such as the bone [143].

There are a number of different dietary factors that can affect gut immunity. Dietary fiber and bioactive components possessing prebiotic activity can enhance SCFA producing *Clostridium*, *Bacteroides*, *Bifidobacterium*, and *Lactobacillus* bacteria population and contribute to the growth and differentiation of peripheral Treg cells. A colitis model produced by the adoptive transfer of $CD4^{+}CD45RB^{hi}$ T cells in $RAG1^{-/-}$ mice that were fed with high fiber diets (e.g., CMF chow) produced SCFA, and increased Treg populations in the colon by inhibition of histone deacetylates [144]. This study provides insight into the possible mechanism of the SCFA, butyrate in regulating the differentiation of Treg cells. Butyrate supplementation upregulates Foxp3, the transcription factor for Treg cells, and expands IL-10 producing Treg cells in the colonic lamina propria. Zhang et al. [145] have reported that butyrate administration decreased Th-17 cell population by downregulating RORγt, a key transcription factor for Th17 cells) in the mesenteric lymph node and cytokine interleukin IL-23 (important for stability and activation of Th17 cells), and increased Treg cells and their cytokine IL-10 in blood in chemically induced (2,4,6-trinitrobenzene sulfonic acid, TNBS) colitis model. Multiple animal studies have further shown that ingestion of phytochemicals with prebiotic activity (e.g., flavonoids, polyphenols) protects animals in colitis model induced by Dextran sodium sulfate (DSS) or TNBS. These phytochemicals reduced RORγt mRNA expression resulting downregulation of Th-17 cell differentiation in the mesenteric lymph node, lamina propria of the colon. The phytochemicals also increased Foxp3 mRNA induced Treg cells differentiation in the mesenteric lymph node, colonic lamina propria and anti-inflammatory cytokines IL-10, TGF-β level in the colon [146-149]. RORγt is the transcription factor of Th-17 cell and Foxp3 is the transcription factor of Treg cells. Taken together, this evidence supports that prebiotics restore the skewed Th-17/Treg balance in the animal model of IBD.

The beneficial effects of prebiotics in human studies with IBD are inconclusive due to smaller sample-sized pilot studies, faulty study design, and variability of effectiveness of the prebiotic compounds. However, supplementation of FOS or, a combination of FOS and probiotics increases bifidogenic bacteria, and *Lactobacilli*, and reduces pro-inflammatory cytokines TNF-α and IL-1β in patients with ulcerative colitis (UC) [150, 151]. Furrie et al. [151] observed that supplementation with a symbiotic (e.g., FOS/inulin with a probiotic) improved sigmoidoscopy inflammatory scores with reduction of inflammatory cytokines TNF-α and IL-1β in patients with ulcerative colitis (UC). Although prebiotic supplementation helps maintain the proper balance of Th17/Treg cells in the gut, the question remains as to whether or not this is the mechanism by which the benefits on bone are mediated.

2.4 The Gut-Bone Axis:

Dysbiosis of gut microbiota has been associated with many diseases including bone loss. This interaction between the gut microbiota with the immune system and its impact on distal bone is known as the gut-bone axis. Earlier concept of the gut-bone axis was based on malabsorption, maldigestion from IBD, celiac disease and the use of glucocorticoid in IBD patients; all of which had a negative impact on bone remodeling [152-154]. Patients with IBD had higher fracture risk (>40%) due to malabsorption of calcium, phosphorus, and vitamin D and compromised intestinal barrier [155]. Patients with celiac disease have defective nutrient absorption, antibodies produced against OPG, low IGF-1 levels, and high leptin levels all contributing to low BMD [156]. Later it was noted that corticosteroid treatment in patients with IBD accelerates bone resorption by dampening osteoblast function, enhancing osteoblast apoptosis, decreasing intestinal calcium absorption, and increasing renal excretion of calcium [153, 157]. The discovery that serotonin, produced by the enterochromaffin cells of the gut, showed the ability to blunt bone formation by downregulating osteoblast production [158]. Both in the human and rodent model, treatment with

selective serotonin reuptake inhibitors (SSRIs) led to low bone mass with high fracture risk [159, 160].

In 2012, pre-clinical mouse studies demonstrated that the gut microbiota can regulate bone mass [161]. Germ-free mice from the C57Bl/6 strain exhibited a higher BMD in trabecular bone than control mice [161]. Li et al. [48] reported that germ-free mice were protected against estrogen deficiency-induced bone loss. However, Yan et al. [162] reported that introducing specific pathogen-free gut microbiota in germ-free mice induced an IGF-1 mediated anabolic effect on bone. Despite the controversy, it is largely accepted that the gut microbes induce alterations in host immunity via their metabolites (e.g., SCFAs) and affect bone remodeling.

Additional insights into the gut-bone axis have been provided through a series of studies utilizing antibiotics to suppress the gut microbiota. Cho et al. [163] and Pytlik et al. [164] showed that suppressing the gut microbiota with antibiotics caused an increase in bone mass [163, 164]. In contrast, studies have also reported antibiotic treatment decreased bone growth promoting factor IGF-1, suppressed SCFA production, altered Firmicutes to Bacteridetes ratio, and overall increased cortical and trabecular bone resorption [162, 165, 166]. The communication between the intestines and the bone is significantly supported by the mice germ-free mice and antibiotic-treated mice models.

Both prebiotics and probiotics have an impact on bone metabolism; however, research on probiotics has accelerated. By definition, probiotics are living microorganisms that provide benefit to the host when taken in a sufficient amount [167]. *Lactobacillus, Enterococcus, Bacillus, Escherichia, and Bifidobacterium* genera are commonly used probiotics. Treating periodontitis with probiotics (e.g., *Bacillus subtilis, Saccharomyces cerevisiae)* as an adjuvant therapy reduced inflammation in the gut and protected alveolar bone resorption in rats [168, 169]. Recent research has shown that *Lactobacillus reuteri* increased BMD in gonadal hormone

deficient female mice [170]. Administration of *L. reuteri* in type-1 diabetes mice model and in vitro study showed amelioration of bone loss by TNF-α suppression and upregulation of Wntb expression in the bone marrow [171]. Probiotics slow down $CD4^+$ T cell production, reduce pro-inflammatory cytokines (e.g., TNF-α, IL-1β, IL-17, RANKL) in the gut and bone marrow and increase Treg cells, enhanced OPG production [168, 172, 173]. Furthermore, probiotics promote barrier function by increasing the production of tight junction proteins of mucosal epithelial lining [174]. Thus, probiotics play a great role in maintaining gut homeostasis, immunoregulation of gut and bone, and bone anabolic effect.

Microbiota-produced metabolites (e.g.: SCFA, indole derivatives, IGF-1, and polyamines) are capable of altering gut immunity and affecting bone metabolism. In bone, butyrate blocks the histone deacetylases (HDAC), suppresses cell proliferation, modifies the pre-osteoclast population, and dampens the *Traf* gene and the master regulator *Nfatc1* for osteoclastogenesis. Additionally, butyrate accelerates Treg cell differentiation from naïve $CD4^+$ T cells in the gut and bone marrow promoting Wnt signaling for bone synthesis. The activated Wnt signaling pathway blocks CD28 signaling so AP-1 coupled NFAT osteoclastic signaling remains blunted, further promoting bone formation in the $CD8^+$ T cells [175]. Effector Treg cells produce anti-inflammatory cytokines IL-10. Treg cells' direct cell-to-cell contact (CTLA-4) mechanisms also antagonize osteoclastogenesis [34]. Another metabolite, tryptophan-derived indole compounds were first reported involved in gut immunity [176]. Indole derivatives bind with pregnane X receptor (PXR) and aryl hydrocarbon receptor (AhR) in the intestine to regulate the gut barrier function and to increase intraepithelial lymphocytes and innate lymphoid cells [177-180]. AhR ligand ablates osteoclast differentiation, reduces the differentiation of dendritic cells and Th-17 cells, and increases $Foxp3^+$ Treg [181, 182]. So, gut-derived metabolites largely favor bone by favorably modulating the immune response.

Our gut has numerous microbes and lymphoid organs where adaptive immune T cells reside. These microbes help in maintaining the balance among the different types of T-cells. Any alteration in Th-17 and Treg balance influence bone homeostasis. Gut dysbiosis causes an increase in Th-17 cells. Interleukin IL-17 released from Th-17 cells in bone marrow catabolizes bone by accelerating the differentiation of osteoclasts [183, 184]. Pacifici et al. [185] reported that it was reported that parathyroid hormone expanded Th-17 cells in the gut of the mice containing segmented filamentous bacteria. In the bone marrow, Th-17 cells number also increased under the influence of chemoattractant CCL20. To track the migration of Th17 cells from gut to bone they blocked sphingosine 1 phosphate (SIP) receptor 1, a receptor involved in the migration. As a result, Th 17 cells decreased in the bone marrow and dampened bone loss. Researchers have also discovered that in the postmenopausal estrogen-deficient state, effector Th 17 subtype increase in the gut, migrates to bone marrow, and secretes bone resorptive pro-inflammatory cytokines TNF-α and IL-17 [186, 187]. Indeed, inference with Th-17 cell or migration of the pro-inflammatory cytokine can restore bone loss. All this evidence strongly posits that immunomodulatory action in the gut and bone are T-cell mediated. The use of probiotics in restoring balance in gut microbiota and their metabolites' ability to manipulate gut and bone immunology have received much attention to view the gut bone axis as a therapeutic field for treating pathological bone loss.

2.5 Prebiotics:

With the increasing appreciation for the role of the gut microbiota in bone health, there has been growing interest in prebiotics. The latest modified definition of prebiotics is non-digestible compounds that, through their metabolism by microorganisms in the gut, modulate the composition and/or activity of the gut microbiota, thus conferring a beneficial physiological effect on the host [188]. In general, prebiotic fibers (e.g., inulin, fructooligosaccharides, pectin, and resist starch) have been shown to convey a number of physiological benefits, including

increasing *Bifidobacteria* and *Lactobacilli*, enhancing intestinal calcium absorption, preventing the overgrowth of pathogenic bacteria (e.g., *E. coli, Salmonella, Campylobacter*), producing important metabolites (e.g., SCFA and indole derivatives), enhancing gut barrier permeability and improving immune function. Other bioactive compounds found in plant-based foods (e.g., flavonoids, organosulfur compounds, phenolic acids, carotenoids) also possess prebiotic functions due to poor absorption and induce similar benefits.

FOSs, inulin, and galactosaccharides (GOSs) are widely studied prebiotics. The health benefits associated with this prebiotics have ranged from improving gut barrier function to modulation of gut microbiota and improved bone health [189, 190]. These fibers are made up of chains of 3 -10 monosaccharides (FOS, GOS) and vary in their degree of polymerization (DP). Short chain fructooligosaccharides (scFOS) has a DP range ranging from 1 to 5 [191]. The DP of inulin normally ranges from 3 to 60 fructan or β (2,1) monomers. Inulin as well as other fructans can be cleaved by endoglycosidase to produce oligosaccharides with a DP of 2-20. In contrast, GOS is a mixture of oligosaccharides (2-10 molecules of galactose, 1 molecule of glucose) that are produced from lactulose by enzymatic reactions. Food sources of GOS include dairy products, beans, and lentils. FOS, however, is found naturally in onion, chicory, asparagus, and banana and can also be produced commercially from inulin and sucrose [192-194]. Inulin-rich foods are leeks, asparagus, onions, wheat, garlic, chicory, oats, soybeans, and artichokes. FOS that contains 2 and 4 β (2,1)-linked fructosyl units can be produced via transfructosylation of sucrose. The FOSs and inulin are highly bifidogenic [195]. FOS tastes sweet and has low calories. The incorporation of FOS in foods like yogurt, biscuits, table spreads, and drinks is increasing due to its nutritive value [196]. The benefit of combining prebiotics is the ability to increase the DP of the supplement, so the varying chain lengths can be fermented and hydrolyzed along the entire length of the lower intestine and thereby maximizing their benefits on the host health [197]. Combining polyphenols with FOS has been shown to increase BMD in animal models [198]. In

ovariectomized rats, the synergistic effect of FOS and dried plum had improved both femur and lumber BMD [198].

As a prebiotic, FOS consumption increases cecum weight reflecting the high fermentation activity of the microbiota. This colonic fermentation of FOS expands *Bacteriodetes* and *Lactobacillus* abundance and produces short-chain fatty acids (SCFAs), which decrease pH and get quickly absorbed into the intestine influencing the metabolism and intestinal immune system of the host [199, 200]. Due to low pH, the acidic environment in the gut prevents colonization of pathogenic bacteria like *Bacteroidetes, Clostridium,* and *Enterococci* as well as assists in mineral absorption [201, 202]. Additionally, SCFA increases the growth and proliferation of epithelial cells augmenting the surface area for absorption of minerals [203, 204]. Besides, FOS-induced butyrate can also act as an energy source for colonocytes, favoring barrier function, mucus production, cytokine production, and protecting the bone indirectly [205, 206].

Prebiotics also help to maintain gut and bone homeostasis. Wen et al. [207] showed that supplementation of stigmasterol, a plant-derived sterol, enhanced butyrate production by gut microbiota and exhibited balance between Treg cells and Th-17 cells suppressing colitis in vitro. It is known that FOS act similarly by increasing SCFA [55]. Kang et al. [208] reported that treatment with FOS suppressed colonic pro-inflammatory cytokines (e.g., INF-γ, TNF-α, IL-6) improving symptoms in a mouse model of colitis. Yan et al. [209] showed that FOS can regulate the Treg and Th-17 cell balance in food allergies. Dietary intake of prebiotics has also proven beneficial for skeletal health. Lucas et al. [34] demonstrated that a high fiber diet produced SCFA, propionate, and butyrate are capable of inhibiting osteoclastic activity by glycolysis. FOS treatment in gastrectomized mice was shown to reverse osteopenia to some extent [210]. Yacon, which is comprised of FOS and inulin, enhanced *Bifidobacterium* and *Lactobacillus*, SCFA, and increased Treg cell numbers [211]. Taken together the literature supports that FOS favorably

influence the gut microbiota, mineral homeostasis, and the immune response, any of which may account for its benefits on bone.

Natural fruits like blueberries, tart cherries, and dried plums are rich in bioactive compounds such as polyphenols and FOS, which have been shown to have prebiotic activity [58, 212-214]. Flavonoids and phenolic acid exert bone protective function. Blueberries ameliorated bone formation biomarkers via p38 MAP kinase (MAPK) activated β-catenin canonical Wnt signaling in young rats by the increasing serum phenolic acid likely derived from gut microbial metabolism [67]. Dried plum elevates level of insulin-like growth factor-1 (IGF-1) and alkaline phosphatase (ALP), both markers of bone formation, and BMD in femur and tibia of ovariectomized rats [215, 216]. Dried plum restored reduced BMD due to sex hormone deficiency in orchidectomized male rats via down-regulation of RANKL, slowing down bone resorption and stimulation of bone formation mediated by IGF-I [212]. As dried plum a rich source of fiber, one of the potential mechanism for regulating bone remodeling might be prebiotic based. In support with that a recent comparison study on prebiotic activity of carbohydrate vs. polyphenol of dried plum in ovariectomized mice revealed that both components changed the gut microbiota and increased cecal SCFA. Carbohydrates persistently inhibited both bone formation and resorption while polyphenol initially reduced bone formation, showing that the components each had a distinct mechanism on bone metabolism [49]. Focusing on the bone immunomodulatory aspect, resveratrol a polyphenolic compound found in grapes and mulberries, is involved in deacetylation of the transcriptional factor STAT3 that is unable to produce RORγt, the promotor for Th-17 cells resulting in decreased differentiation of Th-17 cells [217-219]. Based on the regulatory function of Treg and Th-17 cells on bone cells, these natural prebiotics may be utilized as an effective approach to the prevention of osteoporosis.

2.6 Tart Cherry, Immunity and Bone

Tart cherries, *Prunus cerasus L*, are a type of stone fruit. Montmorency tart cherries, which originated from the Amarelle variety, are the most common variety with dark red skin and lighter-colored flesh [220]. According to a 2017 report, the USA is one of the leading producers of Montmorency tart cherries with Michigan being the state with the highest production followed by Utah, Washington, and New York [221]. Tart cherries are a good source of phenolic acid (7752-10323 μg/g of dried powder form) [222] and also provide a source of FOS (0.33 g/100 g of fresh fruit) [223]. The color, sugar, and phenol content of tart cherries are all influenced by various conditions, including temperature, humidity, and stage of maturation [224]. As the fruit ripens, its anthocyanin concentration, primarily cyanidin-3-glucosylrutinoside and cyanidin-3-rutinoside, increases while its total phenolic content drops [225]. Interestingly, in cold storage, the phenolic content of tart cherry continues to go up. Tart cherry is typically processed into frozen, powder, juice, or concentrate, which changes its chemical composition. Of all the processed products of tart cherry, frozen tart cherry has the highest phenolic and anthocyanin contents [224]. Cherries' phenolic compounds include anthocyanins, flavonoids (anthocyanins, flavan-3-ols, and flavonols), and phenolic acids (i.e., hydroxycinnamic acids and hydroxyl-benzoic acids) [226, 227]. The anti-oxidant efficacy of phenolic acid, and hydroxycinnamic acid, is similar to commercial anti-oxidants (e.g. terbutyl-hydroquinone and butylated hydroxytoluene) [228]. The FOS, the carbohydrate component of TC, is metabolized in the colon.

Recent studies have demonstrated that tart cherry has antioxidant, anti-inflammatory, bone protective, anti-diabetic, anti-lipidemic, cardioprotective, and neurocognitive protective functions [229, 230]. Much of the research has focused on the anti-oxidant and anti-inflammatory activity of the tart cherry [231-234]. In an in vitro study, a 50% acetone extracted concentrated tart cherry juice decreased cyclooxygenase COX-1 enzyme activity [233]. Others have shown that tart cherry reduced COX-2 expression and glial cell activation in the

hippocampus of aged mice [235]. In athletes, tart cherry juice reduces indicators of muscle damage, inflammation, and oxidative stress [236]. Tart cherry supplementation also suppressed oxidative stress and inflammation-induced muscle damage after 3 days of road cycle racing [237]. Tart cherry supplementation has been shown to downregulate inflammatory cytokines (e.g., IL-6, TNF-α) in obese mice [238]. Tart cherry supplementation further reduces plasma uric acid and prevents gout [239, 240].

Tart cherry has also been shown to have osteoprotective effects. Our lab reported that 5% and 10 % tart cherry supplementation in 5-month-old mice restored bone density in the tibia and spine [58]. Both the 5% and 10 % TC supplementation improved trabecular bone mass as well as restored cortical thickness and reduced cortical porosity. Moon et al. [65] showed that tart cherry protected against bone loss in a rheumatoid arthritis mice model by down-regulating the pro-inflammatory cytokine TNF-α and osteoclast-associated genes *Rankl, Trap, Nfatc1*. Consumption of tart cherry juice reduced bone resorption biomarker in postmenopausal women [241]. As is indicated by these studies, a growing body of literature suggests that tart cherry possesses bone protective action. However, the mechanisms of tart cherry on bone remain in question. Therefore, there is a need to further explore the mechanisms to better understand if the gut and gut mucosal immunity involving Treg cells serves as the target for tart cherry.

CHAPTER III

METHODOLOGY

Animal Care and Diet:

Ninety-six, 8-week-old C57BL/6 female mice (Taconic Biosciences, Rensselaer, New York) were acclimated for 2 weeks at Oklahoma State University's environmentally controlled Laboratory Animal Research Facility before initiation of the study. Mice (n=12-16/group) were then randomized to the following treatment groups in a 2 x 3 factorial design with diet (AIN93-M control diet, control diet supplemented with dried tart cherry [TC], or short chain fructooligosaccharides [FOS]) and CD25 antibody (iso-type control or CD25 antibody) as factors. Tart cherries were purchased from Shoreline Fruits at Peterson Farms (Shelby, MI), pitted, freeze-dried and ground into a powder so that it could be incorporated into the diet at a dose of 10% (w/w). This dose of TC was chosen based on previous studies in our lab [58] and others [65] that showed beneficial effects on bone. The source of the FOS used in the study was Nutraflora (FB P-95 Ingredion, Westchester, IL) was also supplemented at a dose of 10% w/w. Throughout the 8-week study, mice received an intraperitoneal injection of either anti-CD25 Ab (500 μg/mice/injection) or the isotype control antibody twice per week. The diets were adjusted to contain similar total energy, carbohydrate, protein, fat, fiber, calcium, and phosphorus as the AIN93-M diet (control) diet (**Table 2**). Mice had free access to food and RO water throughout the study and daily food consumption and weekly body weights were recorded.

Table 2: Diet Formulation

Ingredients	Control Diet (AIN-93M)	Tart Cherry Diet (10%)	FOS Diet (10%)
	(g/kg diet)	(g/kg diet)	(g/kg diet)
Tart cherry[1] or FOS	0.00	100.00	100.00
Carbohydrates			
Cornstarch (g)	465.70	383.60	365.70
Maltodextrin (g)	155.00	155.00	155.00
Sucrose (g)	100.00	100.00	100.00
Tart Cherry/ FOS (g)	0.00	82.10	100.00
Protein			
Casein (g)	140.00	133.50	140.00
Tart Cherry (g)	0.00	6.50	0.00
Fat			
Soy bean oil (g)	40.00	39.40	40.00
Tart Cherry (g)	0.00	0.59	0.00
Fiber			
Cellulose(g)	50.00	48.10	50.00
Tart Cherry (g)	0.00	1.92	0.00
Vitamin mix	10.00	10.00	10.00
Mineral			
Mineral mix[2]	13.40	13.40	13.40
Calcium Carbonate (40.04% Ca)	12.50	12.33	12.50
Calcium from Tart Cherry	0.00	0.06	0.00
Sodium Phosphate, monobasic (25.81 % P)	5.60	5.48	5.60
Potassium Phosphate, monobasic (22.76% P)	2.40	2.36	2.40
Phosphorus from Tart Cherry	0.00	0.09	0.00
Sucrose	1.10	1.28	1.10
Choline Bitartrate	2.50	2.50	2.50
L-cysteine	1.80	1.80	1.80

[1]TC analysis performed by NP Analytical Laboratories (St Louis, MO).[2]Complete mineral mix (TD94049, Harlan-Teklad Laboratories) was used for the control diet and a calcium and phosphorus deficient mineral mix (TD 98057, TD94049, Harlan-Teklad Laboratories) was used for the TC diet.

At the end of 8 weeks, mice were fasted for 3 hrs, anesthetized with a ketamine/xylazine cocktail (100 mg/10 mg per kg BW) followed by whole-body dual-energy x-ray absorptiometry (DXA) scans (GE Lunar PixiMus). Mice were then exsanguinated from the carotid artery and whole blood was collected for quantification of total white blood cells and differential counts or processed for serum assays. Tissues were harvested (i.e., small intestine, femurs, tibiae, and spine), cecal contents were collected and bone marrow and lamina propria cells from the ileum were processed for FACS analyses. All procedures were performed strictly following the guidelines for the ethical care and treatment of animals under the Institutional Animal Care and Use Committee at OSU.

Body Composition Assessment:

At the time of necropsy, whole body DXA scans (GE Medical Systems Lunar, Madison, WI) were performed to determine body composition (i.e., lean mass, fat mass, and body fat percentage), whole body bone mineral area (BMA), content (BMC), and density (BMD).

Microcomputed Tomography Analyses:

The tibia and fifth lumber vertebra were scanned using x-ray microcomputed tomography (μCT40, SCANCO Medical, Switzerland) to evaluate trabecular and cortical bone micro-architecture. The proximal tibial metaphysis and mid-diaphysis of long bones were used to assess trabecular and cortical bone, respectively. Tibia scans were performed at high resolution (2048 x 2048 pixels) and the proximal tibial metaphysis was analyzed by acquiring 256 slices and evaluating 150 slices (900 μm) in the volume of interest (VOI). Semi-automated contours were placed starting 10 slices (60 μm) distal to the growth plate to assess secondary spongiosa within the VOI. Trabecular bone parameters that were assessed included the bone volume expressed as a percentage of the total volume (BV/TV), trabecular number (TbN), trabecular separation (TbSp), trabecular thickness (TbTh), connectivity density (ConnDens) and structural model index (SMI).

The midshaft of the tibia was evaluated by acquiring 50 slices at the midpoint and analyzing 30 slices (180 μm). In terms of cortical bone, cortical porosity, cortical thickness, cortical area, and medullary area were evaluated.

Vertebral samples were analyzed by acquiring images at a resolution of 1024×1024 pixels, 30 μm from the dorsal and caudal growth plates. Similar to the tibial analysis at the proximal metaphysis, semi-automated contours were placed to include the secondary spongiosa within the VOI that included 160 to 180 slices. All analyses were performed at a threshold of 350 and a sigma and support of 1.2 and 2, respectively.

Flow Cytometry:

Single-cell suspensions of lamina propria lymphocytes were prepared from the ileum of the small intestine based on a previously published protocol [242]. The ileum was dissected and flushed with a mixture of RPMI, 2% FBS, and 1mM DTT, cut into small pieces, and incubated with HBSS with 2 mM EDTA at room temperature to remove epithelial cells, followed by a series of three incubations with 0.20 mg/mL collagenase type VIII (Sigma-Aldrich). The cells were then pelleted, resuspended, and filtered through a 70-μm sterile filter before lymphocytes were separated on 40% and 80% Percoll gradients. Cells at the interface of two Percoll gradients were collected and washed in complete media three times. The cells were then fixed (10 million cells/mL) using the mouse Foxp3 fixation buffer (BD Biosciences) and washed once.

For harvesting bone marrow lymphocytes, both ends of the femur were cut and flushed with incomplete Dulbecco's Modified Eagle Medium (DMEM) media. The bone marrow cells were treated with 5 mL of a diluted mixture of lysing buffer (BD Biosciences FACS lysing solution) prepared by diluting 2 mL of the lysing buffer with 3 mL of DMEM media to lyse the erythrocytes and platelets. After centrifuging and decanting lysing buffer, cells were re-suspended in 2 mL complete media of DMEM media, 0.5% BSA, 10mM EDTA with pH 7.4.

Viable cells (2×10^6) from the ileum and bone marrow were first stained with live/dead stain which was a fluorochrome. FACS buffer was added, cells were centrifuged and supernatants were discarded. Then cells were stained with surface marker antibodies (antibodies for CD3, CD4, CD8, IL-17). Next, the permeabilization buffer was added and cells were stained with the intracellular markers (antibody for FOXP3, CD25) following the manufacturer's instructions. Flow cytometry analyses were carried out using BD FACSAria III (BD Biosciences) at the Center for Veterinary Health Sciences flow cytometry laboratory (Oklahoma State University). Data were analyzed with the BD FACS Diva software (version 8.0.1; BD Biosciences).

Fecal SCFA Analyses:

Fecal samples collected per cage at the end of the study were processed in duplicates for SCFA analyses according to previously published protocol [243]. To assess fecal SCFA concentration, samples were freeze-dried and then pulverized into powder. Approximately 150 mg fecal powder was mixed with 250 µl hydrochloric acid, 45 µl internal standard (1 mM 2-ethyl butyric acid in 12% formic acid) followed by two extractions with 1 mL diethyl ether. An aliquot of 500-600 µl of the organic extract was transferred into inserts placed in gas chromatography (GC) vials. Gas chromatographic analysis was performed using an Agilent 6890N GC system with a flame ionizable detector and an automatic liquid sampler (Agilent Technologies Santa Clara, CA). Samples concentration were determined using a 5-point calibration curve, with each standard containing solutions of acetic, propionic, butyric, valeric, isovaleric, isobutyric, caproic, and heptanoic acids (Sigma-Aldrich).

Serum Bone Biomarkers:

To determine alterations in osteoblast activity induced by treatments, serum indicator of bone formation, N-terminal propeptide of type I procollagen (P1NP), was measured by a commercially available EIA kit according to the manufacturer's specifications (Immunodiagnostic

Systems, Inc., Fountain Hills, AZ, USA). During bone formation, collagen type I is synthesized from osteoblastic pro-collagen type I. Pro-collagen type I contains N-terminal extensions, which are removed by specific proteases during the conversion of procollagen to collagen. The inter- and intra-assay coefficients of variations for PINP were 9.2% and 6.4%.

For evaluating bone resorption activity by osteoclasts, serum biomarker C-terminal end of the telopeptide of type I collagen (CTX-1), was assessed by EIA kit (Immunodiagnostic Systems, Inc., Fountain Hills, AZ, USA). During bone remodeling, cathepsin K degrades bone matrix, and CTX-1 is released from intact collagen type 1. CTX-1 was measured as a degradation product involved in bone resorption. The inter- and intra-assay coefficients of variations for CTX-1 were 14.8% and 9.2%.

RNA extraction and Gene Expression Analysis:

Total RNA was extracted from the bone hard tissue and ileum lamina propria using Trizol (Invitrogen, Rockville, MD). Prior to RNA extraction, the flushed tibia (n = 6 samples/group) was pulverized in a liquid nitrogen-containing Freezer/Mill (Spex 6770 Freezer/Mill, Metuchen, NJ, USA). The concentration and purity of RNA were confirmed by optical density measured at 260 and 280 nm using Nanodrop Spectrophotometer (Rockland, SE, USA), and gel electrophoresis was performed to ensure the quality of RNA bands. After DNase treatment, RNA (2 μg; n=6/group) was reverse transcribed (Superscript II, Invitrogen, Carlsbad, CA) to synthesize complementary DNA (cDNA). The real-time quantitative reverse transcription polymerase chain reaction (qRT-PCR) (7300 Real-Time PCR System, Applied Biosystems, Foster City, CA) was performed using SYBR green as the detector (Roche, Penzberg, Germany) and the qRT-PCR results were assessed by the comparative cycle number at threshold (C_T) method with determined housekeeping gene, *Gapdh* or *Hprt* as the invariant control.

In the hard tissue, genes associated with regulating osteoblastogenesis, bone mineralization, and bone matrix protein production will be assessed (e.g., *Runx2, Bmp-2, Col1α1*, *Wnt10b, osterix (Osx), Opn, Ocn (Bglap2), Phex, Mepe, Dmp1*). Additionally, regulators of osteoclastogenesis and osteoclast activity (e.g., *Rankl, Opg)* will also be assessed. In the ileum lamina propria, genes involved in the regulation of T cell differentiation and indicator of their activation (e.g., *IL-10, Tgf-β, CXCR4, CXCL12, CXCL10, CCR7, Vcam*) and SCFA binding receptor (*GPR43*) will be evaluated. The primer sequence of the housekeeping genes and genes of interest for real time qPCR were listed in **Appendix 3**.

Histology:

Vertebral specimens were decalcified with 20% ethylenediaminetetraacetic acid (EDTA) disodium salt for 12-14 days. These specimens as well as sections of the jejunum, and colon were fixed with 10% neutral formalin, and dehydrated (Shandon Citadel 2000 Waltham, MA) using a graded ethanol series and toluene. Tissues were then embedded in paraffin and 5 µm sections were cut on a longitudinal axis by microtome (Leica Biosystems Wetzlar, Germany). Hematoxylin and eosin (H&E) staining was performed to observe cellularity and any indications of inflammatory response within the tissues. Additionally, within the gut specimens, the effects of treatments on structural changes in villi height, villi width, villi area, villi perimeter, and crypt depth were assessed with BZ-X800 software (Keyence Osaka, Japan).

Statistical Analysis:

The SAS Version 9.4 statistical analysis software (SAS Institute Inc., Cary, NC) was used for data analyses. Data was assessed for outlier and normal distribution by the Shapiro Wilks test. If the assumption for normality was broken, data were log-transformed and reassessed for normality. All continuous variables were analyzed by 2-way ANOVA using the generalized linear model (GLM) with CD25 and diet as factors. If data failed to meet the criteria of normal

distribution, they were analyzed with Friedman's test. When the overall P-value was significant for main effects (diet or CD25 antibody) or the interaction, differences between treatment groups were evaluated using *post hoc* test, Fischer's least square means test. Data are expressed as mean ± standard error, and the alpha (α) was set at 0.05.

CHAPTER IV

RESULTS

Body Weight, Food Intake, Body Composition, and Tissue Weight:

Over the course of the 8-week study, the effect of diet and CD25 antibody on body weight was assessed. There was no interaction between dietary treatments and CD25 antibody, but there was a main effect of diet. The FOS treated groups weighed significantly less compared to the TC and Con groups after the first week of treatment (**Figure 3**). This effect of FOS on body weight continued through the remainder of the study so that by 8 wks. the FOS group's body weight was 22% less than the control group *(P<0.01)* and 12% less than the tart cherry group *(P<0.01)*. No CD25 antibody effect was observed on body weight. These alterations in body weight occurred despite the fact that there was no main effect of diet and CD25, or their interaction on food intake **(Table 3).**

To better understand the weight change, body composition was analyzed. No interaction with CD25 and diet was observed on lean mass, fat mass and percent fat, but a main effect of diet was noted. With FOS treatment, there was a decrease in percent fat and fat mass *(P<0.01)* compared to the groups on the Con and TC diets **(Table 3).** Tart cherry had no effect on fat mass or percent fat. There tended (*P=0.0562*) to be a main effect of diet on lean mass. FOS-fed animals tended to have a higher lean mass relative to animals fed TC. In contrast, CD25 antibody treatment exhibited no effect on either lean or fat parameters.

Relative tissue weights revealed that there was a main diet effect on visceral adiposity (i.e., white adipose tissue or WAT) and cecum weight, but not on liver, thymus, spleen and uterine weight **(Table 3).** The relative amount of WAT was significantly reduced *(P<0.01)* in FOS animals compared to the Con and TC groups **(Table 3).** Both TC and FOS supplementation increased cecum weight *(P<0.01)* in contrast to control animals, which is consistent with prebiotic activity **(Table 3).**

Whole Body Bone Density and Tibia Length:

Whole body DXA scans were assessed to determine how BMA, BMC and BMD were altered in response to diet and CD25 alone and in combination. Though there was no interaction effect of CD25 antibody and diet on whole body BMC and BMA, a significant diet effect was observed **(Table 4).** The FOS diet increased whole body BMC *(P<0.01)* and BMA *(P<0.01)* compared with the Con and TC groups **(Table 4).** Furthermore, whole body BMD was significantly increased in FOS animals relative to TC animals. In presence of CD25 antibody, BMA (P<0.05) was reduced in animals, but there were no changes in BMC and BMD **(Table 4).** No CD25 effect or combination effect was noted on tibia length, but a significant diet effect was observed. FOS fed animals had longer tibia than tart cherry or control animals **(Table 4).**

Trabecular and Cortical Bone Microarchitecture:

The microstructural parameters of trabecular and cortical bone were assessed utilizing microCT images of the tibia and lumbar vertebra. There was no interaction or main effect of CD25 antibody on both tibial and vertebral trabecular bone, but a main diet effect was observed on BV/TV (**Figure 4**), Tb. N, Tb. Th and Tb. Sp (**Table 5**). Within the proximal tibial metaphysis (**Figure 4a**) and lumbar vertebra (**Figure 4b**), the trabecular BV/TV was increased (*P<0.01)* in FOS-fed animals. This increase in BV/TV in both tibia and vertebra with FOS treatment occurred in conjunction with an increase in Tb. N and Tb. Th (*P<0.01*) and a decrease in Tb. Sp

(*P<0.01*) compared to the Con and TC groups **(Table 5).** Dietary supplementation with tart cherry did not alter any indices in the tibia, but in the vertebra Tb. Th was reduced (*P<0.01*) compared to control animals.

There was also no interaction or main effect of CD25 antibody on trabecular bone SMI, mean material density (Mean Mat), degree of anisotropy (DA) or connective density trabecular, but there was a main diet effect on these parameters (**Table 5**). The trabecular connectivity density was increased (*P<0.01*) in the FOS treated group compared to the Con and TC group after 8 weeks. There was a decrease *(P<0.01)* in the SMI in both spine and tibia (**Table 5)** with FOS supplementation compared to Con or TC. In contrast, SMI was significantly increased with tart cherry compared to the control group. SMI assesses whether the trabecular struts are oriented in a more rod-like or plate-like manner within the region of interest. FOS supplementation improved the orientation of the trabecular struts to become more plate-like, whereas the TC treatment resulted in a more rod-like structure. FOS consumption also enhanced the bone material density *(P<0.01)* and reduced the degree of anisotropy *(P<0.01)* within the lumbar vertebra and tibia (**Table 5**), whereas tart cherry had no effect. CD25 antibody treatment did not affect the connectivity density, SMI, Mean Mat nor the DA at either skeletal site.

Cortical bone was evaluated at the tibia mid-diaphysis. No interaction or CD25 main effect was overserved in cortical area, thickness, porosity except medullary area (**Table 5**). A main effect of diet was noted on the medullary area, which was increased in response to the FOS diet *(P<0.01)* compared to the Con and TC diet groups.

Serum Bone Biomarkers:

To assess the systemic biomarkers of bone formation and bone resorption in response to diet and CD25 antibody treatment, the bone formation marker, P1NP and the bone resorption marker, CTX-1 were evaluated. There was no interaction or main effect of CD25 or diet on serum

P1NP (**Figure 6a**). Although no main diet effect was noted in serum CTX-1, there was a significant CD25 antibody main effect *(P<0.01)* and interaction (**Figure 6b**). As expected, mice treated with +CD25 exhibited an increase in CTX-1 (P<0.01) compared to mice receiving the isotype control (i.e., –CD25) antibody. Surprisingly, among mice on the –CD25 antibody + TC diet, serum CTX-1 was increased compared to mice receiving the –CD25 antibody on the control and FOS diets. However, neither the TC or FOS altered serum CTX-1 of the mice on +CD25 (**Figure 6b**).

T-Lymphocytes in Gut Ileum and Bone marrow:

Flow cytometry was performed on lymphocytes harvested from the ileum and bone marrow to determine how T cell populations were changing in response to CD25 and dietary treatments alone and in combination. The gating scheme that was used on samples from both tissues is shown **(Figure 7 [a-f]).**

In the ileum, there was a significant main diet effect on the percentage of $CD4^+$ T cells, $CD4^+$ $CD25^+$ $Foxp3^+$ Treg cells, $CD4^+$IL-$17A^+$ Th17 cells and Treg to Th17 cell ratio. FOS diet decreased the percentage of $CD4^+$ T cells *(P<0.05)* **(Figure 8a)** and increased the percentage of Tregs *(P<0.01)* (**Figure 8b)** compared to Con and TC. In presence of CD25 antibody, a decrease in $CD4^+$ $CD25^+$ $Foxp3^+$ Treg (**Figure 8b**) cells and Treg:Th17 ratio (**Figure 8d**) were observed. Additionally, a diet x CD25 antibody interaction effect was noted on the Treg:Th17 ratio (**Figure 8d**). TC increased *(P<0.01)* the Treg:Th17 ratio in absence of CD25 antibody **(Figure 8d**). TC intake downregulated the percentage of Th17 cells *(P<0.01)* (**Figure 8c)** and increased (*P<0.01*) the Treg:Th17 ratio (**Figure 8d)**.

No interaction effect was noted in the absolute number of $CD4^+$ T cells, Treg and Th17 cells in the ileum (**Appendix 2**). However, a main diet effect was observed on absolute count of $CD4^+$ T cells. FOS intake reduced (*P<0.01*) the absolute number of $CD4^+$ T cells compared other

diets. A main CD25 effect was observed on the absolute count of Tregs and Th17 cells in the ileum. CD25 treatment suppressed *(P<0.01)* the total Treg numbers whereas increased *(P<0.01)* the number of Th17 cells in the ileum (**Appendix 2**).

No CD25 x Diet interaction or CD25 effect was noted on the percentage and total number of $CD3^+CD8^+$ T cells in the ileum **(Appendix 1-2)**. Nevertheless, a main diet effect was observed on the relative and absolute count of $CD8^+$ T cells. FOS increased *(P<0.01)* the percentage of $CD8^+$T cells but decreased *(P<0.01)* the total count of $CD8^+$ T cells compared to TC **(Appendix 1-2).**

In the bone marrow, a main diet was exhibited on the percentage of $CD4^+$ T cells, $CD4^+$ $CD25^+$ $Foxp3^+$ Tregs, and Treg:Th17 ratio **(Figure 9 [a-b], d).** FOS supplementation increased the percentage of $CD4^+$ T cells *(P<0.01)* **(Figure 9a)** and decreased the percentage of Treg cells *(P<0.01)* (**Figure 9b)** and the Treg:Th17 ratio *(P<0.01)* **(Figure 9d).** As expected, animals receiving the CD25 antibody exhibited a reduction in the percentage of Treg and the Treg:Th17 ratio (**Figure 9b and 9d**).

No alteration was noted on the absolute number of $CD4^+$ T cells (**Appendix 2**). A main diet effect was noted on the total number of Th17 cells and a main CD25 antibody effect was observed on total count of Treg cells. As anticipated, the Treg cells were suppressed in the bone marrow after CD25 treatment **(Appendix 2).** Surprisingly**,** in the bone marrow, FOS consumption increased *(P<0.01)* the absolute count of Th17 cells which disagree with our anticipation **(Appendix 2).**

In the bone marrow, the percentage of $CD8^+$ T cells was increased *(P<0.01)* with FOS but no alteration was noted in total count of $CD8^+$ T cells **(Appendix 1-2)**.

Fecal Short Chain Fatty Acid Analysis:

Short chain fatty acid analysis of the fecal samples revealed a diet main effect, but no interaction or CD25 main effect (**Table 6**). The FOS fed animals had higher fecal concentrations of acetic, propionic, n-butyric acid *(P<0.01)* and a lower fecal concentration of i-butyric acid *(P<0.01)* compared to the animals fed the Con and TC diets (**Table 6**). Tart cherry also increased acetic, propionic, i-butyric, n-butyric acid concentrations compared to the control diet, but to at a lesser amount than FOS supplementation. However, tart cherry additionally increased fecal i-valeric acid concentrations *(P<0.01)* (**Table 6**).

Relative Gene Expression of Bone Cells Activity in Bone:

As bone phenotype was evident in response to FOS diet, we investigated the alterations in gene expression within the bone tissue (i.e., bone marrow removed) using real time qPCR analysis. *Bmp-2, Runx-2, osterix,* genes, the regulators of osteoblast differentiation as well as bone sialoprotein (*Bsp*), osteocalcin (*Ocn [Bglap2]), col1α1*, osteopontin (*Opn*) genes, the regulators of osteoblast activity were assessed. FOS upregulated *(P<0.01) Bmp-2, osterix, col1α1* genes compared to Con and TC diet (**Table 7**). No effects of FOS were noted on *Runx-2, Bsp* and *Opn* genes (**Table 7**). In presence of CD25 antibody, the relative abundance of *Bmp-2* and *Wnt10b* were downregulated (**Table 7**).

Osteocytes are the most abundant type of bone cells and are responsible for bone metabolism. Although no interaction of diet and CD25 was noted, a main diet effect was observed on *Phex, Dmp-1, Mepe* and *SOST* genes. FOS treatment significantly upregulated *Phex (P<0.01), Dmp-1 (P<0.01),* Mepe *(P<0.01), SOST (P<0.01)* genes (**Table 7**). Tart cherry supplementation also increased the relative abundance of *Phex (P<0.01)* and *Mepe (P<0.01)* mRNA. A main CD25 antibody was observed in *SOST* gene. In presence of CD25 antibody, the *SOST* gene expression *(P<0.01)* was downregulated (**Table 7**).

The RANK-RANKL-OPG axis is an important indicator of bone remodeling and especially the initiation of osteoclastogenesis. No alteration was noted on RANKL, OPG or the RANKL:OPG ratio in response to diet, CD25 alone or their combination (**Table 7**).

Relative Gene Expression of Key Indicators of Inflammation and Other Mediators in Gut Lamina Propria:

In the gut lamina propria, we assessed pro-inflammatory cytokines *Tnf-α, IL-17, IL-6,* chemokines and adhesion molecules *CCR7, CXCL10, CXCR4, CXCL12, Vcam* that are involved in mucosal immune T cell trafficking, anti-inflammatory cytokines *Tgf-β* and *IL-10* and *GPR-43* receptor for SCFA binding (**Table 8**). No CD 25 antibody effect or interaction effect was observed. There was a main diet effect noted on inflammatory gene *IL-17,* chemokines *CXCR4, CXCL12* and adhesion molecule *Vcam. CXCR4* is the receptor for *CXCL12* ligand. As expected, FOS treatment significantly downregulated *IL-17 ($P<0.05$), CXCR4 ($P<0.01$), CXCL12 ($P<0.01$), Vcam ($P<0.01$)* genes compared to Con and TC (**Table 8**). These findings indicate no sign of inflammation which agrees with our anticipation as we designed our study with naïve animals.

Table 3

Food Intake and Anthropometrics

	- CD25			+ CD25			*P-values*		
	Con	**TC**	**FOS**	**Con**	**TC**	**FOS**	**CD25**	**Diet**	**CD25*Diet**
Daily Food Consumption									
Food intake (g/d)	3.24 ± 0.08	3.40 ± 0.11	3.44 ± 0.08	3.40 ± 0.16	3.38 ± 0.14	3.42 ± 0.15	0.6676	0.1332	0.9319
Body Composition									
Lean mass (g)	17.19 ± 0.25	16.68 ± 0.26	17.56 ± 0.33	16.79 ± 0.30	16.92 ± 0.26	17.48 ± 0.26	0.7295	0.0562	0.4634
Fat (g)	10.09 ± 0.51	10.18 ± 0.71	6.60 ± 0.77$^{\#\$}$	10.70 ± 0.69	9.63 ± 0.64	6.76 ± 0.46$^{\#\$}$	0.7673	**<.0001**	0.6312
Percent fat (%)	36.72 ± 1.30	37.11 ± 1.83	26.60 ± 2.34$^{\#\$}$	38.32 ± 1.65	35.78 ± 1.55	27.57 ± 1.38$^{\#\$}$	0.8904	**<.0001**	0.6377
Tissue Weights									
WAT (mg/g)	45.64 ± 1.66	47.46 ± 2.58	34.48 ± 3.39$^{\#S}$	49.55 ± 3.53	50.71 ± 2.90	34.77 ± 2.33$^{\#\$}$	0.3013	**<.0001**	0.8226
Liver (mg/g)	46.33 ± 1.16	43.07 ± 1.42	44.39 ± 1.15	44.74 ± 0.91	44.72 ± 1.03	41.6 ± 1.30	0.3529	0.1076	0.1572
Thymus (mg/g)	2.75 ± 0.18	3.08 ± 0.10	2.75 ± 0.16	2.92 ± 0.14	2.75 ± 0.12	2.75 ± 0.10	0.6369	0.4946	0.1536
Cecum (mg/g)	2.42 ± 0.10	3.19 ± 0.21$^{\#}$	5.74 ± 0.24$^{\#\$}$	2.72 ± 0.12	3.12 ± 0.24$^{\#}$	6.06 ± 0.25$^{\#\$}$	0.4582	**<.0001**	0.2177
Spleen (mg/g)	3.34 ± 0.11	3.41 ± 0.14	3.17 ± 0.23	3.37 ± 0.15	3.5 ± 0.15	3.32 ± 0.17	0.4955	0.3067	0.8772
Uterus (mg/g)	2.74 ± 0.18	2.67 ± 0.22	3.22 ± 0.61	2.78 ± 0.23	3.24 ± 0.24	2.66 ±0.23	0.3398	0.6175	0.3572

Control diet = Con; Tart cherry diet = TC; Fructooligosaccharide diet = FOS.
Data presented as mean ± SEM. *P-values <0.05* are considered statistically different. Tissue weights expressed as mg/g of body weight. # indicates TC or FOS vs. Con (*P<0.05).* $ indicates TC vs. FOS *(P<0.05).*

Table 4

Whole Body Bone Density and Tibia Length

	- CD25			+ CD25			*P-values*		
	Con	**TC**	**FOS**	**Con**	**TC**	**FOS**	**CD25**	**Diet**	**CD25*Diet**
BMA (cm^2)	8.62 ± 0.16	8.84 ± 0.18	9.37 ± 0.10$^{\#\$}$	8.54 ± 0.08	8.44 ± 0.11	9.19 ± 0.09$^{\#\$}$	**0.0479**	**<.0001**	0.4150
BMC (mg)	447.65 ± 10.39	452.94 ± 11.38	486.27 ± 6.41$^{\#\$}$	439.28 ± 6.94	427.33 ± 7.42	476.92 ± 7.72$^{\#\$}$	0.0566	**<.0001**	0.5396
BMD (mg/cm^2)	51.88 ± 0.49	50.61 ± 0.45	51.92 ± 0.31$^{\$}$	51.37 ± 0.58	50.61 ± 0.43	51.44 ± 0.38$^{\$}$	0.4124	**0.0421**	0.8267
Tibia Length (mm)	17.29 ± 0.06	17.19 ± 0.08	17.44 ± 0.09$^{\#\$}$	17.11 ± 0.08	17.05 ± 0.10	17.49 ± 0.07$^{\#\$}$	0.2357	**0.0014**	0.4604

Control diet = Con; Tart cherry diet = TC; Fructooligosaccharide diet = FOS.
Data presented as mean ± SEM. *P-values <0.05* are considered statistically different.
# indicates TC or FOS vs. Con (*P<0.05)*. $ indicates TC vs. FOS *(P<0.05).*

Table 5

Bone Microarchitecture Analysis by MicroCT in Lumbar Vertebra and Tibia

	-CD25			+CD25			*P-values*		
	Con	**TC**	**FOS**	**Con**	**TC**	**FOS**	**CD25**	**Diet**	**CD25*Diet**
Vertebra Trabecular Bone									
TbN ($1/mm^2$)	4.18 ± 0.07	4.20 ± 0.08	5.00 ± 0.07$^{\#\$}$	4.29 ± 0.10	4.46 ± 0.01	4.97 ± 0.11$^{\#\$}$	0.1238	**<.0001**	0.2547
TbTh (µm)	51.58 ± 0.79	49.36 ± 0.57$^{\#}$	55.08 ± 0.37$^{\#\$}$	51.55 ± 1.04	49.95 ± 0.89$^{\#}$	54.88 ± 0.91$^{\#\$}$	0.8579	**<.0001**	0.8764
TbSp (µm)	238.00 ± 4.35	232.86 ± 4.47	196.13 ± 3.44$^{\#\$}$	230.38 ± 6.45	222.88 ± 5.30	198.63 ± 5.68$^{\#\$}$	0.2317	**<.0001**	0.4325
ConnDens ($1/mm^3$)	160.48 ± 7.66	158.07 ± 5.37	240.54 ± 9.97$^{\#\$}$	164.89 ± 7.40	187.15 ± 7.30	241.51 ± 14.92$^{\#\$}$	0.1026	**<.0001**	0.1414
SMI	1.28 ± 0.07	1.46 ± 0.02$^{\#}$	0.73 ± 0.04$^{\#\$}$	1.36 ± 0.06	1.46 ± 0.07$^{\#}$	0.70 ± 0.09$^{\#\$}$	0.8116	**<.0001**	0.5710
MatDen (mg HA/ccm)	1073.95 ± 7.32	1063.88 ± 7.64	1043.19 ± 2.45$^{\#\$}$	1077.51 ± 6.95	1056.17 ± 5.64	1042.52 ± 3.68$^{\#\$}$	0.7423	**<.0001**	0.6341
DA	1.73 ± 0.02	1.72 ± 0.02	1.66 ± 0.02$^{\#\$}$	1.73 ± 0.026	1.69 ± 0.018	1.63 ± 0.013$^{\#\$}$	0.2004	**0.0004**	0.7392
Tibia Trabecular Bone									
TbN ($1/mm^2$)	3.84 ± 0.08	3.76 ± 0.10	4.39 ± 0.10$^{\#\$}$	3.79 ± 0.13	3.64 ± 0.09	4.51 ± 0.08$^{\#\$}$	0.8119	**<.0001**	0.4473
TbTh (µm)	47.22 ± 0.68	45.74 ± 1.31	47.69 ± 0.64$^{\$}$	45.19 ± 0.89	45.40 ± 1.10	48.18 ± 0.65$^{\$}$	0.3937	**0.0330**	0.3581
TbSp (µm)	255.43 ± 5.00	261.88 ± 7.07	219.78 ± 5.54$^{\#\$}$	259.94 ± 9.18	270.14 ± 6.88	212.53 ± 3.98$^{\#\$}$	0.7301	**<.0001**	0.4665
ConnDens ($1/mm^3$)	81.73 ± 4.69	93.10 ± 9.34	145.96 ± 12.34$^{\#\$}$	78.67 ± 7.40	81.66 ± 6.02	154.87 ± 7.22$^{\#\$}$	0.5925	**<.0001**	0.4719
SMI	2.14 ± 0.10	1.99 ± 0.09$^{\#}$	1.83 ± 0.08$^{\#\$}$	2.17 ± 0.06	1.97 ± 0.04$^{\#}$	1.80 ± 0.04$^{\#\$}$	0.8420	**0.0001**	0.9173
MeanMat (mg HA/ccm)	1098.64 ± 4.6	1084.95 ± 7.4	1095.14 ± 1.9$^{\#}$	1086.37 ± 7.6	1077.72 ± 7.8	1103.07 ± 3.5$^{\#}$	0.6818	0.0920	0.1979
DA	2.29 ± 0.02	2.25 ± 0.04	2.10 ± 0.05$^{\#\$}$	2.26 ± 0.07	2.32 ± 0.06	2.17 ± 0.04$^{\#\$}$	0.8025	**0.0051**	0.2600
Tibia Cortical Bone									
BV/TV(%)	96.12 ± 0.06	96.07 ± 0.04	95.9 ± 0.10	96.08 ± 0.11	96.23 ± 0.12	95.93 ± 0.13	0.2053	0.0850	0.7412
CortArea ($µm^2$)	699.00 ± 13.90	695.38 ± 8.79	723.00 ± 8.95	683.13 ± 15.92	698.88 ± 9.42	688.13 ± 13.19	0.1160	0.4859	0.2902
CortTh (µm)	197.38 ± 2.40	199.88 ± 1.73	198.75 ± 3.22	197.14 ± 1.60	197.00 ± 2.27	198.88 ± 2.91	0.6234	0.8093	0.7992
MedullaryArea ($µm^2$)	28.1 ± 0.60	27.7 ± 0.60	30.9 ± 0.70$^{\#\$}$	27.90 ± 0.80	27.60 ± 1.10	29.10 ± 0.70$^{\#\$}$	0.3417	**0.0076**	0.4884
Porosity (%)	3.88 ± 0.06	3.93 ± 0.04	4.1 ± 0.10	3.93 ± 0.11	3.77 ± 0.12	4.07 ± 0.13	0.2124	0.0809	0.7615

Control diet = Con; Tart cherry diet = TC; Fructooligosaccharide diet = FOS. Data presented as mean ± SEM. # for TC or FOS vs. Con (*P<0.05)* and $ for TC vs. FOS (P<0.05). n=8 mice/group

Table 6

Fecal Short Chain Fatty Acid Analysis

	-CD25			+CD25			*Pvalues*		
	Con	**TC**	**FOS**	**Con**	**TC**	**FOS**	**CD25**	**Diet**	**CD25*Diet**
Fecal SCFA (µmol/g)									
acetic	5.93± 0.43	8.70 ± 0.33$^{\#}$	33.96 ± 4.55$^{\#\$}$	6.22 ± 0.76	7.67 ± 0.44$^{\#}$	24.35 ± 3.34$^{\#\$}$	0.0813	**<.0001**	0.0779
propionic	0.66 ± 0.07	1.00 ± 0.04$^{\#}$	5.41 ±1.24$^{\#\$}$	0.70 ± 0.08	0.88 ± 0.04$^{\#}$	3.21 ±0.50$^{\#\$}$	0.3098	**<.0001**	0.2867
i-butyric	0.07 ± 0.01	0.12 ± 0.01$^{\#}$	0.01 ± 0.01$^{\#\$}$	0.07 ± 0.01	0.13 ± 0.01$^{\#}$	0 ± 0$^{\#\$}$	0.1285	**<.0001**	0.3425
n-butyric	0.19 ± 0.01	0.51 ±0.05$^{\#}$	5.54 ± 1.61$^{\#\$}$	0.28 ± 0.06	0.57 ± 0.39$^{\#}$	4.19 ± 0.88$^{\#\$}$	0.3360	**<.0001**	0.0679
i-valeric	0.09 ± 0.02	0.16 ± 0.01	0.08 ± 0.03$^{\$}$	0.10 ± 0.02	0.15 ± 0.10	0.04 ± 0.02$^{\$}$	0.3481	**<.0001**	0.4410
n-valeric	0.07 ± 0.02	0.11 ± 0.07	0.13 ± 0.06	0.09 ± 0.02	0.11 ± 0.01	0.12 ± 0.04	0.3555	0.0805	0.3758

Control diet = Con; Tart cherry diet = TC; Fructooligosaccharide diet = FOS.
Data presented as mean ± SEM. *P-values <0.05* are considered statistically different.
# indicates TC or FOS vs. Con (*P<0.05)*.
$ indicates TC vs. FOS (*P<0.05)*

Table 7

Relative Gene Expression of Bone Cells Activity in Hard Tissue

	-CD25			+CD25			*P-values*		
	Con	TC	FOS	Con	TC	FOS	CD25	Diet	CD25*Diet
Osteoblastogenesis									
Wnt10b	1.00 ± 0.17	1.16 ± 0.16	1.63 ± 0.19	0.76 ± 0.10	0.91 ± 0.13	0.99 ± 0.23	**0.0061**	0.0825	0.5484
Bmp-2	1.00 ± 0.08	1.32 ± 0.13	2.06 ± 0.25$^{\#\$}$	0.89 ± 0.15	0.95 ± 0.11	1.47 ± 0.23$^{\#\$}$	**0.0147**	**0.0006**	0.7756
Runx 2	1.00 ± 0.14	1.39 ± 0.23	1.39 ± 0.17	1.38 ± 0.15	1.35 ± 0.12	1.12 ± 0.21	0.8852	0.5806	0.1806
Osterix	1.00 ± 0.25	1.08 ± 0.09	1.98 ± 0.27$^{\#\$}$	0.97 ± 0.16	1.02 ± 0.14	1.71 ± 0.35$^{\#\$}$	0.6200	**0.0037**	0.7950
Osteoblast Markers									
Bsp	1.00 ± 0.23	1.17 ± 0.37	1.78 ± 0.31	1.10 ± 0.15	1.19 ± 0.15	0.93 ± 0.16	0.2121	0.4246	0.0923
Col1α1	1.00 ± 0.21	1.62 ± 0.30	2.47 ± 0.30$^{\#\$}$	1.19 ± 0.15	1.12 ± 0.10	1.60 ± 0.43$^{\#\$}$	0.1649	**0.0099**	0.0988
Bglap2	1.00 ± 0.18	1.98 ± 0.30	2.15 ± 0.38	1.59 ± 0.22	1.81 ± 0.24	1.87 ± 0.48	0.8661	0.0848	0.3625
Opn	1.00 ± 0.19	1.26 ± 0.19	1.52 ± 0.23	0.85 ± 0.15	1.25 ± 0.26	0.93 ± 0.18	0.1563	0.2700	0.3641
Osteocytes Markers									
Phex	1.00 ± 0.33	1.96 ± 0.44$^{\#}$	2.75 ± 0.28$^{\#\$}$	1.09 ± 0.19	1.30 ± 0.14$^{\#}$	1.89 ± 0.31$^{\#\$}$	0.2533	**0.0002**	0.2136
Dmp1	1.00 ± 0.22	1.42 ± 0.16	1.96 ± 0.22$^{\#\$}$	0.98 ± 0.23	0.87 ± 0.13	1.58 ± 0.24$^{\#\$}$	0.2016	**0.0010**	0.6762
Mepe	1.00 ± 0.22	1.95 ± 0.26$^{\#}$	3.70 ± 0.48$^{\#\$}$	1.22 ± 0.15	1.25 ± 0.18$^{\#}$	2.67 ± 0.38$^{\#\$}$	0.2412	**<.0001**	0.0862
Sost	1.00 ± 0.17	1.21 ± 0.25	2.79 ± 0.35$^{\#\$}$	0.98 ± 0.15	0.79 ± 0.15	1.27 ± 0.23$^{\#\$}$	**0.0059**	**0.0009**	0.0793
Rank: Rankl:Opg Axis									
RankL	1.00 ± 0.16	1.14 ± 0.28	0.90 ± 0.22	0.91 ± 0.1	1.20 ± 0.09	0.81 ± 0.12	0.7798	0.1754	0.8669
Opg	1.00 ± 0.11	0.88 ± 0.15	1.01 ± 0.27	0.74 ± 0.17	1.03 ± 0.2	0.73 ± 0.12	0.3621	0.8459	0.3917
RankL:Opg	1.00 ± 0.27	1.20 ± 0.19	0.72 ± 0.06	1.48 ± 0.37	1.24 ± 0.19	1.63 ± 0.15	0.0820	0.2180	0.4943

Control diet = Con; Tart cherry diet = TC; Fructooligosaccharide diet = FOS; Data presented as mean ± SEM. *P-values <0.05* are considered statistically different. # indicates TC or FOS vs. Con (*P<0.05)*. $ indicates TC vs. FOS (*P<0.05)*

Table 8

Relative Gene Expression of Inflammatory Markers and Other Mediators in Lamina Propria

	-CD25			+CD25			*P-values*		
	Con	TC	FOS	Con	TC	FOS	CD25	Diet	CD25*Diet
Pro-inflammatory Cytokines									
Tnf-α	1.00 ± 0.14	0.77 ± 0.16	1.01 ± 0.14	1.25 ± 0.28	0.88 ± 0.11	1.46 ± 0.39	0.1541	0.1031	0.9370
IL-17	1.00 ± 0.59	0.91 ± 0.24	0.24 ± 0.05$^{\#\$}$	1.23 ± 0.29	0.62 ± 0.14	0.22 ± 0.11$^{\#\$}$	0.9217	**0.0374**	0.6835
IL-6	1.00 ± 0.30	0.93 ± 0.29	0.41 ± 0.12	1.03 ± 0.26	0.96 ± 0.19	0.80 ± 0.45	0.4196	0.0990	0.9767
Chemokines and adhesion Molecule									
CCR7	1.00 ± 0.15	1.09 ± 0.22	0.99 ± 0.27	0.90 ± 0.22	0.99 ± 0.24	0.93 ± 0.33	0.6685	0.9183	0.9972
CXCL10	1.00 ± 0.20	1.05 ± 0.29	0.73 ± 0.14	1.09 ± 0.38	0.90 ± 0.11	0.72 ± 0.29	0.7313	0.7313	0.8867
CXCR4	1.00 ± 0.16	1.01 ± 0.22	0.53 ± 0.09$^{\#\$}$	0.87 ± 0.24	0.97 ± 0.22	0.39 ± 0.11$^{\#\$}$	0.3497	**0.0176**	0.8498
CXCL12	1.00 ± 0.59	1.22 ± 0.32	0.14 ± 0.03$^{\#\$}$	0.97 ± 0.20	1.18 ± 0.29	0.19 ± 0.09$^{\#\$}$	0.9366	**<.0001**	0.9052
Vcam	1.00 ± 0.59	0.91 ± 0.24	0.24 ± 0.05$^{\#\$}$	0.70 ± 0.18	0.62 ± 0.14	0.22 ± 0.11$^{\#\$}$	0.1178	**0.0002**	0.7206
Anti-inflammatory Cytokines									
Tgf-β	1.00 ± 0.20	0.86 ± 0.30	0.80 ± 0.32	0.48 ± 0.29	0.63 ± 0.18	1.27 ± 0.67	0.4797	0.8130	0.4903
IL 10	1.00 ± 0.36	0.86 ± 0.18	0.80 ± 0.15	0.48 ± 0.08	0.63 ± 0.11	1.14 ± 0.37	0.1366	0.1366	0.1397
Receptor for SCFA									
GPR43	1.00 ± 0.11	1.11 ± 0.19	1.00 ± 0.17	1.12 ± 0.19	0.97 ± 0.11	0.87 ± 0.22	0.7101	0.7324	0.6611

Control diet = Con; Tart cherry diet = TC; Fructooligosaccharide diet = FOS; Data presented as mean ± SEM. *P-values <0.05* are considered statistically different. # indicates TC or FOS vs. Con (*P<0.05)*. $ indicates TC vs. FOS (*P<0.05)*

Figure 3. Effect of dietary supplementation with tart cherry (TC) or fructooligosaccharide (FOS) with or without CD25 antibody (+CD25 or ⁻CD25) on body weight over the course of the 8-week study. Data presented as mean ± SEM. *P-values <0.05* are considered statistically different. # indicates main diet effect of diet, FOS (*P<0.05)* compared to control and tart cherry diets. Dashed line represented + CD25 groups and solid lines represented the – CD25 groups

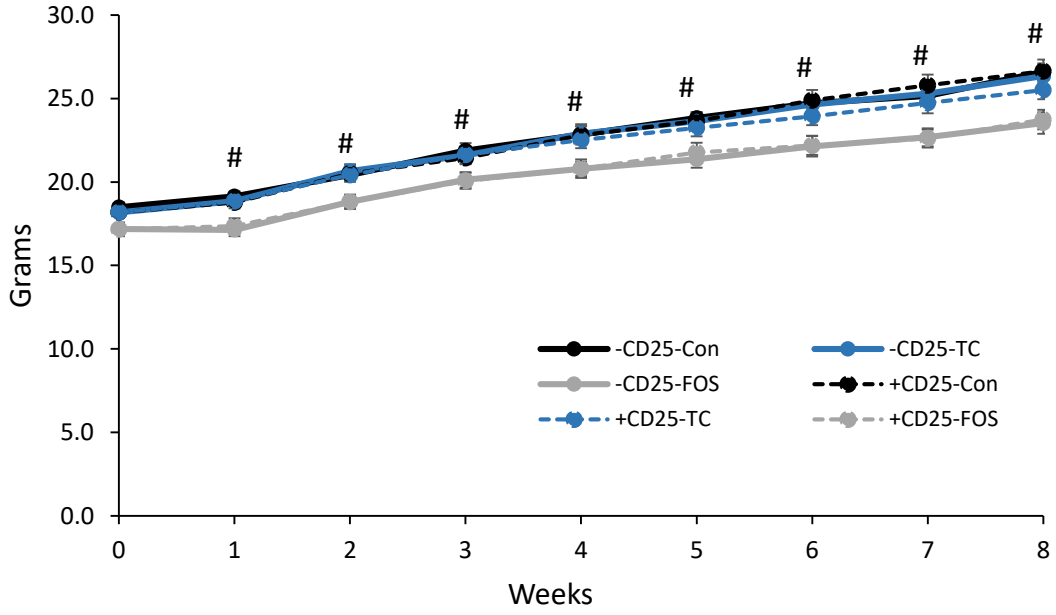
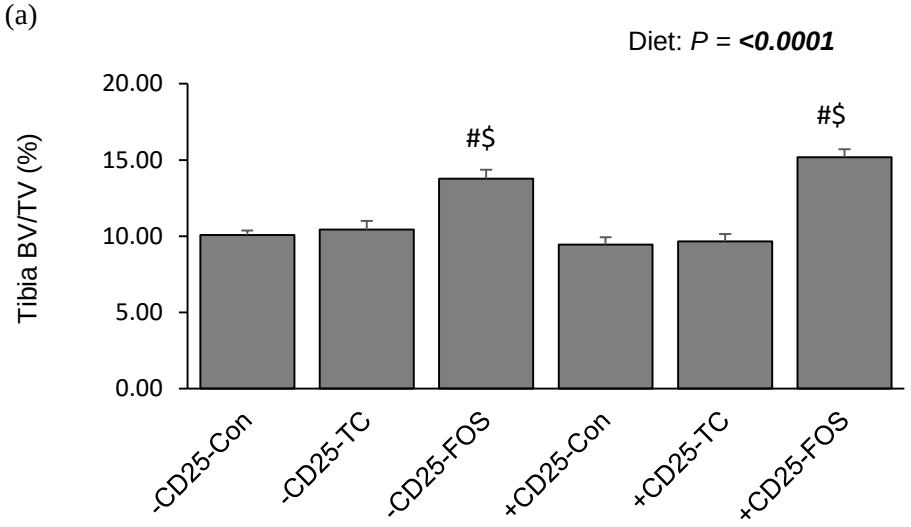


Figure 4. Effect of dietary supplementation with tart cherry (TC) or fructooligosaccharide (FOS) with or without CD25 antibody (+CD25 or ⁻CD25) on BV/TV (%) on (a) tibial bone volume to total volume (BV/TV) and (b) vertebral BV/TV. Data presented as mean ± SEM. *P-values <0.05* are considered statistically different. # indicates main diet effect of FOS (*P<0.05)* compared to control and $ indicates FOS diet (*P<0.05)* compared to tart cherry. N = 8 mice per group;

(a)



(b)

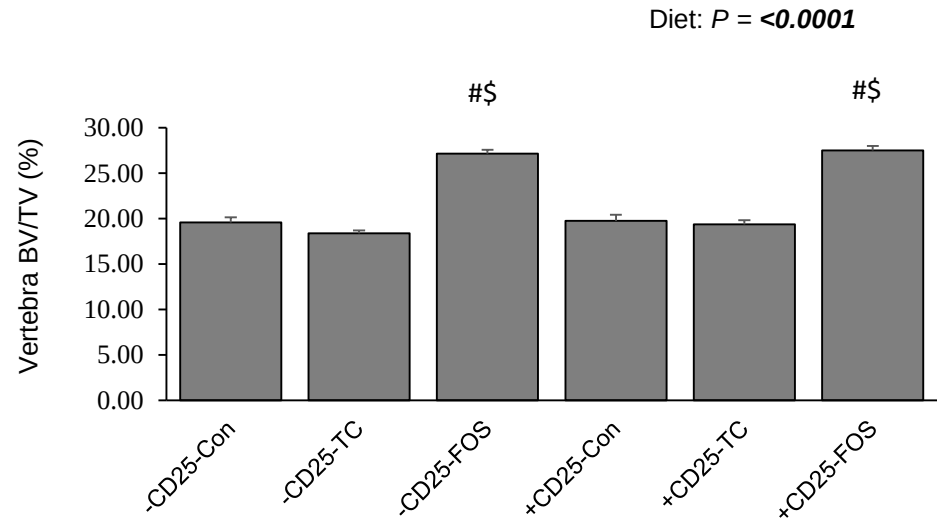
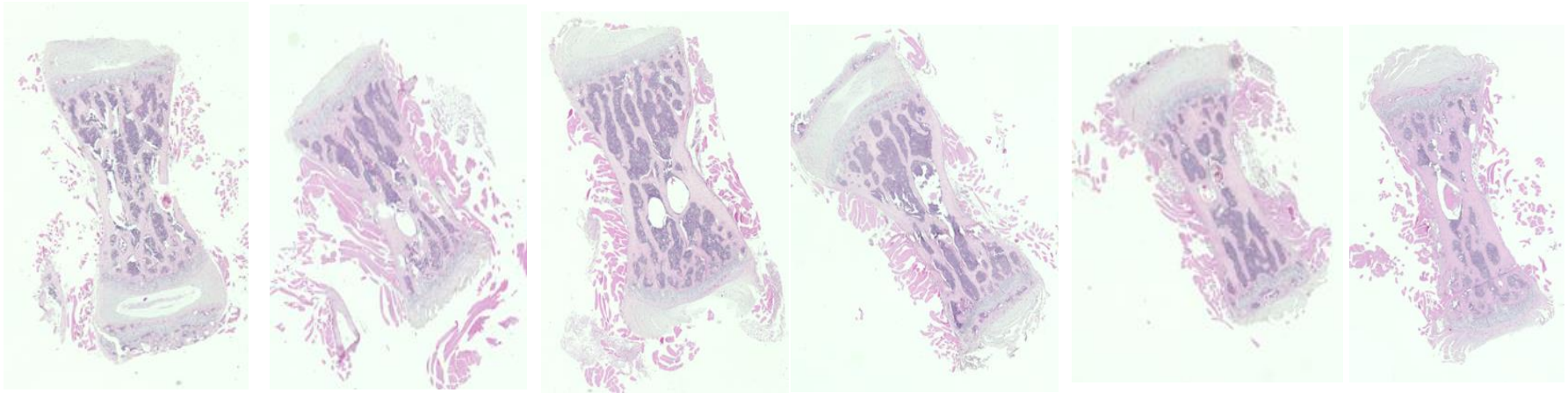


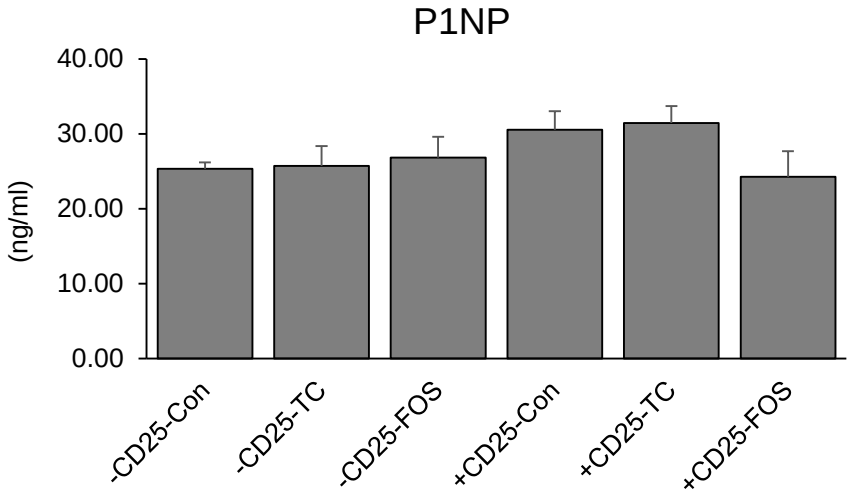
Figure 5. Histological Representative Images of L5 Vertebra.



-CD25-Con -CD25-TC -CD25-FOS +CD25-Con +CD25-TC +CD25-FOS

Figure 6. Effect of dietary supplementation with tart cherry (TC) or fructooligosaccharide (FOS) with or without CD25 antibody +CD25 on serum biomarkers (a) P1NP and (b) CTX-1 after 8 weeks of treatment. Data presented as mean ± SEM. *P-values <0.05* are considered statistically different. # indicates main diet effect of FOS (*P<0.05)* compared to control and $ indicates effect of FOS diet compared to tart cherry (*P<0.05*). Bars that do not share the same superscript letter are statistically different (*P<0.05)* from each other. n = 10 mice per group.

(a)



(b)

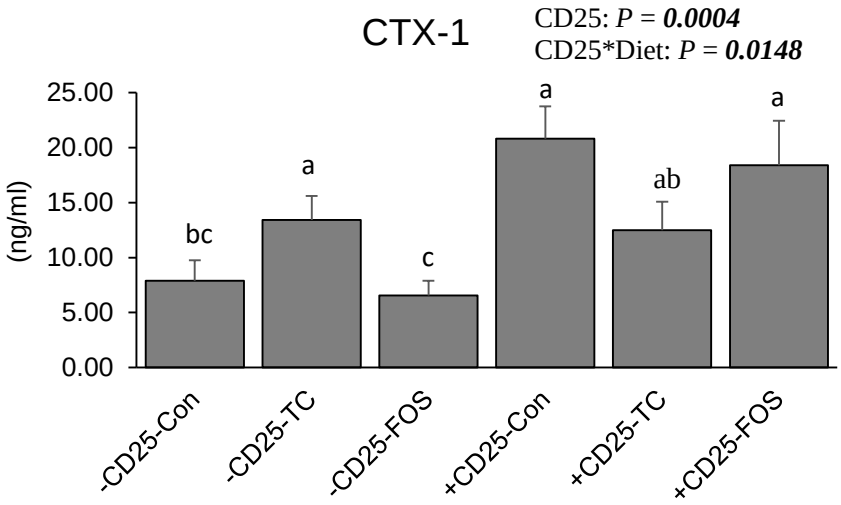


Figure 7. T-lymphocyte count gating strategy by flow cytometry (a) Flow cytometry gating strategy flow chart (b) Lymphocyte gate in the ileum (side scatter Vs. forward scatter) (c) $CD4^+$ T cells Vs. $CD8^+$ T cells population (d) Tregs ($CD4^+CD25^+$ $Foxp3^+$) (e) $CD4^+ROR\gamma^+$T cells (f) Mature Th-17 cells ($CD4^+ROR\gamma^+IL\text{-}17A^+$)

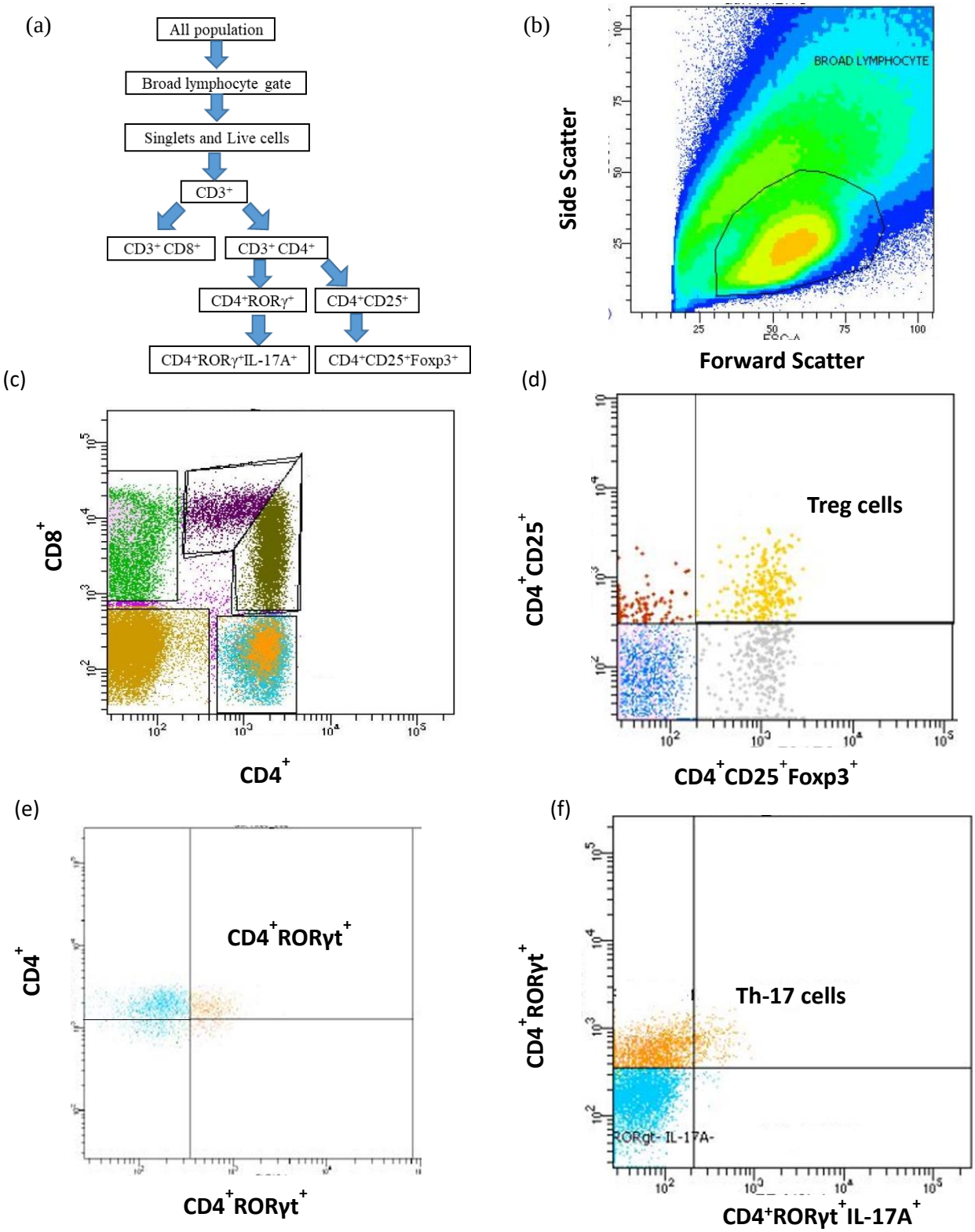


Figure 8. Effect of dietary supplementation with tart cherry (TC) or fructooligosaccharide (FOS) with or without CD25 antibody +CD25 on T-lymphocytes relative count in the ileum over the course of the 8-week study. (a) $CD4^+$cells expressed as percentage from the parent cell population $CD3^+$ (b) Treg ($CD25^+Foxp3^+$) from parent $CD4^+$ (c) Th-17^+ from parent $CD4^+$ (d) Treg and Th-17 cell population ratio. Data presented as mean ± SEM. *P-values <0.05* are considered statistically different. # indicates main diet effect by FOS (*P<0.05)* compared to control and $ indicates for FOS diet compared to tart cherry (*P<0.05*). Bars that do not have the same superscript letter are different (*P<0.05)* from each other.

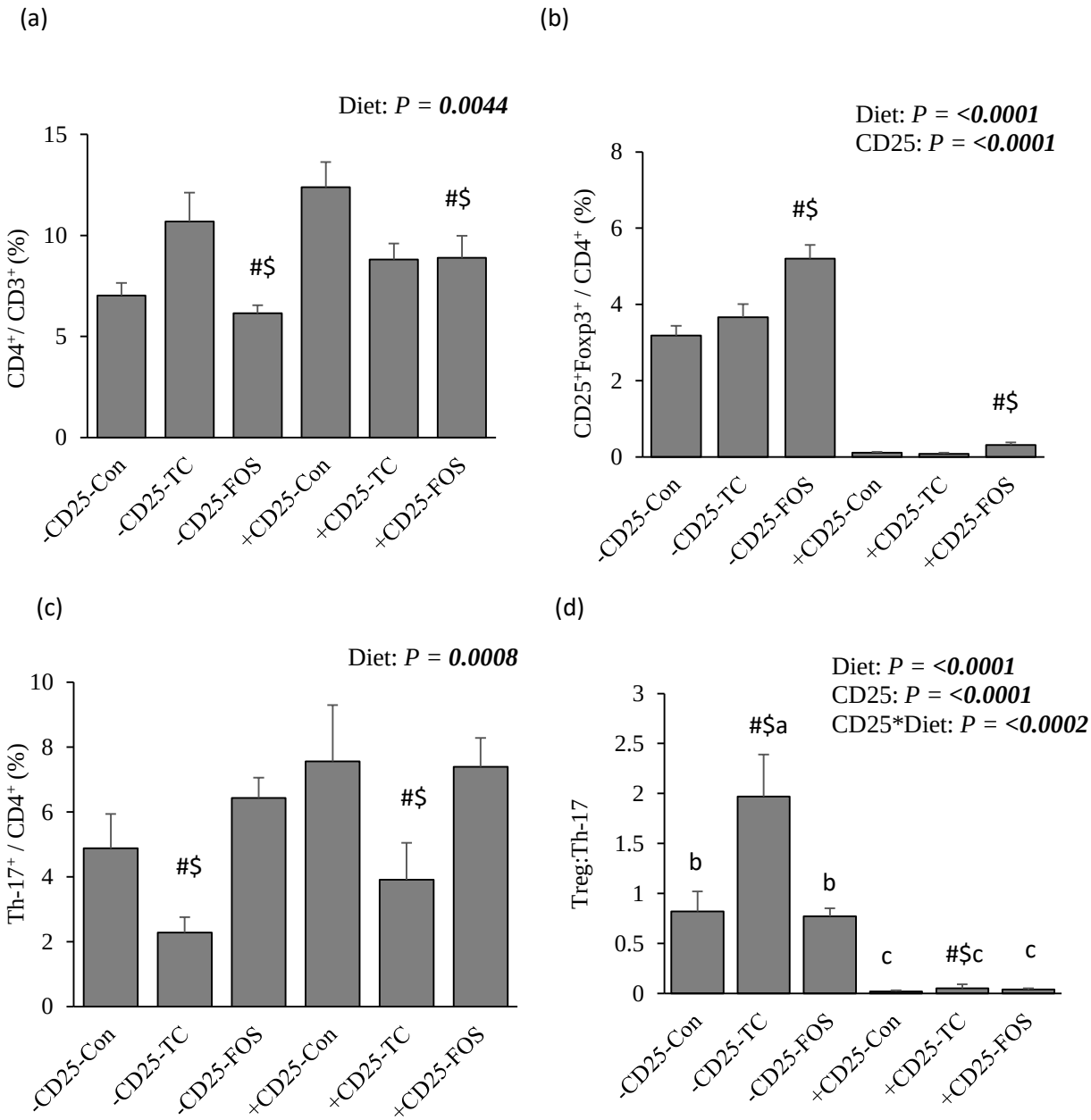


Figure 9. Effect of dietary supplementation with tart cherry (TC) or fructooligosaccharide (FOS) with or without CD25 antibody +CD25 on T-lymphocytes relative count in the bone marrow over the course of the 8-week study. Data presented as mean ± SEM. *P-values <0.05* are considered statistically different. # indicates main diet effect by FOS (*P<0.05)* compared to control and $ indicates for FOS diet compared to tart cherry. A difference between two bars that do not have the same superscript letter is statistically significant (*P<0.05).* (a) $CD4^+$cells expressed as percentage from the parent cell population $CD3^+$ (b) Treg ($CD25^+Foxp3^+$) from parent $CD4^+$ (c) Th-17^+ from parent $CD4^+$ (d) Treg and Th-17 cell population ratio

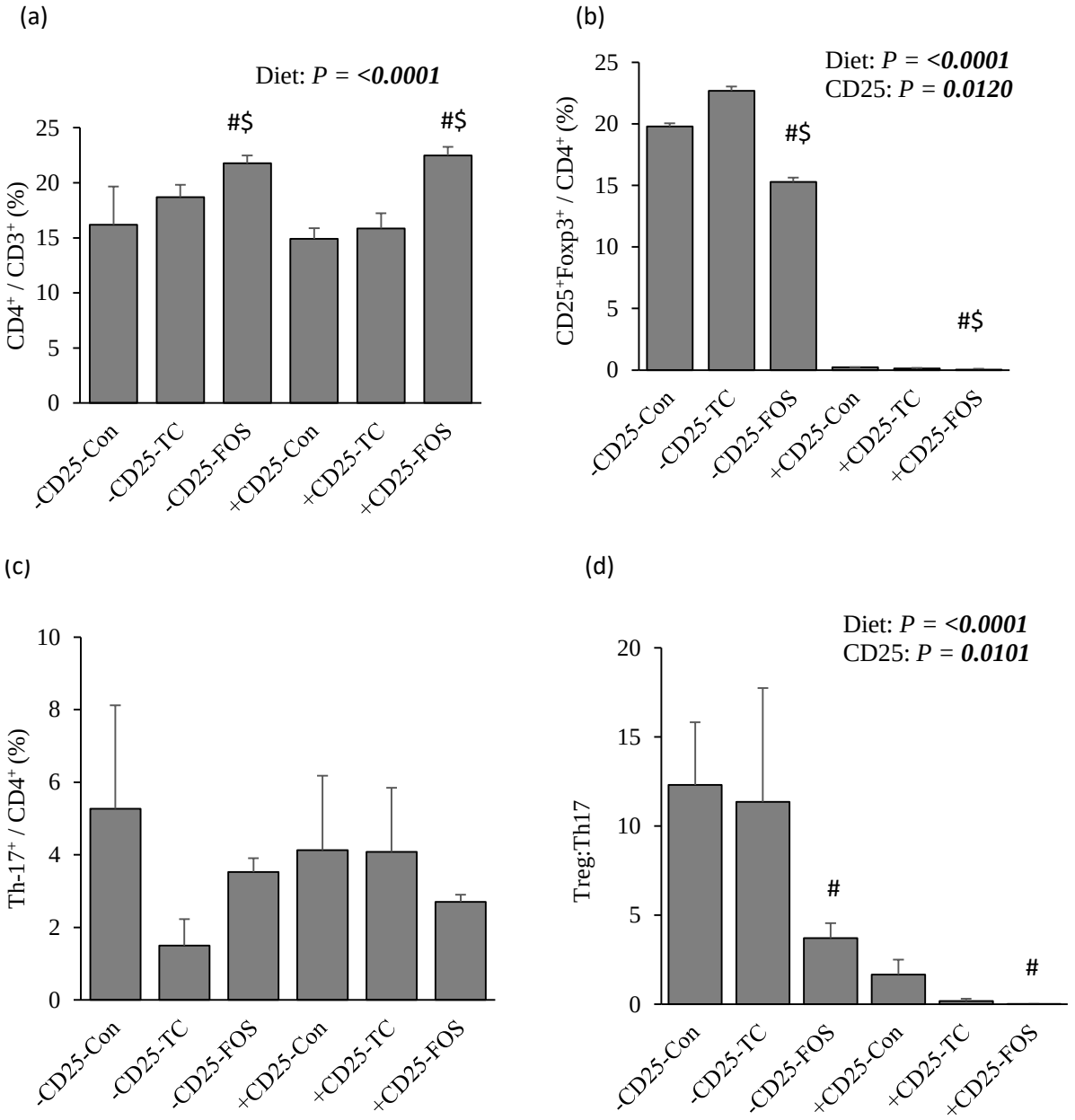
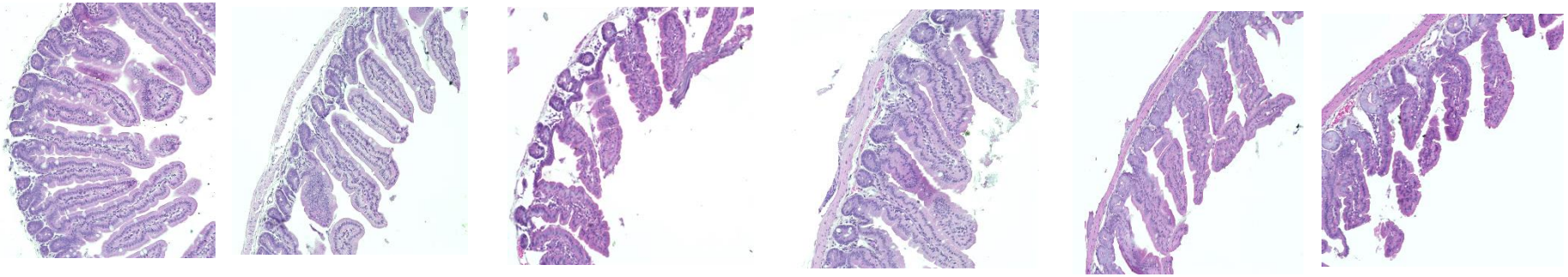


Figure 10. Histological Representative Images of Gut Samples

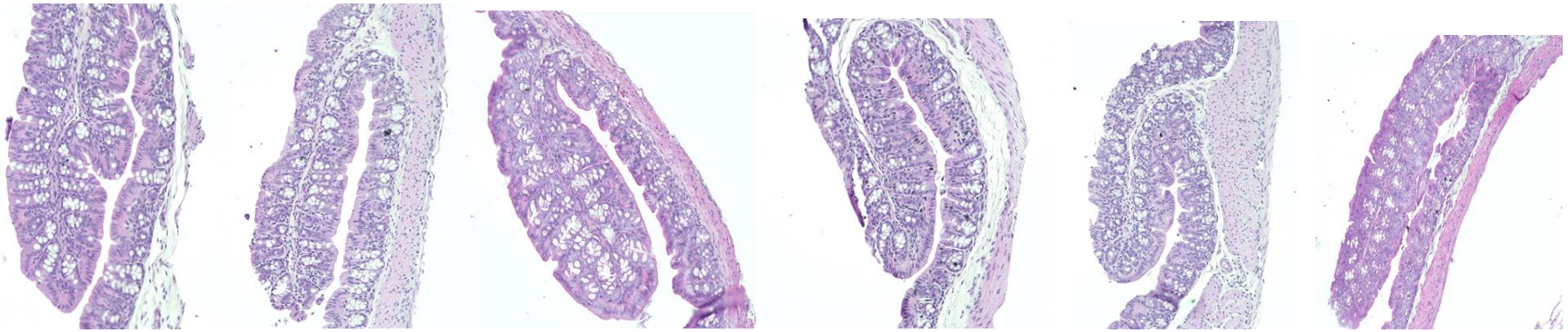
(a) Jejunum

-CD25-Con **-CD25-TC** **-CD25-FOS** **+CD25-Con** **+CD25-TC** **+CD25-FOS**



(b) Colon

-CD25-Con **-CD25-TC** **-CD25-FOS** **+CD25-Con** **+CD25-TC** **+CD25-FOS**



CHAPTER V

DISCUSSION

The purpose of the study was to investigate how two different prebiotics, tart cherry, a multi-component prebiotic, and FOS, a single agent prebiotic affect bone in a mouse model and whether Treg cells are required for this response. Our findings showed that 8 weeks of FOS treatment increased whole body BMC, trabecular bone microarchitecture, and the length of long bones. We provided 10% FOS to young mice that improved vertebral, and tibial BV/TV ratio indicating improved bone structure. However, tart cherry consumption did not alter the bone phenotype. Several clinical and animal studies have revealed that FOS consumption increased bone density [50, 55, 244, 245]. In contrast, other studies in postmenopausal women have not shown benefits of various forms of FOS on BMD [246, 247]. Furthermore, Ohta et al. [248] demonstrated that estrogen deficiency-induced bone loss could be reversed by 6 weeks of consumption of 5% FOS. The results of these ones may differ due to the FOS dose used, the duration of the study, and age of the subjects.

Prebiotics have been shown to increase Treg cells in the mesenteric lymph node and Peyer's patches [249, 250]. Further, prebiotics also indirectly increase dendritic cells in lamina propria which augment differentiation of Treg cells [251, 252]. These Treg cells may affect distal bone by trafficking from gut to bone. As the literature showed, increasing number of dendritic cells correlates with the increasing gut homing receptors [250]. However, it is unknown whether

FOS exhibits bone sparing function via upregulating Treg cells. Probiotics are known to modulate bone metabolism via Treg cells [46, 172, 251]. Tyagi et al. [46] demonstrated that supplementation with a probiotic (i.e., *lactobacillus rhamnosus GG*) or butyrate increased Treg cells in the bone marrow in control. Treg cells in conjunction with $CD8^+$ T cells augmented the expression of the Wnt10b ligand resulting in increased bone mass. A similar effect was also noted by Yu and colleagues [252], in that Treg mediated the effects of intermittent parathyroid hormone on bone. From an immunological standpoint, Tregs can also regulate bone remodeling by inhibiting osteoclastogenesis through a CTLA-4-dependent mechanism or decreasing pro-inflammatory cytokines [253]. In support of this concept, Chen et al. [254] reported that the oligosaccharide lactulose increased the number of Treg cells in the small intestine and decreased pro-inflammatory TNF-α, IL-6, and RANKL in the bone marrow and small intestine. In addition, Tregs can increase osteoblast activity by secreting the anti-inflammatory cytokine, IL-10 or activating the TGF-β mediated Smad pathway and Wnt signaling [46, 255]. Our flow cytometry findings showed that the 8 wks of the FOS supplemented diet increased the percentage of Treg cells in the ileum and improved bone mass. The effects of FOS on bone occurred in the presence and absence of the CD25 antibody, indicating that the mechanism through which FOS affects bone in the young adult mouse differs from that reported with probiotics.

Another possible mechanism through which FOS could favorably affect bone was by increasing SCFA. Earlier studies have shown that FOS increased bone density by enhancing calcium and magnesium absorption in healthy and gastrectomy rats [50, 256, 257]. They found that this increase in Ca and Mg absorption was attributed to the change in pH that occurred in response to increasing SCFA. Later, it was known that FOS also increases SCFA-producing *Bifidobacteria* and SCFA provides an osteogenic effect [55]. In Porwal et al. [55] study, a 1.85g/ kg dose of FOS was given to ovariectomized rats by oral gavage for 12 weeks. The FOS treated rats exhibited an anabolic effect on the trabecular bone with increased SCFA but no alteration was noted on bone catabolic markers and calcium absorption. Likewise, in our study, FOS

increased the fecal SCFAs, acetate, propionate, and n-butyrate. The weight of the cecum in FOS animals also increased in the current study, which may be consistent with increased microbial fermentation activity [192, 258]. Although tart cherry supplementation did not improve bone parameters, it did increase fecal SCFA (i.e. acetic, propionic, and butyric) compared to the control group though to a lesser extent than FOS. Kaur et al. [259] found similar response of tart cherry while assessing the cecal SCFAs. It is known that butyrate increases the Treg population and later Treg-mediated Wnt10b expression is upregulated in pre-osteoblasts [46]. In our study, we observed that the percentage of $CD8^+$ T cells and Treg cells increased in the bone marrow after FOS treatment. However, no alteration was noted in Wnt10b gene expression in response to FOS or tart cherry diet indicating prebiotics might work through increasing SCFA independent of Tregs. Kim et al. [260] showed that sodium butyrate supplementation inhibited osteoclast differentiation by histone deacetylase in a rheumatoid arthritis model. Apart from this, butyrate binds with G-protein coupled receptors GPR-41 and GPR-43, increases glycolysis, manipulates the early phase of osteoclast differentiation, and downregulates the osteoclast function [34]. In our study, we observed an increase in n-butyric acid with FOS and tart cherry and no change with bone resorption serum marker CTX-1, which is an indicator of osteoclastogenesis. In short, diet-induced SCFA may improve bone quality by directly inhibiting osteoclasts or downregulating pro-inflammatory cytokines, or promoting osteoblast differentiation in a Treg cell-independent manner. However, we cannot rule out the role of other gut-derived metabolites such as IGF-1, indole derivatives, serotonin, hydrogen sulfide (H_2S).

Nevertheless, gene expression data from the bone indicate that FOS altered osteoblasts and osteocytes. We observed that osteocyte genes, *Phex*, *Dmp-1*, and *Mepe* were upregulated in the bone tissue with FOS. *Phex* is released by late osteoblasts or early osteocytes, it degrades osteopontin and suppresses fibroblast growth factor (FGF23) [261]. FGF-23 increases urinary phosphate excretion and lowers renal reabsorption of phosphate limiting active vitamin D

synthesis. This further lowers phosphate in the bone disrupting hydroxyapatite formation [262]. *Dmp-1* and *Mepe* are members of sibling proteins that are involved in bone mineralization. *DMP-1* regulates hydroxyapatite nucleation and also influences osteoblast differentiation [263]. *Mepe* is released by mature osteocytes. It inhibits *Phex,* and increases the FGF23 indirectly [264]. *Mepe* inhibits the bone crystal formation as well as reduces the number and activity of osteoclasts [265]. These data indicate that FOS enhanced osteocyte activation. Next, we assessed genes that regulate osteogenesis. We have shown that FOS upregulated *Bmp-2, osterix* and *Col1α1*. These findings indicate that FOS promoted osteoblastic differentiation and activity. Osteocytes regulate osteoblast differentiation in part by the *SOST* gene expression. In the current study, the relative abundance of *SOST* was increased. In response to FOS treatment, indicators of osteoblast activity increased but no change was noted in osteoclast differentiation or activity. Wang et al. [266] and Yan et al. [267] showed that oligosaccharides such as FOS enhanced osteoblastic differentiation and activity in vitro model and in the zebra fish model. Though tart cherry did not exhibit any skeletal response, it upregulated *Phex* and *Mepe* genes compared to the control. In Smith et al. [58] study, in response to tart cherry, the *Bmp-2* gene was upregulated and *Mcsf*, matrix metalloproteinase genes (*Mmp2,8 and 9*) indicators of osteoclast precursor and activity were downregulated. Other prebiotic studies with dried plum showed an increase in *Bmp-4* gene which is a regulator for chondrocyte production as well as a precursor for bone formation and suppressed osteoclast markers such as *Rankl* and *Nfatc1* in response to diet [268, 269]. In summary, based on our study, we might speculate that osteocytes in conjunction with osteoblasts are playing a bigger role in bone in response to the FOS diet.

Previous studies on tart cherry from our lab and others have shown bone protective properties. We reported that supplementing the diet with 5% and 10% tart cherry powder, a product from Van Drunen Farms, increased trabecular bone in the spine and both trabecular and cortical bone in the tibia. With the 10% dose of tart cherry, bone volume exceeded the baseline

control animals indicating potential bone anabolic effect [58]. Our lab has also shown that 10% tart cherry significantly increase trabecular bone to a greater extent than exercise in both young growing animals and old animals (*unpublished data*). In contrast, tart cherry treatment in the current study, a product from Shoreline Fruits from Peterson Farms, did not show any benefits on bone. Another study from our lab showed that tart cherry intake suppresses pro-inflammatory cytokines INF-γ and TNF-α and increased anti-inflammatory cytokine IL-10 in the ileum of ovariectomized mice (*Unpublished data*). Similarly, in our study, tart cherry decreased pro-inflammatory cytokine IL-17 in the lamina propria of young mice. The only notable difference between the previous studies with tart cherry and the current study, was the source of tart cherry. Although the tart cherry used in the present study had some positive findings (e.g., decreased Th17 cells, upregulated *Phex* and *Mepe*, and increased fecal SCFA production), it did not produce a bone phenotype.

Based on these findings, we can conclude that bone was enhanced by FOS supplementation, but not by tart cherry. This response was not Treg mediated as there was no difference in the skeletal response to FOS exhibited by animals treated with CD25 antibody. FOS increased SCFAs production, and increased osteoblast activity and osteocyte activation at the transcriptional level. Future studies are needed to determine whether FOS is altering bone via gut microbiota-derived SCFA, how bone is affected over time, and the role of osteoblasts and osteocytes in this process. In our study, we observed FOS mediated increase in bone in young healthy animals. It remains to be determined whether these benefits occur in the case of aging, estrogen deficiency and pathological condition such as IBD, where the gut bone axis is compromised.

REFERENCES

1. Harvey, N., E. Dennison, and C. Cooper, *Osteoporosis: impact on health and economics.* Nature Reviews Rheumatology, 2010. **6**(2): p. 99-105.
2. Liu, J., et al., *State of the art in osteoporosis risk assessment and treatment.* Journal of Endocrinological Investigation, 2019. **42**(10): p. 1149-1164.
3. Ensrud, K.E., *Epidemiology of fracture risk with advancing age.* Journals of Gerontology Series A: Biomedical Sciences and Medical Sciences, 2013. **68**(10): p. 1236-1242.
4. Pisani, P., et al., *Major osteoporotic fragility fractures: Risk factor updates and societal impact.* World J Orthop, 2016. **7**(3): p. 171-81.
5. Johnell, O. and J.A. Kanis, *An estimate of the worldwide prevalence and disability associated with osteoporotic fractures.* Osteoporos Int, 2006. **17**(12): p. 1726-33.
6. Cooper, C., G. Campion, and L.J. Melton, 3rd, *Hip fractures in the elderly: a world-wide projection.* Osteoporos Int, 1992. **2**(6): p. 285-9.
7. Cummings, S.R. and L.J. Melton, *Epidemiology and outcomes of osteoporotic fractures.* Lancet, 2002. **359**(9319): p. 1761-7.
8. National Osteoporosis Foundation. *What is osteoporosis and what causes it?* 2021; Available from: https://www.nof.org/patients/what-is-osteoporosis/.
9. Sozen, T., L. Ozisik, and N.C. Basaran, *An overview and management of osteoporosis.* Eur J Rheumatol, 2017. **4**(1): p. 46-56.
10. Warriner, A.H., *Which Fractures Are Most Attributable to Osteoporosis?* Journal of Clinical Epidemiology, 2011: p. 46-53.
11. Black, D.M. and C.J. Rosen, *Postmenopausal osteoporosis.* New England Journal of Medicine, 2016. **374**(3): p. 254-262.
12. Pacifici, R., *Estrogen, cytokines, and pathogenesis of postmenopausal osteoporosis.* J Bone Miner Res, 1996. **11**(8): p. 1043-51.
13. Johnston, C.B. and M. Dagar, *Osteoporosis in older adults.* Medical Clinics, 2020. **104**(5): p. 873-884.
14. Rizzoli, R., et al., *Adverse reactions and drug–drug interactions in the management of women with postmenopausal osteoporosis.* Calcified tissue international, 2011. **89**(2): p. 91-104.
15. Lecka-Czernik, B., *PPARs and bone metabolism*. 2006, Hindawi.
16. Demontiero, O., C. Vidal, and G. Duque, *Aging and bone loss: new insights for the clinician.* Therapeutic advances in musculoskeletal disease, 2012. **4**(2): p. 61-76.
17. Tella, S.H. and J.C. Gallagher, *Prevention and treatment of postmenopausal osteoporosis.* The Journal of steroid biochemistry and molecular biology, 2014. **142**: p. 155-170.
18. Pope, T., et al., *Musculoskeletal imaging*. 2014: Elsevier Health Sciences.
19. Riggs, B.L., S. Khosla, and L.J. Melton III, *Sex steroids and the construction and conservation of the adult skeleton.* Endocrine reviews, 2002. **23**(3): p. 279-302.

20. Ji, M.-X. and Q. Yu, *Primary osteoporosis in postmenopausal women.* Chronic diseases and translational medicine, 2015. **1**(01): p. 9-13.
21. Manolagas, S.C., *From estrogen-centric to aging and oxidative stress: a revised perspective of the pathogenesis of osteoporosis.* Endocr Rev, 2010. **31**(3): p. 266-300.
22. Bachrach, L.K., *Acquisition of optimal bone mass in childhood and adolescence.* Trends in Endocrinology & Metabolism, 2001. **12**(1): p. 22-28.
23. Levine, M.A., *Assessing bone health in children and adolescents.* Indian journal of endocrinology and metabolism, 2012. **16**(Suppl 2): p. S205.
24. Meléndez-Ortega, A., *Osteoporosis, falls and exercise.* European Review of Aging and Physical Activity, 2007. **4**(2): p. 61-70.
25. Bonjour, J.-P., et al., *The importance and relevance of peak bone mass in the prevalence of osteoporosis.* Salud publica de Mexico, 2009. **51**: p. s5-s17.
26. Seeman, E., *Bone modeling and remodeling.* Critical Reviews™ in Eukaryotic Gene Expression, 2009. **19**(3).
27. Langdahl, B., S. Ferrari, and D.W. Dempster, *Bone modeling and remodeling: potential as therapeutic targets for the treatment of osteoporosis.* Therapeutic advances in musculoskeletal disease, 2016. **8**(6): p. 225-235.
28. Clarke, B., *Normal bone anatomy and physiology.* Clinical journal of the American Society of Nephrology, 2008. **3**(Supplement 3): p. S131-S139.
29. Hadjidakis, D.J. and I.I. Androulakis, *Bone remodeling.* Annals of the New York Academy of Sciences, 2006. **1092**(1): p. 385-396.
30. Zaiss, M.M., et al., *The gut-bone axis: how bacterial metabolites bridge the distance.* The Journal of clinical investigation, 2019. **129**(8): p. 3018-3028.
31. Collins, F.L., et al., *Immunology of gut-bone signaling,* in *Understanding the Gut-Bone Signaling Axis*. 2017, Springer. p. 59-94.
32. Mitsuoka, T., *Intestinal flora and human health.* Asia Pac J Clin Nutr, 1996. **5**(1): p. 2-9.
33. Silva, Y.P., A. Bernardi, and R.L. Frozza, *The role of short-chain fatty acids from gut microbiota in gut-brain communication.* Frontiers in endocrinology, 2020. **11**: p. 25.
34. Lucas, S., et al., *Short-chain fatty acids regulate systemic bone mass and protect from pathological bone loss.* Nature communications, 2018. **9**(1): p. 1-10.
35. Cummings, J.H., et al., *Short chain fatty acids in human large intestine, portal, hepatic and venous blood.* Gut, 1987. **28**(10): p. 1221-1227.
36. Yan, J., et al., *Gut microbiota induce IGF-1 and promote bone formation and growth.* Proceedings of the National Academy of Sciences, 2016. **113**(47): p. E7554-E7563.
37. Crane, J.L., et al., *IGF-1 signaling is essential for differentiation of mesenchymal stem cells for peak bone mass.* Bone research, 2013. **1**(1): p. 186-194.
38. Powell, D.N., et al., *Indoles from the commensal microbiota act via the AHR and IL-10 to tune the cellular composition of the colonic epithelium during aging.* Proceedings of the National Academy of Sciences, 2020. **117**(35): p. 21519-21526.
39. Jha, S., et al., *Trends in Media Reports, Oral Bisphosphonate Prescriptions, and Hip Fractures 1996-2012: An Ecological Analysis.* J Bone Miner Res, 2015. **30**(12): p. 2179-87.
40. Kim, C.H., J. Park, and M. Kim, *Gut microbiota-derived short-chain Fatty acids, T cells, and inflammation.* Immune Netw, 2014. **14**(6): p. 277-88.
41. Ivanov, I.I., et al., *The orphan nuclear receptor RORγt directs the differentiation program of proinflammatory IL-17+ T helper cells.* Cell, 2006. **126**(6): p. 1121-1133.
42. Kelchtermans, H., et al., *Activated CD4+CD25+ regulatory T cells inhibit osteoclastogenesis and collagen-induced arthritis.* Ann Rheum Dis, 2009. **68**(5): p. 744-50.

43. Kim, Y.G., et al., *Human CD4+CD25+ regulatory T cells inhibit the differentiation of osteoclasts from peripheral blood mononuclear cells.* Biochem Biophys Res Commun, 2007. **357**(4): p. 1046-52.
44. Gattinoni, L., Y. Ji, and N.P. Restifo, *Wnt/β-Catenin Signaling in T-Cell Immunity and Cancer ImmunotherapyWnt/β-Catenin in T-Cell Biology and Tumor Immunotherapy.* Clinical Cancer Research, 2010. **16**(19): p. 4695-4701.
45. Salzman, N.H., *Paneth cell defensins and the regulation of the microbiome: detente at mucosal surfaces.* Gut microbes, 2010. **1**(6): p. 401-406.
46. Tyagi, A.M., et al., *The Microbial Metabolite Butyrate Stimulates Bone Formation via T Regulatory Cell-Mediated Regulation of WNT10B Expression.* Immunity, 2018. **49**(6): p. 1116-1131 e7.
47. Schepper, J.D., et al., *Probiotic Lactobacillus reuteri prevents postantibiotic bone loss by reducing intestinal dysbiosis and preventing barrier disruption.* Journal of Bone and Mineral Research, 2019. **34**(4): p. 681-698.
48. Li, J.-Y., et al., *Sex steroid deficiency–associated bone loss is microbiota dependent and prevented by probiotics.* The Journal of clinical investigation, 2016. **126**(6): p. 2049-2063.
49. Smith, B.J., et al., *Dried Plum's Polyphenolic Compounds and Carbohydrates Contribute to Its Osteoprotective Effects and Exhibit Prebiotic Activity in Estrogen Deficient C57BL/6 Mice.* Nutrients, 2022. **14**(9): p. 1685.
50. Takahara, S., et al., *Fructooligosaccharide consumption enhances femoral bone volume and mineral concentrations in rats.* The Journal of nutrition, 2000. **130**(7): p. 1792-1795.
51. Lobo, A.R., C. Colli, and T.M. Filisetti, *Fructooligosaccharides improve bone mass and biomechanical properties in rats.* Nutrition research, 2006. **26**(8): p. 413-420.
52. Folwarczna, J., et al., *Effects of natural phenolic acids on the skeletal system of ovariectomized rats.* Planta medica, 2009. **75**(15): p. 1567-1572.
53. Chen, J.R., et al., *Diet-derived phenolic acids regulate osteoblast and adipocyte lineage commitment and differentiation in young mice.* Journal of Bone and Mineral Research, 2014. **29**(5): p. 1043-1053.
54. McCabe, L., R.A. Britton, and N. Parameswaran, *Prebiotic and probiotic regulation of bone health: role of the intestine and its microbiome.* Current osteoporosis reports, 2015. **13**(6): p. 363-371.
55. Porwal, K., et al., *A prebiotic, short-chain fructo-oligosaccharides promotes peak bone mass and maintains bone mass in ovariectomized rats by an osteogenic mechanism.* Biomedicine & Pharmacotherapy, 2020. **129**: p. 110448.
56. Wikipedia. *Prunus cerasus*. 2021; Available from: https://en.wikipedia.org/wiki/Prunus_cerasus.
57. Agricultural Marketing Resource Center. *Cherries*. 2018; Available from: https://www.agmrc.org/commodities-products/fruits/cherries.
58. Smith, B.J., et al., *Montmorency tart cherry protects against age-related bone loss in female C57BL/6 mice and demonstrates some anabolic effects.* European Journal of Nutrition, 2019. **58**(8): p. 3035-3046.
59. Chongwatpol, P., et al., *The efficacy of tart cherry supplementation in the prevention of age-related bone loss in C57BL/6 mice.* The FASEB Journal, 2013.
60. Dodier, T., et al., *U.S. Montmorency Tart Cherry Juice Decreases Bone Resorption in Women Aged 65–80 Years.* Nutrients, 2021. **13**(2): p. 544.
61. Mayta-Apaza, A.C., et al., *Impact of tart cherries polyphenols on the human gut microbiota and phenolic metabolites in vitro and in vivo.* J Nutr Biochem, 2018. **59**: p. 160-172.

62. Cherbut, C., C. Michel, and G. Lecannu, *The prebiotic characteristics of fructooligosaccharides are necessary for reduction of TNBS-induced colitis in rats.* The Journal of nutrition, 2003. **133**(1): p. 21-27.
63. Campos, D., et al., *Prebiotic effects of yacon (Smallanthus sonchifolius Poepp. & Endl), a source of fructooligosaccharides and phenolic compounds with antioxidant activity.* Food chemistry, 2012. **135**(3): p. 1592-1599.
64. Alves-Santos, A.M., et al., *Prebiotic effect of dietary polyphenols: A systematic review.* Journal of Functional Foods, 2020. **74**: p. 104169.
65. Moon, N., et al., *Tart cherry prevents bone loss through inhibition of RANKL in TNF-overexpressing mice.* Nutrients, 2018. **11**(1): p. 63.
66. Batlle, E. and J. Massague, *Transforming Growth Factor-beta Signaling in Immunity and Cancer.* Immunity, 2019. **50**(4): p. 924-940.
67. Chen, J.R., et al., *Dietary-induced serum phenolic acids promote bone growth via p38 MAPK/β-catenin canonical Wnt signaling.* Journal of Bone and Mineral Research, 2010. **25**(11): p. 2399-2411.
68. Weaver, C.M., et al., *Galactooligosaccharides improve mineral absorption and bone properties in growing rats through gut fermentation.* Journal of agricultural and food chemistry, 2011. **59**(12): p. 6501-6510.
69. Curtis, E.M., N.C. Harvey, and C. Cooper, *The burden of osteoporosis*, in *Osteoporosis: a lifecourse epidemiology approach to skeletal health*. 2018, CRC Press. p. 1-20.
70. Health, U.D.o. and H. Services, *Bone health and osteoporosis: a report of the Surgeon General.* Rockville, MD: US Department of Health and Human Services, Office of the Surgeon General, 2004. **87**.
71. Searing, L. *The Big Number: 14.1 million Americans 50 and older suffer osteoporosis*. The Washington Post April 12, 2021; Available from: https://www.washingtonpost.com/health/the-big-number-141-million-americans-50-and-older-suffer-osteoporosis/2021/04/09/7ae5f382-9885-11eb-962b-78c1d8228819_story.html.
72. Cooper, C., G. Campion, and L. Melton, 3rd, *Hip fractures in the elderly: a world-wide projection.* Osteoporosis international, 1992. **2**(6): p. 285-289.
73. Cummings, S., *Melton lJ.* Epidemiology and outcomes of osteoporotic fractures. lancet, 2002. **359**: p. 1761.
74. Lane, N.E., *Epidemiology, etiology, and diagnosis of osteoporosis.* American journal of obstetrics and gynecology, 2006. **194**(2): p. S3-S11.
75. Sözen, T., L. Özışık, and N.Ç. Başaran, *An overview and management of osteoporosis.* European journal of rheumatology, 2017. **4**(1): p. 46.
76. on Osteoporosis, N.C.D.P. and D. Prevention, *Osteoporosis prevention, diagnosis, and therapy.* Jama, 2001. **285**(6): p. 785-795.
77. Banu, J., E. Varela, and G. Fernandes, *Alternative therapies for the prevention and treatment of osteoporosis.* Nutrition reviews, 2012. **70**(1): p. 22-40.
78. Wang, T., et al., *Therapeutic potential and outlook of alternative medicine for osteoporosis.* Current drug targets, 2017. **18**(9): p. 1051-1068.
79. Compston, J., *Bone density: BMC, BMD, or corrected BMD?* Bone, 1995. **1**(16): p. 5-7.
80. Bijelic, R., S. Milicevic, and J. Balaban, *The influence of non-preventable risk factors on the development of osteoporosis in postmenopausal women.* Materia socio-medica, 2019. **31**(1): p. 62.
81. Brown, L.B., et al., *Assessment of sex-specific genetic and environmental effects on bone mineral density.* Genetic Epidemiology: The Official Publication of the International Genetic Epidemiology Society, 2004. **27**(2): p. 153-161.
82. Brown, L.B., et al., *Genetic and environmental influences on bone mineral density in pre- and post-menopausal women.* Osteoporosis international, 2005. **16**(12): p. 1849-1856.

83. Alswat, K.A., *Gender disparities in osteoporosis.* Journal of clinical medicine research, 2017. **9**(5): p. 382.
84. Rozenberg, S., et al., *How to manage osteoporosis before the age of 50.* Maturitas, 2020. **138**: p. 14-25.
85. Siris, E.S., et al., *Identification and fracture outcomes of undiagnosed low bone mineral density in postmenopausal women: results from the National Osteoporosis Risk Assessment.* Jama, 2001. **286**(22): p. 2815-2822.
86. Lips, P., *Epidemiology and predictors of fractures associated with osteoporosis.* The American journal of medicine, 1997. **103**(2): p. S3-S11.
87. Kanis, J.A., et al., *The diagnosis of osteoporosis.* Journal of bone and mineral research, 1994. **9**(8): p. 1137-1141.
88. Feldstein, A., et al., *Bone mineral density measurement and treatment for osteoporosis in older individuals with fractures: a gap in evidence-based practice guideline implementation.* Archives of internal medicine, 2003. **163**(18): p. 2165-2172.
89. Bailey, C. and K. Brooke-Wavell, *Exercise for optimising peak bone mass in women: Postgraduate Symposium.* Proceedings of the Nutrition Society, 2008. **67**(1): p. 9-18.
90. Mazziotti, G., E. Canalis, and A. Giustina, *Drug-induced osteoporosis: mechanisms and clinical implications.* The American journal of medicine, 2010. **123**(10): p. 877-884.
91. Whisner, C.M. and L.F. Castillo, *Prebiotics, bone and mineral metabolism.* Calcified Tissue International, 2018. **102**(4): p. 443-479.
92. Collins, F.L., et al., *The potential of probiotics as a therapy for osteoporosis.* Microbiology spectrum, 2017. **5**(4): p. 5.4. 20.
93. Pfeifer, M., et al., *Musculoskeletal rehabilitation in osteoporosis: a review.* Journal of bone and mineral research, 2004. **19**(8): p. 1208-1214.
94. Hsieh, Y.F. and C.H. Turner, *Effects of loading frequency on mechanically induced bone formation.* Journal of Bone and Mineral Research, 2001. **16**(5): p. 918-924.
95. Taichman, R.S., *Blood and bone: two tissues whose fates are intertwined to create the hematopoietic stem-cell niche.* Blood, 2005. **105**(7): p. 2631-2639.
96. Davison, K.S., et al. *Bone strength: the whole is greater than the sum of its parts*. in *Seminars in arthritis and rheumatism*. 2006. Elsevier.
97. Hart, N.H., et al., *Biological basis of bone strength: anatomy, physiology and measurement.* Journal of musculoskeletal & neuronal interactions, 2020. **20**(3): p. 347.
98. Eriksen, E.F., D.W. Axelrod, and F. Melsen, *Bone histomorphometry*. 1994: Raven Press.
99. Dallas, S.L., M. Prideaux, and L.F. Bonewald, *The osteocyte: an endocrine cell… and more.* Endocrine reviews, 2013. **34**(5): p. 658-690.
100. Allen, M.R. and D.B. Burr, *Bone modeling and remodeling*, in *Basic and applied bone biology*. 2014, Elsevier. p. 75-90.
101. Buckwalter, J., et al., *Bone biology.* J Bone Joint Surg Am, 1995. **77**(8): p. 1256-1275.
102. Boyce, B.F. and L. Xing, *Functions of RANKL/RANK/OPG in bone modeling and remodeling.* Archives of biochemistry and biophysics, 2008. **473**(2): p. 139-146.
103. Peruzzi, B. and A. Teti, *The physiology and pathophysiology of the osteoclast.* Clinical Reviews in Bone and Mineral Metabolism, 2012. **10**(2): p. 71-97.
104. Narducci, P., R. Bareggi, and V. Nicolin, *Receptor Activator for Nuclear Factor kappa B Ligand (RANKL) as an osteoimmune key regulator in bone physiology and pathology.* Acta histochemica, 2011. **113**(2): p. 73-81.
105. Bossard, M.J., et al., *Proteolytic activity of human osteoclast cathepsin K. Expression, purification, activation, and substrate identification.* J Biol Chem, 1996. **271**(21): p. 12517-24.
106. Everts, V., et al., *Degradation of collagen in the bone-resorbing compartment underlying the osteoclast involves both cysteine-proteinases and matrix metalloproteinases.* Journal of cellular physiology, 1992. **150**(2): p. 221-231.

107. Boyce, B.F. and L. Xing, *The Rankl/Rank/Opg pathway.* Current osteoporosis reports, 2007. **5**(3): p. 98-104.
108. Everts, V., et al., *The bone lining cell: its role in cleaning Howship's lacunae and initiating bone formation.* Journal of Bone and Mineral Research, 2002. **17**(1): p. 77-90.
109. Bonewald, L. and G. Mundy, *Role of transforming growth factor-beta in bone remodeling.* Clinical orthopaedics and related research, 1990(250): p. 261-276.
110. Durant, D., R. Pereira, and E. Canalis, *Overexpression of insulin-like growth factor binding protein-5 decreases osteoblastic function in vitro.* Bone, 2004. **35**(6): p. 1256-1262.
111. Locklin, R.M., R.O. Oreffo, and J.T. Triffitt, *Effects of TGFβ and bFGF on the differentiation of human bone marrow stromal fibroblasts.* Cell biology international, 1999. **23**(3): p. 185-194.
112. Frost, H.M., *Cybernetic aspects of bone modeling and remodeling, with special reference to osteoporosis and whole-bone strength.* American Journal of Human Biology: The Official Journal of the Human Biology Association, 2001. **13**(2): p. 235-248.
113. Salhotra, A., et al., *Mechanisms of bone development and repair.* Nature reviews Molecular cell biology, 2020. **21**(11): p. 696-711.
114. Hojo, H., S. Ohba, and U.I. Chung, *Signaling pathways regulating the specification and differentiation of the osteoblast lineage.* Regen Ther, 2015. **1**: p. 57-62.
115. Stein, G.S. and J.B. Lian, *Molecular mechanisms mediating proliferation/differentiation interrelationships during progressive development of the osteoblast phenotype.* Endocr Rev, 1993. **14**(4): p. 424-42.
116. Lin, X., et al., *The Bone Extracellular Matrix in Bone Formation and Regeneration.* Front Pharmacol, 2020. **11**: p. 757.
117. Cremers, S., P. Garnero, and M.J. Seibel, *Biochemical markers of bone metabolism*, in *Principles of Bone Biology*. 2008, Elsevier. p. 1857-1881.
118. Zurick, K.M., C. Qin, and M.T. Bernards, *Mineralization induction effects of osteopontin, bone sialoprotein, and dentin phosphoprotein on a biomimetic collagen substrate.* Journal of biomedical materials research Part A, 2013. **101**(6): p. 1571-1581.
119. Baht, G.S., G.K. Hunter, and H.A. Goldberg, *Bone sialoprotein–collagen interaction promotes hydroxyapatite nucleation.* Matrix Biology, 2008. **27**(7): p. 600-608.
120. Gordon, J.A., et al., *Bone sialoprotein expression enhances osteoblast differentiation and matrix mineralization in vitro.* Bone, 2007. **41**(3): p. 462-473.
121. Jilka, R.L., *The relevance of mouse models for investigating age-related bone loss in humans.* Journals of Gerontology Series A: Biomedical Sciences and Medical Sciences, 2013. **68**(10): p. 1209-1217.
122. Nakashima, T., et al., *Evidence for osteocyte regulation of bone homeostasis through RANKL expression.* Nature medicine, 2011. **17**(10): p. 1231-1234.
123. Winkler, D.G., et al., *Osteocyte control of bone formation via sclerostin, a novel BMP antagonist.* The EMBO journal, 2003. **22**(23): p. 6267-6276.
124. Yuan, B., et al., *Aberrant Phex function in osteoblasts and osteocytes alone underlies murine X-linked hypophosphatemia.* The Journal of clinical investigation, 2008. **118**(2): p. 722-734.
125. Addison, W.N., et al., *MEPE-ASARM peptides control extracellular matrix mineralization by binding to hydroxyapatite: an inhibition regulated by PHEX cleavage of ASARM.* Journal of Bone and Mineral Research, 2008. **23**(10): p. 1638-1649.
126. Feng, J.Q., et al., *Loss of DMP1 causes rickets and osteomalacia and identifies a role for osteocytes in mineral metabolism.* Nature genetics, 2006. **38**(11): p. 1310-1315.
127. Takayama, Y. and K. Mizumachi, *Effect of lactoferrin-embedded collagen membrane on osteogenic differentiation of human osteoblast-like cells.* Journal of bioscience and bioengineering, 2009. **107**(2): p. 191-195.

128. Rios-Arce, N.D., et al., *Epithelial Barrier Function in Gut-Bone Signaling.* Adv Exp Med Biol, 2017. **1033**: p. 151-183.
129. Mowat, A.M., *Anatomical basis of tolerance and immunity to intestinal antigens.* Nature Reviews Immunology, 2003. **3**(4): p. 331-341.
130. Wolff, M.J., et al., *TH17, TH22 and Treg cells are enriched in the healthy human cecum.* PLoS One, 2012. **7**(7): p. e41373.
131. Ueda, Y., et al., *Commensal microbiota induce LPS hyporesponsiveness in colonic macrophages via the production of IL-10.* Int Immunol, 2010. **22**(12): p. 953-62.
132. Komano, H., et al., *Homeostatic regulation of intestinal epithelia by intraepithelial gamma delta T cells.* Proceedings of the National Academy of Sciences, 1995. **92**(13): p. 6147-6151.
133. Mowat, A.M. and W.W. Agace, *Regional specialization within the intestinal immune system.* Nature Reviews Immunology, 2014. **14**(10): p. 667-685.
134. Johansson, M.E., et al., *Composition and functional role of the mucus layers in the intestine.* Cellular and molecular life sciences, 2011. **68**(22): p. 3635-3641.
135. Hatayama, H., et al., *The short chain fatty acid, butyrate, stimulates MUC2 mucin production in the human colon cancer cell line, LS174T.* Biochemical and biophysical research communications, 2007. **356**(3): p. 599-603.
136. Mangan, P.R., et al., *Transforming growth factor-β induces development of the TH17 lineage.* Nature, 2006. **441**(7090): p. 231-234.
137. Korn, T., et al., *IL-17 and Th17 Cells.* Annual review of immunology, 2009. **27**: p. 485-517.
138. Wu, W., et al., *Microbiota-specific Th17 cells: Yin and Yang in regulation of inflammatory bowel disease.* Inflammatory bowel diseases, 2016. **22**(6): p. 1473-1482.
139. Chen, B., et al., *Adhesive bacteria in the terminal ileum of children correlates with increasing Th17 cell activation.* Frontiers in Pharmacology, 2020. **11**: p. 588560.
140. Wei, Y., et al., *High salt diet stimulates gut Th17 response and exacerbates TNBS-induced colitis in mice.* Oncotarget, 2017. **8**(1): p. 70.
141. Ivanov, I.I., et al., *Induction of intestinal Th17 cells by segmented filamentous bacteria.* Cell, 2009. **139**(3): p. 485-498.
142. Wu, H.-J., et al., *Gut-residing segmented filamentous bacteria drive autoimmune arthritis via T helper 17 cells.* Immunity, 2010. **32**(6): p. 815-827.
143. Rescigno, M., *Intestinal dendritic cells.* Adv Immunol, 2010. **107**: p. 109-38.
144. Furusawa, Y., et al., *Commensal microbe-derived butyrate induces the differentiation of colonic regulatory T cells.* Nature, 2013. **504**(7480): p. 446-450.
145. Zhang, M., et al., *Butyrate inhibits interleukin-17 and generates Tregs to ameliorate colorectal colitis in rats.* BMC Gastroenterol, 2016. **16**(1): p. 84.
146. Lv, Q., et al., *Alpinetin exerts anti-colitis efficacy by activating AhR, regulating miR-302/DNMT-1/CREB signals, and therefore promoting Treg differentiation.* Cell death & disease, 2018. **9**(9): p. 1-25.
147. Zou, Y., et al., *Baicalin attenuates TNBS-induced colitis in rats by modulating the Th17/Treg paradigm.* Archives of Pharmacal Research, 2015. **38**(10): p. 1873-1887.
148. Xu, Z., et al., *Epigallocatechin-3-gallate-induced inhibition of interleukin-6 release and adjustment of the regulatory T/T helper 17 cell balance in the treatment of colitis in mice.* Experimental and Therapeutic Medicine, 2015. **10**(6): p. 2231-2238.
149. Lim, S.-M., et al., *Mangiferin corrects the imbalance of Th17/Treg cells in mice with TNBS-induced colitis.* International Immunopharmacology, 2016. **34**: p. 220-228.
150. Casellas, F., et al., *Oral oligofructose-enriched inulin supplementation in acute ulcerative colitis is well tolerated and associated with lowered faecal calprotectin.* Alimentary pharmacology & therapeutics, 2007. **25**(9): p. 1061-1067.

151. Furrie, E., et al., *Synbiotic therapy (Bifidobacterium longum/Synergy 1) initiates resolution of inflammation in patients with active ulcerative colitis: a randomised controlled pilot trial.* Gut, 2005. **54**(2): p. 242-249.
152. Ali, T., et al., *Osteoporosis in inflammatory bowel disease.* The American journal of medicine, 2009. **122**(7): p. 599-604.
153. Bernstein, C.N. and W.D. Leslie, *The pathophysiology of bone disease in gastrointestinal disease.* European journal of gastroenterology & hepatology, 2003. **15**(8): p. 857-864.
154. Bernstein, C.N., W.D. Leslie, and M.S. Leboff, *AGA technical review on osteoporosis in gastrointestinal diseases.* Gastroenterology, 2003. **124**(3): p. 795-841.
155. Loftus Jr, E.V., et al., *Long-term fracture risk in patients with Crohn's disease: a population-based study in Olmsted County, Minnesota.* Gastroenterology, 2002. **123**(2): p. 468-475.
156. Larussa, T., et al., *Bone mineralization in celiac disease.* Gastroenterology research and practice, 2012. **2012**.
157. Guidelines, A.C.o.R.T.F.o.O., *Recommendations for the prevention and treatment of glucocorticoid-induced osteoporosis.* Arthritis & Rheumatism, 1996. **39**(11): p. 1791-1801.
158. Yadav, V.K., et al., *Lrp5 controls bone formation by inhibiting serotonin synthesis in the duodenum.* Cell, 2008. **135**(5): p. 825-837.
159. Bliziotes, M., *Update in serotonin and bone.* The Journal of Clinical Endocrinology & Metabolism, 2010. **95**(9): p. 4124-4132.
160. Mödder, U.I., et al., *Relation of serum serotonin levels to bone density and structural parameters in women.* Journal of Bone and Mineral Research, 2010. **25**(2): p. 415-422.
161. Sjogren, K., et al., *The gut microbiota regulates bone mass in mice.* J Bone Miner Res, 2012. **27**(6): p. 1357-67.
162. Yan, J., et al., *Gut microbiota induce IGF-1 and promote bone formation and growth.* Proc Natl Acad Sci U S A, 2016. **113**(47): p. E7554-E7563.
163. Cho, I., et al., *Antibiotics in early life alter the murine colonic microbiome and adiposity.* Nature, 2012. **488**(7413): p. 621-626.
164. Pytlik, M., J. Folwarczna, and W. Janiec, *Effects of doxycycline on mechanical properties of bones in rats with ovariectomy-induced osteopenia.* Calcified tissue international, 2004. **75**(3): p. 225-230.
165. Guss, J.D., et al., *Alterations to the gut microbiome impair bone strength and tissue material properties.* Journal of Bone and Mineral Research, 2017. **32**(6): p. 1343-1353.
166. Rios-Arce, N.D., et al., *Post-antibiotic gut dysbiosis-induced trabecular bone loss is dependent on lymphocytes.* Bone, 2020. **134**: p. 115269.
167. Schepper, J.D., et al., *Probiotics in gut-bone signaling.* Understanding the gut-bone signaling axis, 2017: p. 225-247.
168. Messora, M.R., et al., *Probiotic therapy reduces periodontal tissue destruction and improves the intestinal morphology in rats with ligature-induced periodontitis.* Journal of periodontology, 2013. **84**(12): p. 1818-1826.
169. Garcia, V., et al., *Effect of the probiotic Saccharomyces cerevisiae on ligature-induced periodontitis in rats.* Journal of periodontal research, 2016. **51**(1): p. 26-37.
170. Britton, R.A., et al., *Probiotic L. reuteri treatment prevents bone loss in a menopausal ovariectomized mouse model.* Journal of cellular physiology, 2014. **229**(11): p. 1822-1830.
171. Zhang, J., et al., *Loss of bone and Wnt10b expression in male type 1 diabetic mice is blocked by the probiotic Lactobacillus reuteri.* Endocrinology, 2015. **156**(9): p. 3169-3182.
172. Ohlsson, C., et al., *Probiotics protect mice from ovariectomy-induced cortical bone loss.* PloS one, 2014. **9**(3): p. e92368.

173. Pan, H., et al., *A single bacterium restores the microbiome dysbiosis to protect bones from destruction in a rat model of rheumatoid arthritis.* Microbiome, 2019. **7**(1): p. 1-11.
174. Krishna Rao, R. and G. Samak, *Protection and restitution of gut barrier by probiotics: nutritional and clinical implications.* Current Nutrition & Food Science, 2013. **9**(2): p. 99-107.
175. Tyagi, A.M., et al., *The microbial metabolite butyrate stimulates bone formation via T regulatory cell-mediated regulation of WNT10B expression.* Immunity, 2018. **49**(6): p. 1116-1131. e7.
176. Schiering, C., et al., *Feedback control of AHR signalling regulates intestinal immunity.* Nature, 2017. **542**(7640): p. 242-245.
177. Hubbard, T.D., I.A. Murray, and G.H. Perdew, *Indole and tryptophan metabolism: endogenous and dietary routes to Ah receptor activation.* Drug Metabolism and Disposition, 2015. **43**(10): p. 1522-1535.
178. Garidou, L., et al., *The gut microbiota regulates intestinal CD4 T cells expressing RORγt and controls metabolic disease.* Cell metabolism, 2015. **22**(1): p. 100-112.
179. Venkatesh, M., et al., *Symbiotic bacterial metabolites regulate gastrointestinal barrier function via the xenobiotic sensor PXR and Toll-like receptor 4.* Immunity, 2014. **41**(2): p. 296-310.
180. Cervantes-Barragan, L., et al., *Lactobacillus reuteri induces gut intraepithelial CD4+ CD8αα+ T cells.* Science, 2017. **357**(6353): p. 806-810.
181. Naruse, M., et al., *Inhibition of osteoclast formation by 3-methylcholanthrene, a ligand for arylhydrocarbon receptor: suppression of osteoclast differentiation factor in osteogenic cells.* Biochemical pharmacology, 2004. **67**(1): p. 119-127.
182. Park, R., S. Madhavaram, and J.D. Ji, *The role of Aryl-Hydrocarbon Receptor (AhR) in osteoclast differentiation and function.* Cells, 2020. **9**(10): p. 2294.
183. Sato, K., et al., *Th17 functions as an osteoclastogenic helper T cell subset that links T cell activation and bone destruction.* The Journal of experimental medicine, 2006. **203**(12): p. 2673-2682.
184. Takayanagi, H., et al., *T-cell-mediated regulation of osteoclastogenesis by signalling cross-talk between RANKL and IFN-γ.* Nature, 2000. **408**(6812): p. 600-605.
185. Yu, M., et al., *PTH induces bone loss via microbial-dependent expansion of intestinal TNF+ T cells and Th17 cells.* Nature communications, 2020. **11**(1): p. 1-17.
186. Cenci, S., et al., *Estrogen deficiency induces bone loss by enhancing T-cell production of TNF-α.* The Journal of clinical investigation, 2000. **106**(10): p. 1229-1237.
187. Tyagi, A.M., et al., *Estrogen deficiency induces the differentiation of IL-17 secreting Th17 cells: a new candidate in the pathogenesis of osteoporosis.* 2012.
188. Bindels, L.B., et al., *Towards a more comprehensive concept for prebiotics.* Nature reviews Gastroenterology & hepatology, 2015. **12**(5): p. 303-310.
189. Carlson, J.L., et al., *Health effects and sources of prebiotic dietary fiber.* Current developments in nutrition, 2018. **2**(3): p. nzy005.
190. Rolim, P.M., *Development of prebiotic food products and health benefits.* Food Science and Technology, 2015. **35**: p. 3-10.
191. Bornet, F., et al., *Nutritional aspects of short-chain fructooligosaccharides: natural occurrence, chemistry, physiology and health implications.* Digestive and liver disease, 2002. **34**: p. S111-S120.
192. Sabater-Molina, M., et al., *Dietary fructooligosaccharides and potential benefits on health.* Journal of physiology and biochemistry, 2009. **65**(3): p. 315-328.
193. Singh, R.S. and R.P. Singh, *Production of fructooligosaccharides from inulin by endoinulinases and their prebiotic potential.* Food Technology and Biotechnology, 2010. **48**(4): p. 435.

194. Park, Y. and M. Almeida, *Production of fructooligosaccharides from sucrose by a transfructosylase from Aspergillus niger.* World Journal of Microbiology and Biotechnology, 1991. **7**(3): p. 331-334.
195. Van de Wiele, T., et al., *Inulin-type fructans of longer degree of polymerization exert more pronounced in vitro prebiotic effects.* Journal of applied microbiology, 2007. **102**(2): p. 452-460.
196. Kolida, S., K. Tuohy, and G.R. Gibson, *Prebiotic effects of inulin and oligofructose.* British Journal of Nutrition, 2002. **87**(S2): p. S193-S197.
197. Coxam, V., *Inulin-type fructans and bone health: state of the art and perspectives in the management of osteoporosis.* British journal of nutrition, 2005. **93**(S1): p. S111-S123.
198. Arjmandi, B.H., et al., *Combining fructooligosaccharide and dried plum has the greatest effect on restoring bone mineral density among select functional foods and bioactive compounds.* Journal of medicinal food, 2010. **13**(2): p. 312-319.
199. Gibson, G.R. and M.B. Roberfroid, *Dietary modulation of the human colonic microbiota: introducing the concept of prebiotics.* The Journal of nutrition, 1995. **125**(6): p. 1401-1412.
200. Peng, M., et al., *Effectiveness of probiotics, prebiotics, and prebiotic-like components in common functional foods.* Comprehensive reviews in food science and food safety, 2020. **19**(4): p. 1908-1933.
201. Wang, Y., et al., *Fructo-oligosaccharides enhance the mineral absorption and counteract the adverse effects of phytic acid in mice.* Nutrition, 2010. **26**(3): p. 305-311.
202. Gudiel-Urbano, M. and I. Goni, *Effect of fructooligosaccharide on nutritional parameters and mineral bioavailability in rats.* Journal of the Science of Food and Agriculture, 2002. **82**(8): p. 913-917.
203. Roberfroid, M., *Dietary fiber, inulin, and oligofructose: a review comparing their physiological effects.* Critical Reviews in Food Science & Nutrition, 1993. **33**(2): p. 103-148.
204. Raschka, L. and H. Daniel, *Mechanisms underlying the effects of inulin-type fructans on calcium absorption in the large intestine of rats.* Bone, 2005. **37**(5): p. 728-735.
205. Hosono, A., et al., *Dietary fructooligosaccharides induce immunoregulation of intestinal IgA secretion by murine Peyer's patch cells.* Bioscience, biotechnology, and biochemistry, 2003. **67**(4): p. 758-764.
206. Korzenik, J.R. and D.K. Podolsky, *Evolving knowledge and therapy of inflammatory bowel disease.* Nature reviews Drug discovery, 2006. **5**(3): p. 197-209.
207. Wen, S., et al., *Stigmasterol restores the balance of Treg/Th17 cells by activating the butyrate-PPARγ axis in colitis.* Frontiers in immunology, 2021: p. 4201.
208. Kang, S., et al., *Butyl-fructooligosaccharides modulate gut microbiota in healthy mice and ameliorate ulcerative colitis in a DSS-induced model.* Food & function, 2022. **13**(4): p. 1834-1845.
209. Yan, X., et al., *Fructooligosaccharides protect against OVA-induced food allergy in mice by regulating the Th17/Treg cell balance using tryptophan metabolites.* Food & Function, 2021. **12**(7): p. 3191-3205.
210. Ohta, A., et al., *Dietary fructooligosaccharides prevent postgastrectomy anemia and osteopenia in rats.* The Journal of nutrition, 1998. **128**(3): p. 485-490.
211. Marcon, L.D.N., et al., *Yacon (Smallanthus sonchifolius)-based product increases fecal short-chain fatty acids and enhances regulatory T cells by downregulating RORγt in the colon of BALB/c mice.* Journal of Functional Foods, 2019. **55**: p. 333-342.
212. Franklin, M., et al., *Dried plum prevents bone loss in a male osteoporosis model via IGF-I and the RANK pathway.* Bone, 2006. **39**(6): p. 1331-1342.

213. Parkar, S.G., D.E. Stevenson, and M.A. Skinner, *The potential influence of fruit polyphenols on colonic microflora and human gut health.* International journal of food microbiology, 2008. **124**(3): p. 295-298.
214. Jiao, X., et al., *Blueberry polyphenols extract as a potential prebiotic with anti-obesity effects on C57BL/6 J mice by modulating the gut microbiota.* The Journal of Nutritional Biochemistry, 2019. **64**: p. 88-100.
215. Hooshmand, S., et al., *Comparative effects of dried plum and dried apple on bone in postmenopausal women.* British Journal of Nutrition, 2011. **106**(6): p. 923-930.
216. Arjmandi, B.H., et al., *Dried plums improve indices of bone formation in postmenopausal women.* Journal of women's health & gender-based medicine, 2002. **11**(1): p. 61-68.
217. Limagne, E., et al., *Sirtuin-1 activation controls tumor growth by impeding Th17 differentiation via STAT3 deacetylation.* Cell reports, 2017. **19**(4): p. 746-759.
218. Alesci, A., et al., *Resveratrol and immune cells: A link to improve human health.* Molecules, 2022. **27**(2): p. 424.
219. Malaguarnera, L., *Influence of Resveratrol on the Immune Response.* Nutrients, 2019. **11**(5).
220. Papp, N., et al., *Main quality attributes and antioxidants in Hungarian sour cherries: identification of genotypes with enhanced functional properties.* International journal of food science & technology, 2010. **45**(2): p. 395-402.
221. Mansoori, S., A. Dini, and S.C. Chai, *Effects of tart cherry and its metabolites on aging and inflammatory conditions: Efficacy and possible mechanisms.* Ageing Research Reviews, 2021. **66**: p. 101254.
222. Alba C, M.-A., M. Daya, and C. Franck, *Tart Cherries and health: Current knowledge and need for a better understanding of the fate of phytochemicals in the human gastrointestinal tract.* Critical reviews in food science and nutrition, 2019. **59**(4): p. 626-638.
223. Jovanovic-Malinovska, R., S. Kuzmanova, and E. Winkelhausen, *Oligosaccharide profile in fruits and vegetables as sources of prebiotics and functional foods.* International journal of food properties, 2014. **17**(5): p. 949-965.
224. Ferretti, G., et al., *Cherry antioxidants: from farm to table.* Molecules, 2010. **15**(10): p. 6993-7005.
225. Karaaslan, M., et al., *Synthesis and accumulation of anthocyanins in sour cherries during ripening in accordance with antioxidant capacity development and chalcone synthase expression.* European Food Research and Technology, 2016. **242**(2): p. 189-198.
226. Chaovanalikit, A. and R. Wrolstad, *Anthocyanin and polyphenolic composition of fresh and processed cherries.* Journal of Food Science, 2004. **69**(1): p. FCT73-FCT83.
227. Damar, İ. and A. Ekşi, *Antioxidant capacity and anthocyanin profile of sour cherry (Prunus cerasus L.) juice.* Food chemistry, 2012. **135**(4): p. 2910-2914.
228. Seeram, N.P., L.D. Bourquin, and M.G. Nair, *Degradation products of cyanidin glycosides from tart cherries and their bioactivities.* Journal of agricultural and food chemistry, 2001. **49**(10): p. 4924-4929.
229. Blando, F. and B.D. Oomah, *Sweet and sour cherries: Origin, distribution, nutritional composition and health benefits.* Trends in food science & technology, 2019. **86**: p. 517-529.
230. Csiki, Z., et al., *Orally delivered sour cherry seed extract (SCSE) affects cardiovascular and hematological parameters in humans.* Phytotherapy Research, 2015. **29**(3): p. 444-449.
231. Matchynski, J.J., et al., *Combinatorial treatment of tart cherry extract and essential fatty acids reduces cognitive impairments and inflammation in the mu-p75 saporin-induced mouse model of Alzheimer's disease.* Journal of Medicinal Food, 2013. **16**(4): p. 288-295.

232. Wojdyło, A., et al., *Evaluation of sour cherry (Prunus cerasus L.) fruits for their polyphenol content, antioxidant properties, and nutritional components.* Journal of Agricultural and Food Chemistry, 2014. **62**(51): p. 12332-12345.
233. Ou, B., et al., *Processed Tart Cherry Products—Comparative Phytochemical Content, in vitro Antioxidant Capacity and in vitro Anti-inflammatory Activity.* Journal of food science, 2012. **77**(5): p. H105-H112.
234. Šarić, A., et al., *Improved antioxidant and anti-inflammatory potential in mice consuming sour cherry juice (Prunus Cerasus cv. Maraska).* Plant foods for human nutrition, 2009. **64**(4): p. 231-237.
235. Thangthaeng, N., et al., *Tart cherry supplementation improves working memory, hippocampal inflammation, and autophagy in aged rats.* Age, 2016. **38**(5): p. 393-404.
236. Gao, R. and P.D. Chilibeck, *Effect of tart cherry concentrate on endurance exercise performance: a meta-analysis.* Journal of the American College of Nutrition, 2020. **39**(7): p. 657-664.
237. Bell, P.G., et al., *Montmorency cherries reduce the oxidative stress and inflammatory responses to repeated days high-intensity stochastic cycling.* Nutrients, 2014. **6**(2): p. 829-843.
238. Seymour, E.M., et al., *Regular tart cherry intake alters abdominal adiposity, adipose gene transcription, and inflammation in obesity-prone rats fed a high fat diet.* Journal of medicinal food, 2009. **12**(5): p. 935-942.
239. Mahmoud, F., et al., *Sour cherry (Prunus cerasus) seed extract increases heme oxygenase-1 expression and decreases proinflammatory signaling in peripheral blood human leukocytes from rheumatoid arthritis patients.* International immunopharmacology, 2014. **20**(1): p. 188-196.
240. Bell, P.G., et al., *Montmorency tart cherry (Prunus cerasus L.) concentrate lowers uric acid, independent of plasma cyanidin-3-O-glucosiderutinoside.* journal of functional foods, 2014. **11**: p. 82-90.
241. Dodier, T., et al., *US Montmorency Tart Cherry Juice Decreases Bone Resorption in Women Aged 65–80 Years.* Nutrients, 2021. **13**(2): p. 544.
242. Ojo, B.A., et al., *Wheat germ supplementation increases lactobacillaceae and promotes an anti-inflammatory gut milieu in C57BL/6 mice fed a high-fat, high-sucrose diet.* The Journal of Nutrition, 2019. **149**(7): p. 1107-1115.
243. Scortichini, S., et al., *Development and validation of a GC-FID method for the analysis of short chain fatty acids in rat and human faeces and in fermentation fluids.* Journal of Chromatography B, 2020. **1143**: p. 121972.
244. Holloway, L., et al., *Effects of oligofructose-enriched inulin on intestinal absorption of calcium and magnesium and bone turnover markers in postmenopausal women.* British Journal of Nutrition, 2007. **97**(2): p. 365-372.
245. Tahiri, M., et al., *Effect of short-chain fructooligosaccharides on intestinal calcium absorption and calcium status in postmenopausal women: a stable-isotope study.* The American journal of clinical nutrition, 2003. **77**(2): p. 449-457.
246. Slevin, M.M., et al., *Supplementation with calcium and short-chain fructo-oligosaccharides affects markers of bone turnover but not bone mineral density in postmenopausal women.* The Journal of nutrition, 2014. **144**(3): p. 297-304.
247. Kim, Y.-Y., et al., *The effect of chicory fructan fiber on calcium absorption and bone metabolism in Korean postmenopausal women.* Nutritional sciences, 2004. **7**(3): p. 151-157.
248. Ohta, A., et al., *A combination of dietary fructooligosaccharides and isoflavone conjugates increases femoral bone mineral density and equol production in ovariectomized mice.* The Journal of nutrition, 2002. **132**(7): p. 2048-2054.

249. Hartog, A., et al., *A potential role for regulatory T-cells in the amelioration of DSS induced colitis by dietary non-digestible polysaccharides.* The Journal of nutritional biochemistry, 2015. **26**(3): p. 227-233.
250. Pujari, R. and G. Banerjee, *Impact of prebiotics on immune response: from the bench to the clinic.* Immunology and Cell Biology, 2021. **99**(3): p. 255-273.
251. Dar, H.Y., et al., *Bacillus clausii inhibits bone loss by skewing Treg-Th17 cell equilibrium in postmenopausal osteoporotic mice model.* Nutrition, 2018. **54**: p. 118-128.
252. Yu, M., et al., *Regulatory T cells are expanded by Teriparatide treatment in humans and mediate intermittent PTH-induced bone anabolism in mice.* EMBO reports, 2018. **19**(1): p. 156-171.
253. Yuan, F.-L., et al., *Regulatory T cells as a potent target for controlling bone loss.* Biochemical and biophysical research communications, 2010. **402**(2): p. 173-176.
254. Chen, X., et al., *Lactulose Suppresses Osteoclastogenesis and Ameliorates Estrogen Deficiency-Induced Bone Loss in Mice.* Aging and disease, 2020. **11**(3): p. 629-641.
255. Zhao, L., S. Jiang, and B.M. Hantash, *Transforming growth factor β1 induces osteogenic differentiation of murine bone marrow stromal cells.* Tissue Engineering Part A, 2010. **16**(2): p. 725-733.
256. Ohta, A., et al., *Calcium and magnesium absorption from the colon and rectum are increased in rats fed fructooligosaccharides.* The Journal of nutrition, 1995. **125**(9): p. 2417-2424.
257. Ohta, A., et al., *Dietary fructooligosaccharides prevent osteopenia after gastrectomy in rats.* The Journal of nutrition, 1998. **128**(1): p. 106-110.
258. Hooshmand, S., S. Juma, and B.H. Arjmandi, *Combination of genistin and fructooligosaccharides prevents bone loss in ovarian hormone deficiency.* Journal of medicinal food, 2010. **13**(2): p. 320-325.
259. Kaur, A., et al., *Montmorencytart cherry supplementation improved markers of glucose homeostasis but has modest effects on indicators of gut health in mice fed a Western diet.* Nutr Res, 2022. **99**: p. 66-77.
260. Kim, D.S., et al., *Attenuation of rheumatoid inflammation by sodium butyrate through reciprocal targeting of HDAC2 in osteoclasts and HDAC8 in T cells.* Frontiers in Immunology, 2018. **9**: p. 1525.
261. Barros, N.M., et al., *Proteolytic processing of osteopontin by PHEX and accumulation of osteopontin fragments in Hyp mouse bone, the murine model of X-linked hypophosphatemia.* Journal of Bone and Mineral Research, 2013. **28**(3): p. 688-699.
262. Beck-Nielsen, S.S., et al., *FGF23 and its role in X-linked hypophosphatemia-related morbidity.* Orphanet Journal of Rare Diseases, 2019. **14**(1): p. 1-25.
263. Narayanan, K., et al., *Dual functional roles of dentin matrix protein 1: implications in biomineralization and gene transcription by activation of intracellular Ca2+ store.* Journal of Biological Chemistry, 2003. **278**(19): p. 17500-17508.
264. Bonewald, L.F., *The role of the osteocyte in bone and nonbone disease.* Endocrinology and Metabolism Clinics, 2017. **46**(1): p. 1-18.
265. Staines, K.A., V.E. MacRae, and C. Farquharson, *The importance of the SIBLING family of proteins on skeletal mineralisation and bone remodelling.* The Journal of endocrinology, 2012. **214**(3): p. 241-255.
266. Wang, C., et al., *Structural characterization of a novel oligosaccharide from Achyranthes bidentata and its anti-osteoporosis activities.* Industrial Crops and Products, 2017. **108**: p. 458-469.
267. Yan, C., et al., *A fructooligosaccharide from Achyranthes bidentata inhibits osteoporosis by stimulating bone formation.* Carbohydrate polymers, 2019. **210**: p. 110-118.
268. Smith, B., et al., *Effects of dried plum supplementation on bone metabolism in adult C57BL/6 male mice.* Calcified tissue international, 2014. **94**(4): p. 442-453.

269. Rendina, E., et al., *Dietary supplementation with dried plum prevents ovariectomy-induced bone loss while modulating the immune response in C57BL/6J mice*. The Journal of nutritional biochemistry, 2012. **23**(1): p. 60-68.

APPENDICES

Appendix 1.

$CD8^+$ T- Lymphocyte Percentage Count in Ileum and Bone Marrow

	-CD25			+CD25			*Pvalues*		
	Con	TC	FOS	Con	TC	FOS	CD25	Diet	CD25*Diet
Ileum T-Lymphocytes									
$CD3^+CD8^+$ (%)	71.41 ± 2.18	67.69 ± 1.88	77.3 ± 0.89$^{\#\$}$	65.96 ± 0.96	67.15 ± 2.98	77.14 ± 1.32$^{\#\$}$	0.1582	**<.0001**	0.2602
Bone Marrow T-Lymphocytes									
$CD3^+CD8^+$ (%)	29.46 ± 4.67	34.47 ± 1.90	44.27 ± 0.78$^{\#\$}$	32.75 ± 1.90	31.15 ± 3.58	46.08 ± 1.34$^{\#\$}$	0.6487	**<.0001**	0.4231

Control diet = Con; Tart cherry diet = TC; Fructooligosaccharide diet = FOS.
Data presented as mean ± SEM. *P-values <0.05* are considered statistically different.
A difference between two groups that do not have the same superscript letter is statistically significant (*P<0.05)*
# indicates TC or FOS vs. Con (*P<0.05)*. $ indicates TC vs. FOS (*P<0.05)*

Appendix 2.

T- Lymphocyte Absolute Count in Ileum and Bone Marrow

	-CD25			+CD25			*Pvalues*		
	Con	TC	FOS	Con	TC	FOS	CD25	Diet	CD25*Diet
Ileum T-Lymphocytes (Thousands)									
$CD3^+CD4^+$	51.84 ± 13.98	62.28 ± 11.04	24.06 ± 3.79$^{\#\$}$	70.74 ± 18.81	81.33 ± 9.58	29.71 ± 5.27$^{\#\$}$	0.0537	**0.0004**	0.8326
$CD3^+CD8^+$	418.64 ± 96.31	409.76 ± 47.02	291.78 ± 28.64$^{\$}$	425.02 ± 90.49	566.37 ± 77.96	330.04 ± 46.53$^{\$}$	0.5198	**0.0113**	0.4676
$CD4^+$: $CD8^+$ Ratio	0.10 ± 0.01^{c}	0.18 ± 0.03^{ab}	0.08 ± 0.01$^{\#\$c}$	0.19 ± 0.02^{a}	0.13 ± 0.01^{bc}	0.11 ± 0.01$^{\#\$c}$	**0.0287**	**0.0013**	**0.0140**
Treg ($CD4^+CD25^+Foxp3^+$)	0.97 ± 0.53	2.33 ± 0.65	1.24 ± 0.21	0 ± 0	0 ± 0	0.11 ± 0.07	**<.0001**	0.0939	0.2198
Th-17 ($CD4^+$Th-17^+)	2.44 ± 1.95	1.96 ± 0.87	1.65 ± 0.37	4.85 ± 2.26	4.08 ± 1.70	3.40 ± 0.84	**0.0177**	0.3771	0.8290
Bone Marrow T-Lymphocytes (Thousands)									
$CD3^+CD4^+$	47.22 ± 19.65	66.62 ± 20.08	29.93 ± 4.25	57.79 ± 18.02	56.57 ± 11.57	32.13 ± 4.12	0.2415	0.2763	0.8632
$CD4^+CD8^+$	66.69 ± 27.85	129.76 ± 41.62	61.29 ± 8.34	134.37 ± 39.88	112.84 ± 29.98	66.83 ± 8.78	0.1323	0.5629	0.6161
CD4+: CD8+ Ratio	1.54 ± 0.89	0.56 ± 0.05	0.49 ± 0.01	0.48 ± 0.02	0.47 ± 0.03	0.50 ± 0.04	**0.0411**	0.6825	0.1885
Treg ($CD4^+CD25^+Foxp3^+$)	6.13 ± 3.30	7.36 ± 4.16	4.13 ± 1.05	0 ± 0	0 ± 0	0 ± 0	**<.0001**	0.8074	0.0914
Th-17 ($CD4^+$Th-17^+)	0 ± 0	0 ± 0	1 ± 0$^{\#\$}$	0 ± 0	0 ± 0	0.87 ± 0.12$^{\#\$}$	0.8365	**<.0001**	0.9537

Control diet = Con; Tart cherry diet = TC; Fructooligosaccharide diet = FOS.
Data presented as mean ± SEM. *P-values <0.05* are considered statistically different.
A difference between two groups that do not have the same superscript letter is statistically significant (*P<0.05)*
# indicates TC or FOS vs. Con (*P<0.05)*. $ indicates TC vs. FOS (*P<0.05)*

Appendix 3.

Primer Sequence List for qRT-PCR

Symbol	Name	Sequence
Hprt-1	Hypoxanthine-guanine phosphoribosyl transferase	QF 5'- GCCTAAGATGAGCGCAAGTTG -3' QR 5'- TACTAGGCAGATGGCCACAGG - 3'
Gapdh	Glucose 6 phosphate dehydrogenase	QF 5'- GAACGCAAAGCTGAAGTGAGACT -3' QR 5'- TCATTACGCTTGCACTGTTGGT - 3'
Wnt10b	Wingless-type MMTV integration site family, member 10b	QF 5'- ATGCGGATCCACAACAACAG-3' QR 5'- TTCCATGGCATTTGCACTTC- 3'
Bmp2	Bone morphogenetic protein 2	QF 5'- GGACATCCGCTCCACAAA -3' QR 5'- GGCGCTTCCGCTGTTT-3'
Runx2	Runt-related transcription factor 2	QF 5'- CGACAGTCCCAACTTCCTGT-3' QR 5'- CGGTAACCACAGTCCCATCT -3'
Osx	Osterix	QF 5'- GAAGTTCACCTGCCTGCTCTGT -3' QR 5'- CGTGGGTGCGCTGATGT -3'
Bsp	Bone sialoprotein	QF 5'- ACACCCCAAGCACAGACTTTTG -3' QR 5'- TCCTCGTCGCTTTCCTTCACT -3'
Col1α1	Type I collagen called the pro-α 1	QF 5'- CGTCTGGTTTGGAGAGAGCAT -3' QR 5'- GGTCAGCTGGATAGCGACATC -3'
Ocn (Bglap2)	Osteocalcin (Bone gamma-carboxyglutamate protein 2	QF 5'- TGAGCTTAACCCTGCTTGTGACGA -3' QR 5'- AGGGCAGCACAGGTCCTAAATAGT -3'
Opn	Osteopontin	QF 5'- ACTCCAATCGTCCCTACAGTCG -3' QR 5'- TGAGGTCCTCATCTGTGGCAT -3'
Phex	Phosphate regulating endopeptidase X linked	QF 5'- GGCATGACTGCTGTAAGATCAGAT -3' QR 5'- AGCTCCATTGACATAAGGCACT -3'
Dmp1	Dentin matrix acidic phospho protein 1	QF 5'- CTGTCATTCTCCTTGTGTTCCT -3' QR 5'- CAAATCACCCGTCCTCTCTTC -3'
Mepe	Matrix extracellular phospho glycoprotein	QF 5'- CCCCAAGAGCAGCAAAGGTA -3' QR 5'- CTCCGCTGTGACATCCCTTTA -3'
SOST	Sclerostin	QF 5'- ACCGGGCGGAGAATGG -3' QR 5'- GCTGTACTCGGACACATCTTTGG -3'

Rankl	Receptor activator of nuclear factor kappa-B ligand	QF 5'- TCTGCAGCATCGCTCTGTTC -3' QR 5'- AGCAGTGAGTGCTGTCTTCTGATATT- 3'
Opg	Osteoprotegerin	QF 5'- TCCCGAGGACCACAATGAAC -3' QR 5'- TGGGTTGTCCATTCAATGATGT -3'
Tnfα	Tumor necrosis factor alpha	QF 5'- CTGAGGTCAATCTGCCCAAGTAC -3' QR 5'- CTTCACAGAGCAATGACTCCAAAG-3'
IL-17	Interleukin 17	QF 5'- ATCCCTCAAAGCTCAGCGTGTC -3' QR 5'- GGGTCTTCATTGCGGTGGAGAG-3'
IL-6	Interleukin 6	QF 5'- GAGGATACCACTCCCAACAGACC -3' QR 5'- AAGTGCATCATCGTTGTTCATACA -3'
CCR7	C-C Motif Chemokine Receptor 7	QF 5'- GGTGGCTCTCCTTGTCATTTTC -3' QR 5'- GTGGTATTCTCGCCGATGTAGTC -3'
CXCL10	C-X-C Motif Chemokine Ligand 10	QF 5'- AGTGCTGCCGTCATTTTCTG -3' QR 5'- ATTCTCACTGGCCCGTCAT-3'
CXCR4	C-X-C Motif Chemokine Receptor 4	QF 5'- TCAGTGGCTGACCTCCTCTT -3' QR 5'- CTTGGCCTTTGACTGTTGGT-3'
CXCL12	C-X-C Motif Chemokine Ligand 12	QF 5'- CAAGCATCTGAAAATCCTCAACAC -3' QR 5'- TCTTCAGCCGTGCAACAATC -3'
Vcam1	Vascular Cell Adhesion Molecule 1	QF 5'- TGAACCCAAACAGAGGCAGAGT -3' QR 5'- GGTATCCCATCACTTGAGCAGG -3'
Tgf-β	Transforming growth factor beta	QF 5'- CCCTATATTTGGAGCCTGGA -3' QR 5'- CTTGCGACCCACGTAGTAGA -3'
IL-10	Interleukin 10	QF 5'- GGTTGCCAAGCCTTATCGGA -3' QR 5'- ACCTGCTCCACTGCCTTGCT -3'
GPR-43	G protein-coupled receptor 43	QF 5'- TTCCCATGGCAGTCACCAT -3' QR 5'- GGGCTGCGTGAGCATGAT -3'

QF= Forward Primer; QR= Reverse Primer

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