INVESTIGATION OF THE GUT MICROBIOTA AS A MEDIATOR AND THERAPEUTIC TARGET FOR SECONDARY OSTEOPOROSIS

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A DISSERTATION

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

Physiology - Doctor of Philosophy

2019

ABSTRACT

INVESTIGATION OF THE GUT MICROBIOTA AS A MEDIATOR AND THERAPEUTIC TARGET FOR SECONDARY OSTEOPOROSIS

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Osteoporosis is a disease characterized by low bone mass and increased fracture risk. It is currently estimated that over 300 million people are impacted by osteoporosis. This thesis will primarily focus on secondary osteoporosis, which is due to consequences of diseases or treatments for disease. With current treatments having several adverse side effects, there is an increased need to develop novel therapies for osteoporosis. One area of research that has gained attention recently involves the influence of the gut microbiota on bone health. My thesis work investigated the role of the gut microbiota in two different models of bone loss. First, I investigated how 2 weeks of broad-spectrum antibiotics and subsequent intestinal microbiome repopulation (dysbiosis) affected bone density. Intestinal microbiome repopulation, 4-weeks post-antibiotic treatment, resulted in an increase in dysbiosis, increased intestinal permeability and notably reduced femoral trabecular bone volume (~30%). Treatment with a mucus supplement (MDY) prevented the post-antibiotic induced barrier break as well as bone loss, indicating a mechanistic link between increased intestinal permeability and bone loss. A link between the microbiome composition and bone density was demonstrated by supplementing the mice with probiotic bacteria. Specifically, *Lactobacillus reuteri*, reduced the post-antibiotic elevation of the *Firmicutes:Bacteroidetes* ratio and prevented femoral and vertebral trabecular bone loss. Consistent with causing bone loss, post-antibiotic induced dysbiosis decreased osteoblast and increased osteoclast activities, changes that were prevented by both

Lactobacillus reuteri and MDY. These data underscore the importance of microbial dysbiosis in the regulation of intestinal permeability and bone health as well as identify *Lactobacillus reuteri* and MDY as novel therapies for preventing these adverse effects.

In the second part of this thesis, I present an in vivo murine model to study the role of the gut microbiota in glucocorticoid-induced osteoporosis (GIO). Glucocorticoids (GCs) directly induce osteoblast and osteocyte apoptosis but can affect other organs including the intestine. Our lab and others identified that the microbiota contributes to the regulation of bone density; however, the role of the gut in mediating GIO has never been examined. We report that GC treatment alters the microbiota composition. To determine the contribution of the microbiota to GIO pathogenesis, we treated adult male mice for 8-weeks with GC (prednisolone) in the presence or absence of broad-spectrum antibiotic treatment (ABX) to deplete the microbiota. Strikingly, depletion of the microbiota prevented GC-induced bone loss, establishing the requirement of the microbiota in the pathogenesis GIO. We next supplemented GC treated mice with an oral probiotic (*Lactobacillus reuteri*, LR; 10^8 CFU/day) for the duration of GC treatment. LR treatment prevented bone loss, suggesting a role for beneficial bacteria in modulating GIO. Interestingly, we found that GC treatment causes intestinal barrier leaks and raises serum endotoxin levels, a response that was prevented by both LR and ABX treatments. Accordingly, enhancement of barrier function, with a mucus supplement, prevented both GC-induced barrier dysfunction and GIO, establishing a mechanistic link between intestinal barrier function and GIO. Taken together, these data highlight the previously unappreciated role of the gut microbiota and intestinal barrier function in GIO pathogenesis and identify the gut as a novel therapeutic target for preventing GIO.

ACKNOWLEDGEMENTS

Completing my Ph.D. would have never been possible without the support and encouragement of so many people. I would like to extend my gratitude to everyone that has helped me during this process.

I would like to extend my deepest and sincerest gratitude to my mentors Laura McCabe and Narayanan Parameswaran. You took a chance on me by allowing me to join the lab 5 years ago. Thank you so much for your time, knowledge, help with my project and last but not least patience. Your patience has allowed me to better myself not only as a scientist but also as a person, and its something I can never repay. I would also like to thank you for supporting my career goals and for your guidance helping me prepare for whatever the future has to bring.

I would like to thank my committee members for their willingness to meet at any time and answer my questions to further develop my project; Dr. Susanna Mohr, Dr. Adam Mosier and Dr. Robert Wiseman. I very much appreciate all of the time and dedication that you have devoted to my Ph.D. career.

I would also like to extend my thanks and love to pervious members of our lab especially Fraser and Regina. Without your early guidance and training I would not be where I am today. I would also like to thank them for the making the lab atmosphere such a memorable and enjoyable experience and for becoming the best of friends. To current lab members; Nick, thanks for all of the feedback on my dissertation and for always being ready to help.

I want to give a special thanks to Naiomy. We joined the lab at similar times and have gone through the Ph.D. program together. I want to thank you for helping me become a better scientist and person with our daily conversations over the past 5 years. Without your encouragement I could have never achieved all that I have. Even though we are going our separate ways and I will miss our conversations and am very excited to continue our friendship.

I would like to thank my many friends for all the support throughout my graduate career. I especially want to thank Connor, Ben, Andrea (Mom), Octavio, and Will for always being there and allowing me relax during times of stress. Thanks for sticking around my weird self and making the last 5 years a time that I will never forget. I would also like to thank the cats: Dan, Cleary, Balto and Smaug.

To my sports teams, thanks for never winning a championship during my time in graduate school. The lack of a good team to root for, gave me more time to study, read and write.

To Jessi, I would like to thank you for sticking around these past 5 years. I know me being in graduate school and you following your dreams in Chicago have made for some interesting times in our relationship. However, your friendship and support have been amazing and I don't know where I would be without your love and encouragement.

Most importantly I would like to thank my family. None of this would be possible without you. To my parents, words can't explain how thankful I am for all of the things you have taught and done for me during my lifetime. It is not an understatement that without your guidance, support and help I would have not finished this graduate program. Thank you for always supporting me in all of my endeavors and I hope I make you proud.

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KEY TO ABBREVIATIONS

5-HT: 5-hyroxytrptamine receptors

ABX: Antibiotic

ACR: The American College of Rheumatology

ALP: Alkaline phosphatase

BMC: Bone mineral content

BMD: Bone mineral density

BMPs: Bone morphogenic proteins

c-fms: Colony-stimulating factor-1 receptor

CTX-1: C-terminal telopeptides of type 1 collagen

DKK1: Dickkopf-1

DMP-1: Dentin matrix protein 1

EC: Escherichia coli

FDA: Food and drug administration

FGFs: Fibroblast growth factors

FPPS: Farnesyl pyrophosphate synthase

GC: Glucocorticoid

GH: Growth hormone

GIO: Glucocorticoid induced osteoporosis

GIP: Gastric inhibitory polypeptide

GLP-1: Glucagon-like peptide-1

GSK-3β: Glycogen synthase kinase 3

IBD: Inflammatory bowel disease

IBS: Irritable bowel syndrome

IGF-1: Insulin like growth factor-1

IGFBP: IGF-binding proteins

IL-1: Interleukin 1

IL-6: Interleukin-6

IOF: International osteoporosis foundation

IOM: Institute of Medicine

IU: International units

LGG: Lactobacillus rhamnosus GG

LR: Lactobacillus reuteri 6475

LRP5/6: Lipoprotein receptor related proteins

M-CSF: Macrophage colony stimulating factor

MMPs: Matrix metalloproteases

OPG: Osteoprotegerin

PGE-2: Prostaglandin E2

PPARγ2: Proliferator-activated receptor γ 2

PTH: Parathyroid hormone

RANK: Receptor activator of nuclear factor κ B

RANKL: Receptor activator of nuclear factor kappa-β ligand

Runx2: Runt-related transcription factor 2

SCFAs: Short chain fatty acids

SERMs: Selective estrogen receptor modulators

Spp: Species

TGF- β: Transforming growth factor β

TRAP: Tartrate-resistant acid phosphatase

CHAPTER 1: LITERATURE REVIEW

This thesis project investigated the role of the gut microbiota in two different models of bone loss. Those models include antibiotic-induced dysbiosis (Chapter 2) and glucocorticoid-induced osteoporosis (Chapter 3). In addition, I also examined how gut supplementation with probiotics or barrier enhancers can prevent bone loss. In chapter 1, I will provide an overview of the skeletal system, as well as outline skeletal pathophysiologies including glucocorticoid-induced osteoporosis. I will then discuss how alterations to the gut microbiome may be playing a role in bone remodeling. Lastly, I will explain the potential for probiotics as therapies in different models of bone loss.

1.1 The Skeletal System

The skeletal system provides structure for the human body and is composed of bone, ligaments, tendons, and cartilage. In this section, I will discuss the normal functions of bone, its cellular components and the process of bone remodeling.

1.1.1 Functions of the Bone

Bone is a highly specialized and dynamic organ that provides internal support and protection for the human body. It is a complex tissue with essential roles in mechanical, protective, chemical, hematological and immunological functions necessary for survival. During bone development the skeletal extracellular matrix becomes mineralized, conferring marked rigidity and strength, which leads to its mechanical properties. Its rigidity and strength provide areas for muscle attachment ultimately allowing for locomotion. Mineralization of skull and thoracic chest bones protect the brain and thoracic organs respectively. With regard to its chemical properties, bone functions as a significant source of inorganic ions, actively participating in calcium homeostasis. The hematological function of bone arises from marrow stem cells that reside in the medulla of the bone (1).

1.1.2 Bone Structure and Anatomy

Bone structure is comprised of organic and inorganic components. The organic matrix is composed of 95% type 1 collagen, while the remaining 5% is composed of proteoglycans. The inorganic component, deposited in the organic matrix, is primarily made up of phosphate and calcium salts in the form of hydroxyapatite. Morphologically, bone is composed of cortical (compact) and trabecular bone. Cortical bone is heavily calcified and densely packed with collagen fibrils to fulfill a structural and protective role (2). In contrast, trabecular bone is less calcified, highly porous and metabolically more active than the cortical bone (2). Overall the adult skeleton is approximately 80% cortical bone and 20% trabecular bone; however, the proportions of bone type vary depending on the skeletal site. For example, vertebrae are rich in trabecular bone but have thinner cortical bone; the long bones, however, have thicker cortices and less trabecular bone (Fig 1.1).

Bone growth is a process in which bones increase in size and become mineralized during childhood and adolescence, with bone mass increasing from approximately 80 g at birth to 3000 g at peak mass (2). The periosteum lines the outer surface of the bone while the endosteum lines the inner surface. The periosteum and endosteum are thin vascularized connective tissue, which work in coordination to regulate cortical thickness and size (3). Longitudinal growth and development of long bones (such as the femur) occur via endochondral ossification while the growth of bone diameter is by periosteal apposition (4). During endochondral bone growth mesenchymal chondrocytes (cartilage cells) proliferate and produce extracellular matrix. These cells eventually stop proliferating and undergo further maturation and become hypertrophic. Hypertrophic

chondrocytes secrete matrix that is rich in type X collagen, which then rapidly calcifies. At the same time, this matrix becomes vascularized, and mesenchymal pre-osteoblasts are directed to differentiate into mature osteoblasts that lay down mineralized bone matrix (4). This process occurs at the growth plate and leads to bone lengthening. Longitudinal growth and development end with mineralization of the growth plate during young adulthood.

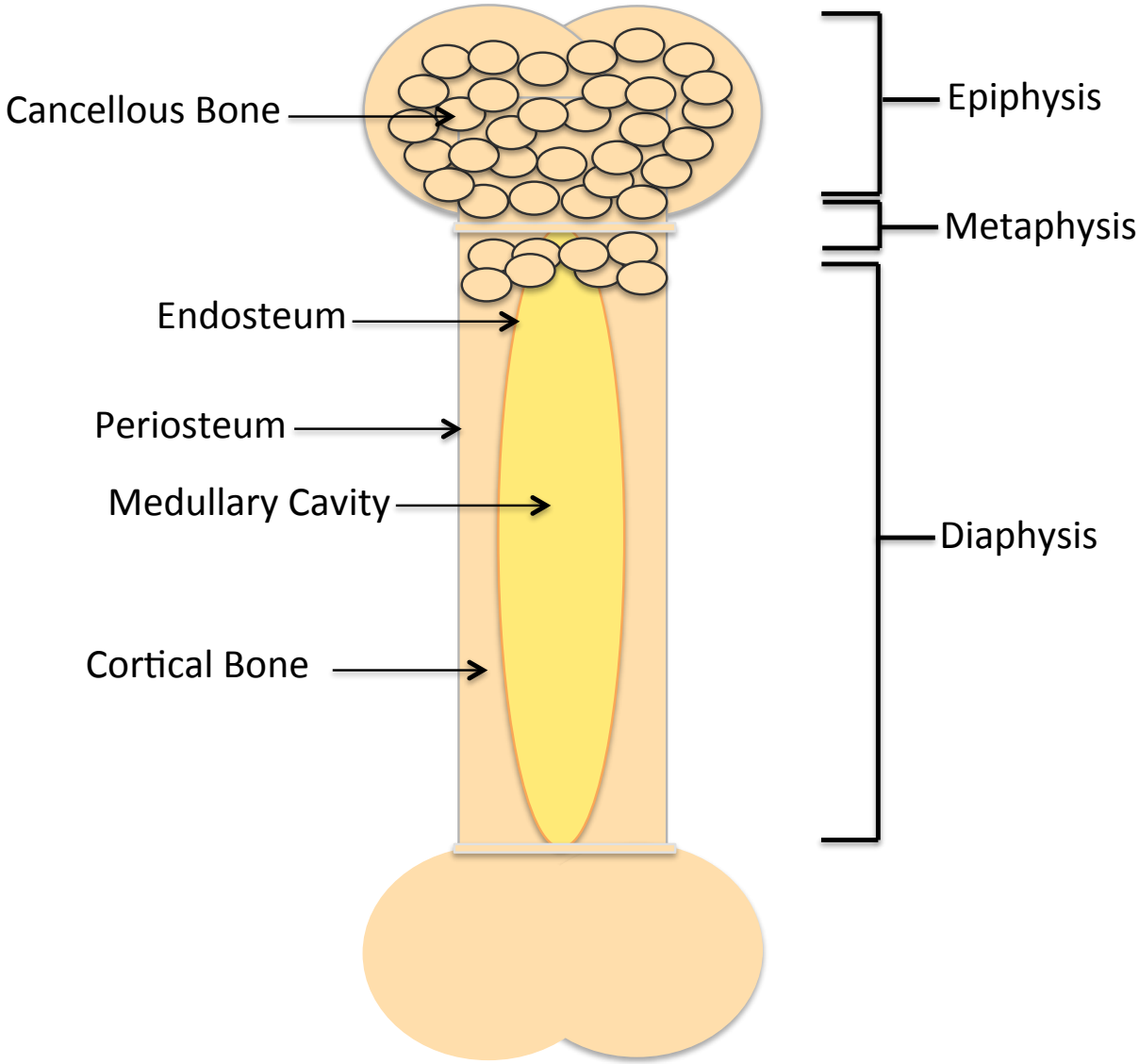

Figure 1.1: Schematic Representation of a Long Bone
Long bones are composed of two types of bone. Dense compact (cortical) bone that surrounds the medullary cavity making up the diaphysis, while the epiphysis and metaphysis are comprised of spongy cancellous (trabecular) bone. The periosteum and endosteum are thin fibrous sheaths that cover the outside and inside of long bone respectively.

1.1.3 Cellular Components of Bone

1.1.3.1 Osteoblast

Osteoblasts are cells responsible for the generation of bone matrix and for facilitating its mineralization. Derived from mesenchymal stem cells, osteoprogenitor cells can differentiate into adipocytes, fibroblasts, chondrocytes, and myoblasts. Appropriate activation pathways are necessary for osteoblastogenesis. Any alterations to this pathway can result in several skeletal pathologies. This section will cover some of the primary mechanisms of osteoblastogenesis.

The first step in osteoblast differentiation is the selection of osteoprogenitor cells from mesenchymal stem cells. As mentioned above, mesenchymal stem cells have many fates and are directed to their specific fate by transcription factors. Differentiation into osteoblasts is dependent on the canonical Wnt/β-catenin pathway and bone morphogenic proteins (BMPs). This signaling is an essential event in bone development as it promotes osteoblast differentiation from mesenchymal progenitors at the expense of adipogenesis (5,6)(Discussed in further detail later in this chapter).

Runt-related transcription factor 2 (Runx2) is a key transcription factor and master regulator of osteoblast differentiation (7). This was demonstrated in Runx2 null mice that did not develop osteoblasts (8). During the early phase of osteoblast differentiation, Runx2 regulates expression of genes encoding for osteocalcin, receptor activator of nuclear factor kappa β ligand (RANKL), sclerostin and dentin matrix protein 1 (DMP-1). In addition to Runx2, osterix is a secondary transcription factor essential for further osteoblast differentiation towards pre-osteoblasts (7). Pre-osteoblasts can express low levels of alkaline phosphatase (ALP), which is an early marker for mature osteoblasts. After

differentiation is complete, mature osteoblasts express alkaline phosphatase and type 1 collagen, which is necessary for the synthesis of the bone matrix (7). At the end of their lifespan, osteoblasts undergo the following fates: apoptosis, mature to become osteocytes or become inactive bone lining cells that form a single layer of cells along the bone surface.

1.1.3.2 Osteocyte

Mature osteoblasts embedded in the bone matrix are defined as osteocytes. The process of becoming an osteocyte involves osteoblasts being surrounded by their secreted extracellular matrix. Consisting mostly of type I collagen with lesser amounts of matrix proteins, this unmineralized material is often called osteoid. These osteoblasts present in the osteoid are termed osteoid osteocytes or pre-osteocytes (9). As the mineralization process continues the osteocytes become fully embedded within the mineralized bone matrix. This process leads to a syncytium of osteocytes in which the cells are connected via thin cell processes that allows for communication between neighboring osteocytes. These processes reside in channels called canaliculi, which allow for nutrients and oxygen from blood vessels to reach the osteocytes as well as signaling molecules from one osteocyte to the next. This anatomy allows for osteocytes deep within the bone matrix to communicate with cells on the bone surface (9).

Due to their location, these cells can sense stress forces placed on the bone and respond accordingly. Thus, osteocytes serve as mechanosensors and play a significant role in the repair of bone microdamage (10). Osteocytes respond to mechanical loading by sending signals to osteoblasts and osteoclasts that modulate their activity (11). Recent studies have shown that in response to stress forces osteocytes release factors such as

RANKL, sclerostin, nitric oxide and insulin-like growth factor-1 (IGF-1) that modulate bone remodeling (11).

1.1.3.3 Osteoclast

Unlike osteoblasts, osteoclasts are giant, multinucleated cells (typically 10-20 nuclei) derived from the hematopoietic stem cell lineage. They share a common differentiation pathway with macrophages up until the final differentiation stage (1). Starting from hematopoietic stem cells, macrophage colony stimulating factor (M-CSF) drives the commitment towards a common progenitor via activation of their colony-stimulating factor-1 receptor (c-fms) (12). M-CSF receptor activation stimulates proliferation of osteoclast precursors and upregulates receptor activator of nuclear factor κ B (RANK) expression. With both c-fms and RANK receptor activation, precursors are fully committed to the osteoclast lineage.

In order for bone resorption to occur, osteoclasts must be in physical contact with the bone matrix. To do this, integrins expressed by osteoclasts bind with specific amino acid sequences within proteins (osteopontin and sialoprotein) on the surface of the bone matrix (13). After adhesion, osteoclasts undergo cytoskeletal reorganization and assembly of dynamic structures called podosomes (13,14). Integrin signaling and podosomes allow for osteoclasts to create a sealing zone (15). Once sealed the osteoclasts create a low pH environment via the fusion of acidic vesicles and membrane-bound proton pumps. This environment dissolves the inorganic crystalline hydroxyapatite (4). Further secretion of lytic enzymes such as cathepsin K, tartrate-resistant acid phosphatase (TRAP) and matrix metalloproteases (MMPs) begin to digest the organic matrix (2,4,16). After resorption, the remaining degraded ions and collagen fibrils are digested by cathepsins, and the residues

from this final digestion are internalized and transported across the cell and released into the extracellular fluid (13).

1.1.4 Bone Remodeling

Bone remodeling is a dynamic process that balances the formation and resorption of bone. This is not only important for repair of microdamage but also helps maintain strength. This process is also crucial for regulating serum calcium levels by releasing mineral from the bone matrix as required. In the adult human, remodeling replaces approximately 5-10% of the skeleton each year (2,17). Bone remodeling occurs through activities of a group of cells called the bone-remodeling unit (BRU). There are 4 cell types that make up the BRU: 1) osteoblast, the cell that produces the organic bone matrix and aids in its mineralization (4); 2) osteoclasts, responsible for the degradation and resorption of bone (4); 3) osteocyte, a mature osteoblast-derived cell that lies within the bone matrix and acts as a mechanosensor as well as an endocrine cell (9); and 4) the bone lining cells, that help couple the processes involved in bone remodeling (18). There are four phases of the bone remodeling cycle: initiation/activation, resorption, reversal and formation (Fig 1.2)(17).

1.1.4.1 Initiation/Activation Phase

The initiation/activation phase can be induced by different signals including mechanical strain sensed by osteocytes or by cytokines or systemic factors in the bone environment. These factors include IGF-1, tumor necrosis factor-α (TNF- α), interleukin-6 (IL-6) and parathyroid hormone (PTH), all of which activate the bone-lining cells. As a result, bone-lining cells increase their expression of RANKL, which activates RANK

receptors on the membranes of pre-osteoclasts. This interaction initiates pre-osteoclast fusion and differentiation toward mature multinucleated osteoclasts.

1.1.4.2 Resorption Phase

The resorption phase begins as bone-lining cells expose the bone matrix where mature osteoclasts can attach (19–21). To accomplish this, osteoclasts polarize and adhere to the bone surface and begin to dissolve bone. Resorption occurs in two steps: 1) acidification to dissolve the inorganic component of the bone matrix and 2) the release of lysosomal enzymes responsible for the breakdown of the organic component of bone (12). Once the bone resorption process is completed, osteoclasts undergo apoptosis (4,18).

1.1.4.3 Reversal Phase

After the resorption phase is complete, the reversal phase begins. The first step of this phase involves macrophage-like cells moving into the resorption area to remove debris such as old demineralized collagen (13). During this process, osteoblast precursors move into the resorption pit area.

1.1.4.4 Formation Phase

Resorption leads to the release of several factors stored within the bone matrix, including BMPs, fibroblast growth factors (FGFs) and transforming growth factor β (TGF-β). These growth factors are responsible for the recruitment of pre-osteoblasts to the resorbed area (12). Once recruitment and maturation of osteoblasts is complete, the formation phase begins. Mature osteoblasts first secrete non-calcified osteoid, which is composed of mostly type 1 collagen along with specific bone proteins such as osteopontin and osteocalcin (4). To promote mineralization of the secreted osteoid, osteoblasts release matrix vesicles. These vesicles allow for initial mineral deposition by concentrating

phosphate and calcium ions. After the bone formation is complete, osteoblasts undergo apoptosis, become bone-lining cells or become trapped in the mineralized bone matrix where they undergo further differentiation into osteocytes. The entire remodeling process usually takes around 200 days to complete and is highly regulated with resorption and formation being tightly coupled, leading to the same amount of bone tissue at the beginning and end of each cycle (Fig 1.2).

1.1.4.5 RANK/RANKL/OPG Triad

Direct contact between osteoblasts and osteoclasts is critical for coupling of bone formation and resorption. Key regulators of bone remodeling depend on interactions between RANK, RANKL, and osteoprotegerin (OPG). RANK is a cell surface receptor present on the cell membrane of premature and mature osteoclasts. Activation of RANK receptor together with M-CSF receptor stimulates osteoclast differentiation and activity (Fig 1.3). RANKL activates the RANK-signaling cascade (in osteoclasts) and is secreted by stromal cells, immune cells, osteoblasts, and osteocytes. In addition, OPG is secreted by osteoblasts and osteocytes and is a soluble decoy receptor that blocks RANKL, thereby inhibiting osteoclast differentiation and activity. There are also other cells that can alter the balance of the RANK-RANKL-OPG triad. This includes activated T cells that have been shown to secrete RANKL to promote osteoclastogenesis and bone resorption (22,23). In addition, levels of hormones and cytokines, including sex steroids, PTH, interleukin 1 (IL-1) and prostaglandin E2 (PGE-2), can affect the secretion of RANKL, OPG and M-CSF thus, altering the balance of bone formation and resorption (2,4,24).

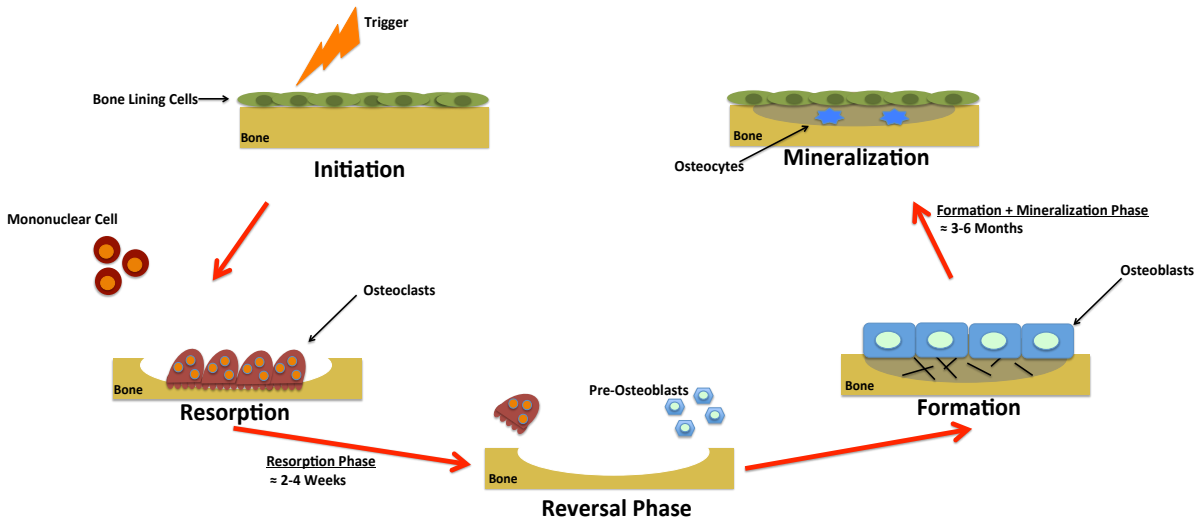

Figure 1.2: The Bone Remodeling cycle
A representative image of the bone remodeling cycle. Certain triggers leading to recruitment and differentiation of osteoclasts initiate bone remodeling. Mature osteoclasts resorb old bone followed by the recruitment and differentiation of pre-osteoblasts. Mature osteoclasts start the formation process by laying down new osteoid, which is subsequently mineralized to form new bone.

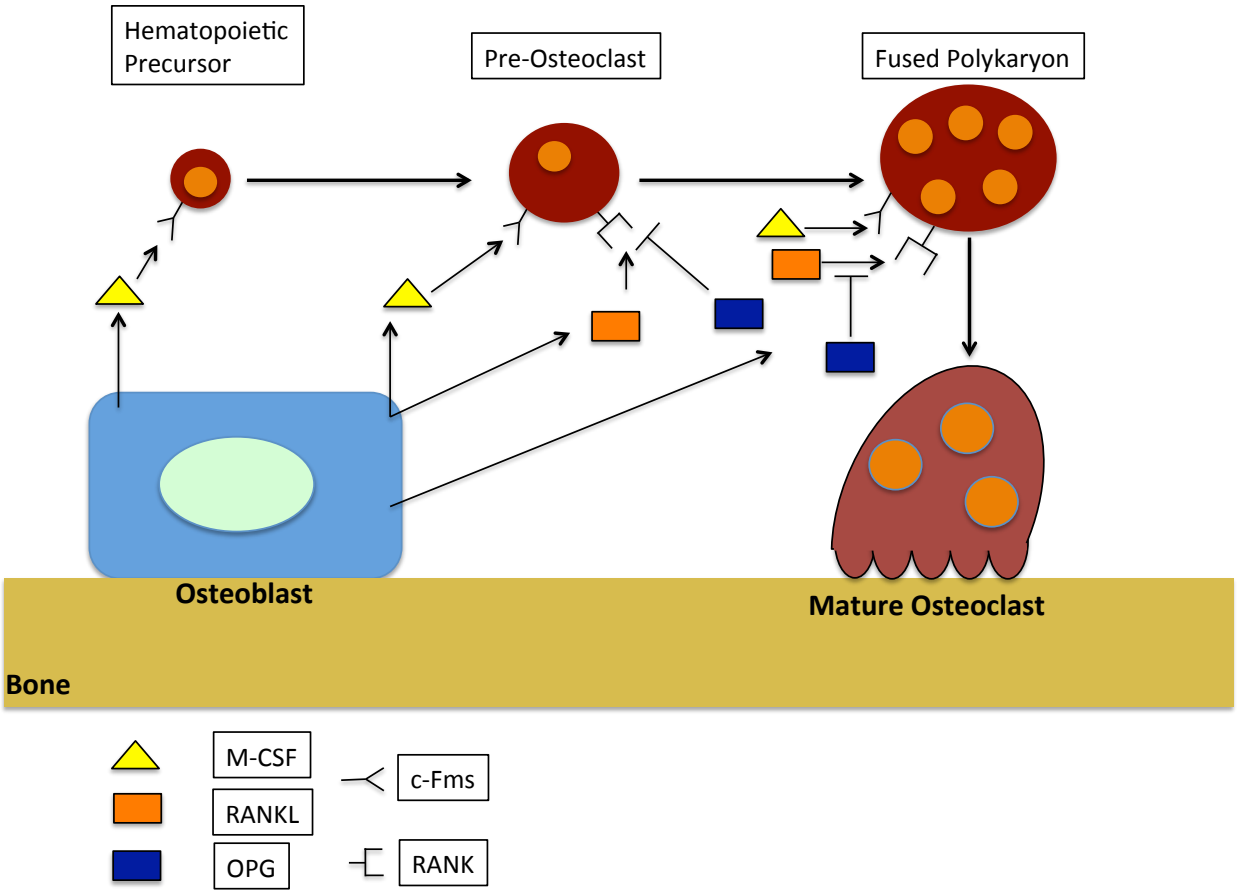

Figure 1.3: RANK/RANKL/OPG Triad
Hematopoietic precursors from the bone marrow are recruited to site of bone remodeling. In the presence of MCSF and RANKL, cells undergo proliferation and differentiation into multinucleated osteoclasts. This process is controlled via soluble decoy receptor osteoprotegerin (OPG) released from osteoblasts to compete with RANKL for RANK binding.

1.2 Bone Pathophysiology

Disruption of the remodeling process can result in a variety of bone disorders (25). Some of these include osteomalacia, rickets, rheumatoid arthritis, and osteoarthritis. However, the most common bone disease and pathophysiology this thesis will focus on is osteoporosis.

1.2.1 Osteoporosis Definition

Osteoporosis is a disease characterized by low bone mass resulting from changes in bone microarchitecture and deterioration of bone tissue. This leads to a decrease in bone strength and increased risk of fracture (26,27). The National Osteoporosis Foundation defines osteoporosis as a bone mineral density (BMD) value that statistically falls 2.5 standard deviations below the mean of a young adult.

1.2.2 Classification of Osteoporosis

Osteoporosis can be classified as either primary or secondary depending on the mechanisms involved in bone loss. The International Osteoporosis Foundation (IOF) has four different subgroups of classification for bone health based on T-score: 1) Normal bone: BMD or bone mineral content (BMC) not more than 1 standard deviation below the mean of a young adult. 2) Low bone mass (osteopenia): BMD/BMC between 1 and 2.5 standard deviations below the mean of a young adult. 3) Osteoporosis: BMD or BMC 2.5 standard deviations below the mean of a young adult and 4) Severe Osteoporosis: BMD or BMC 2.5 standard deviations below the mean of a young adult with one or more fragility fractures (26).

1.2.2.1 Primary Osteoporosis

Primary osteoporosis, the most common form, is a result of normal physiological processes such as aging or menopause. The IOF has further classified primary osteoporosis into type I or type II (26). Type I is known as postmenopausal osteoporosis which is caused by the deficiency of estrogen. This lack of estrogen leads to an increase in pro-inflammatory cytokines, which in turn increases bone resorption (high turnover osteoporosis)(28). Type I mainly affects trabecular bone causing increased fractures at the wrist and vertebral sites (26). Type II is described as senile osteoporosis and is related to cortical and trabecular bone loss due to aging (29,30).

1.2.2.2 Secondary Osteoporosis

Secondary osteoporosis is less common than primary and is caused by specific medical treatments or conditions that interfere with bone metabolism. Secondary osteoporosis is a frequent pathology associated with many inflammatory conditions such as rheumatoid arthritis or inflammatory bowel disease (IBD). These disease conditions are characterized by high bone turnover due to elevated levels of pro-inflammatory cytokines (31). However, the most common cause of secondary osteoporosis is due to side effects of corticosteroid treatments, (>5 mg prednisolone daily or equivalent for >3 months)(31) which will be discussed in further detail later in this chapter.

1.2.3 Epidemiology of Osteoporosis

It is estimated that there are more than 300 million patients worldwide who have osteoporosis (32). In the United States and Europe, approximately 30% of all postmenopausal women suffer from this disease (33). At least 50% of women (1 in 2) and 15-30% of men (1 in 5) over the age of 50 will sustain an osteoporosis-related fracture

during their remaining lifetime (34,35). Fractures can have severe consequences on the patient's quality of life. For example during the first year following a hip fracture the mortality rate is 36% for men and 21% for women (33). According to statistics, the cost of sustaining an osteoporosis-related fracture is approximately $19 billion per year, a value that is predicted to double in the next 25 years highlighting the economic consequences of this disease.

1.2.4 Current Osteoporosis Therapies

1.2.4.1 Non-Pharmacological Treatments

Non-pharmacological approaches are the first line of treatment to prevent osteoporosis. These therapies can be split into lifestyle and dietary changes.

1.2.4.1.1 Lifestyle Changes

Baseline lifestyle changes for prevention of osteoporosis include increased physical activity, cessation of smoking, alcohol reduction and reduced sodium intake. Increased weight bearing exercises will increase the mechanical load placed on bones, thus allowing for osteocytes to increase bone remodeling (36). Exercise intervention also reduces the occurrence of falls (36). Cessation of smoking and reduction of alcohol are beneficial as both have been linked to adverse skeletal effects and are associated with an increased risk of fracture (37). Reduction in sodium is also used, as high sodium diets can have negative impacts on calcium balance by increasing urinary calcium excretion (36).

1.2.4.1.2 Dietary Supplements

In addition to lifestyle changes, dietary supplements such as vitamin D and calcium can be used to prevent osteoporosis (29). These dietary supplements are essential for improving bone density (29). The Institute of Medicine (IOM) suggests that men and

women over the age of 50 should consume between 1,000 and 1,200 mgs of calcium daily (38). However, the side effects of increasing calcium consumption include increased risk for kidney stones and myocardial infarction (39). Increased vitamin D intake is also used to prevent osteoporosis. The IOM recommends men and women over 50 years of age intake 600 international units (IU) per day and for those patients over the age of 70, 800 IU per day (38).

1.2.4.2 Pharmacological Treatments

If non-pharmacologic treatments fail, patients can explore pharmacologic approaches, which include anti-resorptive and anabolic therapies.

1.2.4.2.1 Anti-Resorptive Treatments

Anti-resorptive interventions account for the bulk of osteoporotic treatments and include estrogen replacement therapy, selective estrogen receptor modulators (SERMS), bisphosphonates and monoclonal antibodies directed towards RANKL (Denosumab).

1.2.4.2.1.1 Estrogen Treatment

Treatments with pharmacologic doses of estrogen have beneficial effects on bone by increasing trabecular bone volume and cortical thickness (40–42). Estrogen treatment is usually administered as a daily transdermal patch or pill. However, the use of estrogen treatment has waned considerably due to concerns about an increased risk of cancer (43).

1.2.4.2.1.2 Selective Estrogen Receptor Modulators

Raloxifene is a SERM used for the management of osteoporosis and is often prescribed to women who cannot utilize estrogen therapy due to an increased risk of breast cancer (44). SERM's have tissue-specific effects and can act either as an estrogen agonist in bone or as an antagonist in breast tissue. Activation of estrogen signaling in bone

increases bone remodeling thereby preventing osteoporosis (44). However, Raloxifene may increase the risk of venous thromboembolic disease and stroke (44).

1.2.4.2.1.3 Bisphosphonates

Bisphosphonates, unlike estrogen and SERMs, are chemically stable, inorganic pyrophosphate analogs with extremely high affinity for the mineral component of bone (45). This high affinity allows bisphosphonates to reach high concentrations within the skeleton. Newer generation bisphosphonates have nitrogen side chains, which allow them to adhere more tightly to the hydroxyapatite mineral than previous generations (45,46). Once embedded into bone mineral, actively resorbing osteoclasts take up bisphosphonates, which inhibit farnesyl pyrophosphate synthase (FPPS)(47,48). Inhibition of FPPS induces lipid modification within osteoclasts, ultimately leading to apoptosis (47). While this treatment does decrease bone loss, it does not increase bone formation. Long-term use of bisphosphonates has been linked to rare cases of osteonecrosis of the jaw and increased risk of irregular or non-union fractures (49).

1.2.4.2.1.4 Monoclonal Antibodies

Denosumab is a monoclonal antibody that limits osteoclast-mediated bone resorption (27,50,51). Denosumab binds to RANKL, preventing RANK activation, ultimately disrupting osteoclastogenesis. In a clinical trial involving 7868 women with a T-score between -2.5 and -4.0 at the lumbar spine or hip, treatment with Denosumab every six months for three years reduced vertebral fractures by 68%, hip fractures by 40%, and nonvertebral fractures by 20% compared to placebo controls (52). Although Denosumab has shown promise, treatment is expensive and has side effects including bone and muscle pain, low blood calcium levels and increased risk of infection (50,53).

1.2.4.2.2 Anabolic Treatments

While all of the above treatments target osteoclasts to prevent bone resorption they share a common defect in that they do not directly affect osteoblasts and bone formation. This means that although patients undergoing treatments do not lose more bone, they are still at an increased risk for fracture. Currently anabolic treatments for osteoporosis are limited to intermittent PTH treatment.

1.2.4.2.2.1 Intermittent Parathyroid Hormone

Teriparatide is a recombinant PTH treatment that is the only FDA-approved skeletal anabolic agent (54,55). Daily treatment involves subcutaneous injections that increase both bone formation and resorption, with formation outweighing resorption particularly in the first 6 to 12 months of treatment (55). However, due to the potential for osteosarcoma formation, the FDA has limited the treatment to 24 months in a lifetime (55).

1.2.4.2.3 Emerging Therapies for Osteoporosis

With current osteoporosis treatments presenting with multiple side effects, the need for new therapies is warranted. Emerging approaches include a FDA approved monoclonal antibody drug that inhibits sclerostin (Romosozumab). Sclerostin is secreted by osteocytes and inhibits osteoblast activity. Treatment with Romosozumab increased bone mineral density and decreased the risk of vertebral fractures in postmenopausal women after 12 months (56). Additional approaches include integrin antagonists that can prevent bone resorption by inhibiting osteoclast attachment to the extracellular matrix (57). Recently, probiotics have demonstrated the ability to prevent bone loss (58). Probiotics are a novel potential therapeutic agent that is promising due to lack of side

effects. The beneficial effects of probiotics on bone density will be described in detail later in this chapter (Sections 1.6 and 1.7).

1.2.5 Risk Factors for Osteoporosis

Many factors increase the risk of developing osteoporosis. Some include self-controlled factors such as a sedentary lifestyle, excess alcohol or smoking, low calcium and vitamin D intake and low body mass index. However, many more risk factors are uncontrollable such as gender, age, family history, ethnicity, diseases, and certain medications. As the aging process occurs, the chance of decreased bone density increases, due to complications from diseases or treatments for those diseases such as glucocorticoids. As mentioned previously, glucocorticoid treatment is the most common cause of secondary osteoporosis.

1.3 Glucocorticoid Induced Osteoporosis

1.3.1 Glucocorticoid Effects on Bone

Synthetic glucocorticoids (GCs) are anti-inflammatory drugs used to treat a variety of disorders including autoimmune, pulmonary and gastrointestinal diseases. Although the need for GCs anti-inflammatory effects in these various conditions is clear, their use is fraught with a host of potential side effects. One organ system specifically affected by GC treatment is the skeleton as seen with glucocorticoid-induced osteoporosis (GIO)(59). Cushing et al., first reported GIO in 1932 when they described bone loss in patients with high levels of cortisol due to a tumor in the pituitary gland (60). The GIO issue became more clinically relevant in 1949 when pharmacological doses of GCs were introduced due to their potent anti-inflammatory and immunosuppressive effects. Interestingly, physiologic secretion of the GC hormone cortisol is essential for the differentiation and function of osteoblasts and osteoclasts, whereas pharmacological doses inhibit bone formation (61). GC treatment can directly affect bone cells as well as affect other systems that rapidly induce bone loss. This leads to 30-50% of patients undergoing long-term GC treatment sustaining a fracture (62). Although GC treatment causes bone loss and fractures, many patients receiving treatment are not evaluated for their skeletal health. Therefore, patients often do not receive osteoporosis preventive or therapeutic agents when needed (63–65).

1.3.2 Effects of Glucocorticoids on Osteoblasts

GC treatment has multiple adverse effects on osteoblasts. GCs act via cytosolic GC receptors (61,66) impairing osteoblast differentiation/function and increasing apoptosis resulting in decreased bone formation (67).

1.3.2.1 Osteoblast Differentiation

Pharmacological doses of GCs directly affect osteoblast precursors, driving their differentiation towards the adipocyte lineage instead of the osteoblast lineage (68–70). GC redirection of mesenchymal cells is due to induction of nuclear factors of the CCAAT enhancer binding protein family and peroxisome proliferator-activated receptor γ 2 (PPARγ2)(67,71–73). Together, these play an essential role in directing stem cells towards the adipocyte lineage.

Additional mechanisms by which GCs inhibit osteoblast differentiation is by opposing the Wnt/β-catenin pathway, a key regulator of osteoblastogenesis (67,74–79). Under normal conditions, Wnt-7/10b binds to specific frizzled receptors, and two co-receptors called low-density lipoprotein receptor-related proteins (LRP-5/6). This leads to inhibition of glycogen synthase kinase 3 (GSK-3β) activity, which allows stabilized β-catenin to translocate into the nucleus where it associates with transcription factors that regulate gene expression and promote osteoblastogenesis (6). However, GC treatment opposes Wnt/β-catenin signaling by enhancing the expression of Dickkopf-1 (DKK1)(59). DKK1 is an antagonist that prevents Wnt from binding to its frizzled-LRP 5/6-receptor complex (80). In addition, treatment with GCs maintain GSK-3β levels in an active state. Together, both of these mechanisms lead to inactivation of β-catenin, preventing its translocation into the nucleus and thus inhibiting osteoblastogenesis (76,79–81). In addition to its effects on Wnt signaling, GCs inhibit mature osteoblasts from synthesizing type 1 collagen (67). As mentioned previously, type 1 collagen is a major component of the bone extracellular matrix and prevention of its synthesis has an impact on the amount of

bone matrix available for mineralization (68). This decrease in collagen synthesis occurs via transcriptional and post-transcriptional mechanisms (82).

1.3.2.2 Osteoblast Apoptosis

In addition, to negatively regulating osteoblast differentiation and activity, GC treatment increases apoptosis of osteoblasts through activation of caspase 3, a common downstream effector of several apoptotic-signaling pathways (61,83–87). Studies have shown that GC receptor activation and translocation to the nucleus is associated with a decrease in the Bcl-2/Bax protein ratio (84,88). After GC receptor activation and translocation, Bax activation initiates the release of cytochrome c from the mitochondria, ultimately leading to activation of the caspase cascade (66). Active Caspase 3 contributes to apoptosis by cleaving essential nuclear, plasma membrane and mitochondrial proteins (89). GC induced activation of this pathway leads to apoptosis of osteoblasts and osteocytes in the femoral and vertebral trabecular bone in both human patients and animal models (59,87,90–95). This mechanism is thought to be a key component in the pathophysiology of GIO.

1.3.3 Effects of Glucocorticoids on Osteocytes

As previously mentioned, osteocytes are mature osteoblasts which have become embedded into the bone matrix and play a significant role in microdamage repair (96,97). However, similar to osteoblasts, GC treatment also induces apoptosis of osteocytes, leaving the osteocyte lacunae empty (86). In addition to initiating apoptosis, GCs modify the elastic modulus surrounding the lacunae making the lacunae larger (98). Together, these mechanisms disrupt the osteocyte-canalicular network impairing the ability for the remaining osteocytes to detect signals to initiate bone remodeling (97). Disruption of the

network can also alter fluid flow, adversely affecting the material properties of the surrounding bone (88).

1.3.4 Effects of Glucocorticoids on Osteoclasts

In the initial phase of GIO, there is a substantial increase in bone resorption (99). This is due to GCs increasing overall expression of RANKL and decreasing OPG, leading to increased osteoclast differentiation and resorptive activity (99). Furthermore, GC treatment has been shown to up regulate the expression of M-CSF, which is critical for the survival and proliferation of osteoclast precursors (99). Recent studies have shown that GCs may suppress the expression of anti-osteoclastogenic cytokines, such as interferon β, thereby promoting osteoclastogenesis (100).

1.3.5 Role of Endocrine Factors in Glucocorticoid Induced Bone Loss

1.3.5.1 Calcium Absorption

GC treatment affects nearly every system in the body; including decreasing calcium absorption from the gastrointestinal tract by inhibiting vitamin D action (59,101). Furthermore, GCs also inhibit renal tubular calcium reabsorption (59). As a consequence of decreased blood calcium following GC treatment, secondary chronic hyperparathyroidism (increased parathyroid hormone, PTH) develops (62,99,102). Although acute increases in PTH have been shown to have anabolic bone effects, chronic increases, as in the case of GC treatment lead to catabolic bone effects (Fig 1.4)(62,102). In addition, GCs have been shown to enhance the sensitivity to PTH by increasing the number of receptors on osteoblasts (103).

1.3.5.2 Gonadal Hormones

GCs influence the production and action of hormones that can regulate bone metabolism. GC treatment has been shown to reduce gonadotropin, leading to a decrease in sex steroid levels. This is noteworthy as estrogens and androgens influence the growth and maintenance of the mammalian skeleton and deficiency in these steroids during menopause or old age contribute to the development of osteoporosis (Fig 1.4)(104). Due to this, the administration of sex steroids has been used in the management of hypogonadal patients receiving GCs (105).

1.3.5.3 Growth Hormones

GC treatment can modulate the growth hormone (GH)-IGF-1 axis (106). Under normal conditions, GH and IGF-1 induce longitudinal growth and maintain levels of bone remodeling (2,107). Previous studies have shown that GC treatment decreases secretion of GH and decreases IGF-1 transcription in osteoblasts (106,108,109)(Fig 1.4). However, serum concentrations of GH and IGF-1 have been reported to be normal in patients receiving GCs (110–112). Despite normal levels, IGF-1 activity is decreased in patients undergoing GC treatment, due to increased IGF-1 inhibitor (113). This inhibitory factor could be one of the many IGF-binding proteins (IGFBP). Additional studies have shown that GCs increase circulating levels of IGFBP-1, which limits the activity of IGF-1 and is associated with glucocorticoid-induced fetal growth retardation (114).

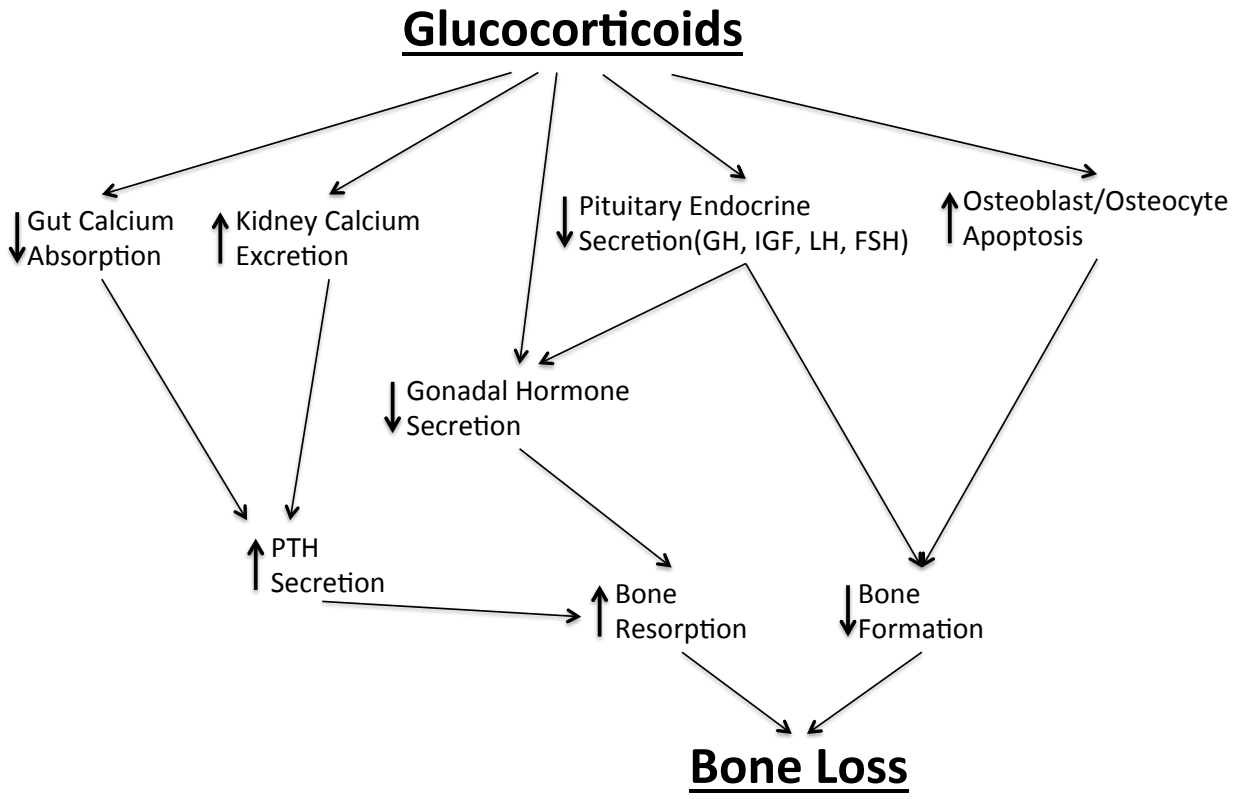

Figure 1.4: Effect of steroids on bone and calcium metabolism
Pharmacological doses of glucocorticoid inhibit gastrointestinal absorption and increase renal excretion of calcium. Decreases in serum calcium levels and perhaps failure to transport calcium into parathyroid cells cause an increase in the release of parathyroid hormone (PTH). PTH increases the number of bone sites undergoing bone remodeling leading to a net increase in bone resorption. Inhibition of secretion of gonadal hormones caused by treatment with glucocorticoids further augments bone resorption. Glucocorticoids decrease the differentiation and increased apoptosis of osteoblasts and osteocytes, and inhibit bone formation. The combination of all these processes is thought to be the main reasons for glucocorticoid-induced osteoporosis.

1.3.6 Treatment of Glucocorticoid induced Osteoporosis

The osteoporosis therapies discussed above (section 1.2.4) are used for both primary and secondary osteoporosis. This section will focus specifically on treatments for secondary GIO. The American College of Rheumatology (ACR) and the Royal College of Physicians published guidelines that advocate for the following measures for prevention and treatment of GIO: 1) general health awareness, 2) administration of sufficient calcium and vitamin D, 3) reduction of dose of GC to minimum and 4) therapeutic intervention with bisphosphonates and other agents if needed (105). The ultimate goal of these measures is to prevent fractures. Some appropriate prevention measures include increasing daily calcium (1500 mg/day) and vitamin D (800 IU/day) intake (115). Depending on the patients BMD and other risk factors, the use of vitamin D and calcium may be sufficient, particularly if the dose of GCs is lower than 7.5 mg per day and is administered for less than three months (105,115). However, for those patients exposed to GCs for longer than three months, the ACR recommends a proactive approach. This includes lifestyle changes such as tobacco cessation, alcohol reduction and increased exercise in conjunction with bisphosphonate therapy.

Postmenopausal women exposed to GCs are at even higher risk of bone loss. As estrogen deficiency combined with GCs effects on bone health exacerbate bone resorption (59). In this case, the use of anti-resorptive therapy and oral bisphosphonates can be used to blunt bone loss (44,116,117). Studies have shown that a combined treatment of estrogen and bisphosphonates can have additive therapeutic effects on osteocyte survival (118). Furthermore, the ACR suggests that treatment with bisphosphonates should be started in

male patients who are receiving a daily dose of prednisone (>5 mgs) with T-scores of 1.0 standard deviation below the mean of a young adult (115).

In addition to anti-resorptive agents, pulsed PTH is used as an anabolic agent that effectively reduces vertebral and non-vertebral fractures (119,120). Studies have shown that PTH protects against osteoblast apoptosis and increases osteoblast number. Thus, PTH treatment has been proposed for treating postmenopausal women with rheumatoid arthritis taking prednisone (120–124). This treatment has shown promise, as daily treatment with 1-34 hPTH increased vertebral BMD and modestly increased bone mass in the hip region (123,124).

1.4 The Gut-Bone Axis

It was first thought that the interaction between the gut and bone was primarily attributed to gastrointestinal absorption of minerals such as calcium and phosphorous. However, the gut can also secrete endocrine factors such as incretins that can affect bone remodeling. In this section, I will cover mechanisms by which the gut can affect bone health.

The intestinal epithelium plays a significant role in maintaining host health with its ability to digest and absorb nutrients. It also provides a barrier from the outside world by preventing translocation of harmful products or bacterial pathogens from getting into the systemic circulation. To accomplish this, the gastrointestinal tract is comprised of a single continuous layer of intestinal epithelial cells linked together by tight junction proteins (Fig 1.5). Nutrients are absorbed via transcellular and paracellular pathways (Fig 1.5). Within the epithelial layer are specialized cells (goblet cells), which secrete a mucus layer, essential for limiting the ability of the luminal gut bacteria from accessing host cells. This luminal commensal bacteria, or gut microbiota, is thought to be home to ~100 trillion microbes comprising ~1000 species and 28 different phyla (125). Of these phyla, the microbiome composition is dominated by *Bacteroidetes*, *Firmicutes*, *Actinobacteria*, *and Proteobacteria* (125). In response to this bacterial presence, the intestine can secrete defensins and other antimicrobial products in order to maintain a healthy environment (Fig 1.5).

The preservation of the epithelial barrier is critical as disruption can affect: 1) nutrient absorption; 2) pathogen translocation into the bloodstream; and 3) alteration of the gut microbiome composition (dysbiosis)(126). Furthermore, disruption in barrier

function is associated with gastrointestinal diseases such as IBD, celiac disease and colon cancer (127–129). However, whether barrier dysfunction is due to alterations to the gut microbiome in these diseases or a consequence of outside disease signals still needs to be researched. Barrier dysfunction can lead to increased systemic inflammation that can contribute to bone loss. Indeed, in diseases such as IBD, characterized by dysbiosis and barrier break, bone loss is often present in patients (130). Furthermore, we recently demonstrated that post-ABX dysbiosis and barrier dysfunction can cause significant bone loss in mouse models.

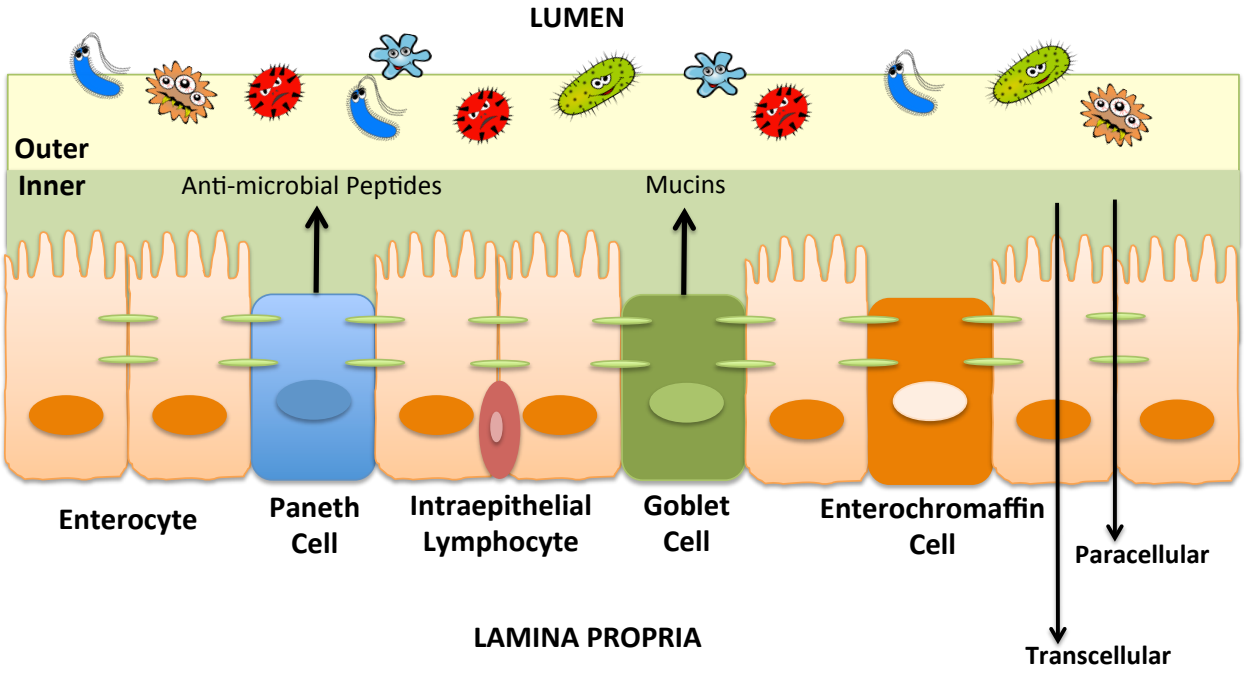

Figure 1.5: Simplified schematic representation of the intestinal layer
The intestinal epithelial barrier is made up of numerous cell types. Enterocytes, the absorptive cell, have microvilli on their apical surface further increasing their surface area for digestion. In between enterocytes are goblet cells, enterochromaffin cells, paneth cells, and intraepithelial lymphocytes. Goblet cells produce mucus which forms a protective layer over the epithelial barrier. The mucus layer consists of two layers; a loose stirred outer layer and an adherent inner layer. Intraepithelial lymphocytes have many functions including forming a first line of defense against pathogens in the lumen, suppressing excess inflammation and helping ensure integrity of the epithelial barrier. Paneth cells produce antimicrobial peptides, defensins and lysozymes for additional protection. Enterochromaffin cells are a type of enteroendocrine cell that are crucial in motility and secretion. They also are involved in secretion of the neurotransmitter serotonin.

1.4.1 Bone Mineral Absorption and the Gut-Bone Axis

Intestinal absorption of calcium is a critical physiologic process for maintaining bone mineralization and serum calcium homeostasis. Early in life, this process allows for the achievement of peak bone mass and, later in life, the maintenance of bone mass. Calcium homeostasis involves the intestine, kidney, bone and parathyroid glands, which work in unison to maintain serum calcium within a narrow range. To do this, calcium absorption occurs through a transcellular pathway (metabolically driven) or paracellular pathway (passive non-saturable)(131). The transcellular pathway moves calcium from the mucosal to serosal side, which occurs against the concentration gradient, mostly in the duodenum and jejunum. This is an active saturable process that is regulated by nutritional and physiological factors, including vitamin D (132). The paracellular pathway is a non-saturable/passive transport that occurs across the majority of the intestine and is dependent on luminal calcium concentration (133). The luminal pH mainly determines the level of soluble calcium that is available for absorption. This begins with the acidic environment in the stomach, which dissolves calcium salts into ions. In the intestine when pH decreases, calcium solubility increases. This makes the duodenum that has a pH of 6.0, the site of maximum calcium solubility (134).

1.4.1.1 Regulation of Mineral Absorption

As mentioned above, calcium homeostasis involves input from the intestine, kidneys, bone and parathyroid gland. Parathyroid hormone (PTH) is one of the most important regulators of calcium homeostasis. Under conditions of hypocalcemia, PTH release is increased which stimulates: 1) bone resorption; 2) decreases urinary calcium loss by stimulating renal calcium reabsorption; and 3) stimulates 1,25-

dihydroxycholecalciferol (the active form of vitamin D, Calcitriol) in the small intestine to increase intestinal calcium absorption. These three mechanisms work together to increase serum calcium levels. In the case of hypercalcemia, PTH release is decreased, and calcitonin levels are increased. Calcitonin is produced by the parafollicular cells of the thyroid gland and reduces blood calcium levels by two primary mechanisms; 1) prevents the activity of osteoclasts by mediating the loss of the ruffled border, stops motility and inhibits the secretion of proteolytic enzymes needed for degradation of bone mineral matrix; and 2) prevents reabsorption of calcium by the kidneys. Maintaining calcium homeostasis is very important for bone as alterations can have drastic effects on bone remodeling and overall bone health.

1.4.2 Gut-Derived Endocrine Factors that Regulate Bone Remodeling

Another proposed mechanism of how the gut regulates bone is through secretion of hormones such as incretins, serotonin or modulation of growth factors.

1.4.2.1 Regulation of Bone Remodeling by Serotonin

The primary peripheral site of serotonin synthesis is the enterochromaffin cell in the gastrointestinal tract (135)(Fig 1.5). It plays a significant role in regulating sensory and motor gastrointestinal reflexes. Additionally, there is some evidence that serotonin can act through different mechanisms to influence bone remodeling. However, since serotonin is also released as a neurotransmitter in the central nervous system, its actions on bone metabolism can vary, based on its site of synthesis (136,137). In the central nervous system, serotonergic neurons act in the hypothalamus to suppress sympathetic effects on bone. Regular sympathetic input inhibits bone formation; therefore, brain serotonin has been suggested to have a net positive effect on bone health (137). However, gut-derived

serotonin has been shown to inhibit bone growth by attenuating osteoblast proliferation (136,138,139). Additionally, there is evidence that osteoblasts, osteoclasts, and osteocytes can all synthesize serotonin to modulate bone metabolism (137). Early studies performed by Sjögren K et al., investigating the role gut microbiota on bone showed that germ-free female mice displayed increased bone density compared to their conventionally raised cohorts (136). The germ-free mice also displayed decreased serotonin levels suggesting serotonin as a mechanism for how the gut microbiota could affect bone density (136).

1.4.2.2 Incretins and Bone Remodeling

Incretins are metabolic intestinal peptide hormones secreted in response to food ingestion. Gastric inhibitory polypeptide (GIP) and glucagon-like peptide-1 (GLP-1) were initially identified for their effects on nutrient absorption. GIP is secreted by K-cells from the mucosa of the duodenum and jejunum, while GLP-1 is secreted by L-cells in the distal jejunum, ileum, and colon (140,141). Recently findings show that these hormones promote bone formation by stimulating osteoblast differentiation and longevity via activation of their respective receptors (140). GIP has been shown to attenuate osteoclast activity, ultimately leading to an increase in bone mass (140). Furthermore, in humans, GIP treatment (4pmol/kg/min for 15 minutes, followed by 2 pmol/kg/min for 45 minutes) decreases serum c-terminal telopeptides of type 1 collagen (CTX-1), suggesting a role in prevention of bone resorption (142). In addition, alterations to the gut microbiome composition have been shown to alter incretin levels and to improve glucose tolerance, and barrier function (143).

1.4.2.3 Growth Hormones and Bone Remodeling

Typically, GH and IGF-1 are fundamental in achieving normal longitudinal bone growth and mass during development (144). IGF-1 specifically is considered essential for longitudinal bone growth, skeletal maturation, and acquisition of bone mass during development as well as maintenance during adult life (145). This has been supported by decreases in serum IGF-1 correlating with a decrease in BMD in a variety of conditions such as old age and post-menopause (146–150). Recently the gut microbiome has been shown to increase IGF-1 levels to promote bone growth and remodeling (151). Yan et al., showed that increases in short-chain fatty acids (SCFAs) by microbes that ferment fiber, increase serum IGF-1 levels and increase bone density, suggesting yet another mechanism by which the microbiota can affect bone health (151).

1.4.4 Gut Microbiota Dysbiosis

As mentioned above, changes to the intestinal microbiome can have harmful effects on human health (152). Alterations, imbalances or maladaptation to the gut microbial communities is called dysbiosis. Dysbiosis usually occurs when common potentially beneficial species become underrepresented and outcompeted by other potentially harmful species. This can lead to overgrowth of pathogenic bacteria, which can alter microbial homeostasis to the extent that destroys the protective epithelial barrier (153,154). Dysbiosis can be caused by a variety of environmental factors such as diet, geography, disease, or medical intervention (antibiotics)(152,155–159). Antibiotics deplete microbial abundance and alter microbial composition by decreasing bacterial diversity (160). Streptomycin treatment, for example, reduces *Firmicute* levels and depletes levels of *Lactobacillus* species (spp), *Streptococcus* spp and *Enterococcus* spp (161). In addition,

studies have shown that dysbiosis is linked to diseases such as IBD, irritable bowel syndrome (IBS), obesity, diabetes and rheumatoid arthritis (162–165). Recently, dysbiosis has been linked to alterations in bone health (166–168). However, further research is needed to fully understand specific changes to the gut microbiota that affect bone density.

1.4.5 Gut Microbiome Regulation of Bone Remodeling

Several approaches can be used to study the role of the gut microbiota in bone remodeling. The most direct approach is by using germ-free mice, while another approach is by depletion of the gut microbes with antibiotics.

1.4.5.1 Bone Density in Germ Free Mice

Sjögren et al., provided early evidence that the intestinal microbiota could affect bone density (136). In their study, conventional mice, germ-free mice and germ-free mice colonized with a normal microbiota were used to investigate the role of the microbiota in bone health. Bone mass was observed to be higher in germ-free mice compared to that of the conventional mice. Additionally, germ-free mice had reduced number of osteoclasts per bone surface, decreased frequency of CD4+ T cells and osteoclast precursors in their bone marrow. These events were normalized by colonization of the germ-free mice with a conventional microbiota. The exact role that the microbiota plays in the development of bone is not without controversy, as subsequent studies have shown no difference in bone density between conventional mice and germ-free mice (169,170). Additional studies have also identified that while initial colonization acutely reduces bone density, long-term colonization increases bone formation (151). Inconsistencies in results can be attributed to differences in the gut microbiome samples used, sex, age and strain of mice. Furthermore, germ-free mice suffer from developmental defects, as they lack a fully mature immune

system. Germ-free mice present with lower levels of CD4+T cells in the lamina propria and smaller Peyer's patches compared to wild-type mice (171,172). In the bone, levels of TNFα and RANKL are lower in germ-free mice (172). Overall, studies done in germ-free mice should be carefully interpreted due to the role the immune system plays in bone remodeling.

1.4.5.2 Antibiotic Depletion of the Gut Microbiome and its Effects on Bone Density

Antibiotics offer another approach to study the role of the gut microbiome and bone density. In humans, antibiotics are used to treat bacterial infections. However, researchers can use antibiotics to examine the role of the gut microbiome as they; 1) deplete the intestinal microbiome, or 2) alter the microbial community structure following cessation of treatment (161,167,173). In a study completed by Cho I et al., female C57BL/6J mice were chronically treated at the time of weaning with separate antibiotics in their drinking water (penicillin, vancomycin, chlortetracycline). Compared to control mice, every antibiotic treatment group displayed an increase in BMD after three weeks; however, the difference in BMD disappeared after seven weeks of continued treatment (174). Whereas, in another study treating 2-month old BALB/c female mice for six weeks with an antibiotic cocktail (ampicillin, vancomycin, metronidazole, and neomycin) resulted in a decrease in bone formation that was related to reduced IGF-1, which is critical for postnatal bone growth (175,176). Scholz-Ahrens et al., looked at ovariectomized (estrogen deficient) rats to determine the effect of antibiotic treatment on trabecular bone. They showed that compared to sham control, eight weeks of estrogen deficiency caused a significant decrease trabecular number and thickness. This bone loss was significantly prevented in

ovariectomized rats chronically treated with antibiotics (177). In a different study, chronic antibiotic treatment of 4-week old C57BL/6J male mice with ampicillin and neomycin for 16 weeks decreased femoral bone bending (178). Antibiotic effects on the bone before birth have also been examined in both male and female mice. Cox et al., exposed mice to low dose penicillin either before birth or from weaning. After 20 weeks of treatment, female mice displayed a small but significant increase in BMC and BMD; however, opposite results were seen in male mice (179). Conversely, I show in chapter 2 of this thesis, that in 12-week-old BALB/c male mice, 4 weeks of microbiota repopulation post antibiotic treatment has a detrimental effect on femoral and vertebral trabecular bone volume (167). This study is the first to directly examine the effect of natural microbiota repopulation following acute antibiotics on skeletally mature bone health. Together, these studies suggest that chronic and acute antibiotic treatments can affect bone health in a sex- and age-dependent manner. Direct comparison between studies is complicated due to differences in treatment duration, sex, genetic strain, age, and antibiotics used. More research needs to be done looking into the microbial fingerprint to identify which specific microbial communities are beneficial or harmful to bone health.

There is strong evidence for a role of probiotic supplementation in modulating bone health. Numerous studies have revealed that modulating the intestinal microbiota with probiotic bacteria can have a beneficial effect on bone in a variety of disease models. This will be discussed in detail in the next chapter.

1.5 Probiotics in Gut-Bone Signaling

This section represents an accepted manuscript ahead of print in the book "Probiotics in Gut-Bone Signaling" published by Advances in experimental medicine and biology.

1.5.1 Introduction

Each year more than 2 million fractures occur because of osteoporosis (180). Numerous therapies have been developed for the prevention and treatment of osteoporosis. As a first approach, patients are asked to make changes to their lifestyle (i.e., exercise, cessation of smoking) and diet (including vitamin D and calcium supplementation) (181). For patients at a higher risk of fractures, pharmacologic treatments (drugs and biologics) are used to inhibit bone resorption or stimulate bone formation (182). Despite the many treatment options, we have yet to stop the increase in osteoporosis fractures. This may be in part due to patient concerns about side effects (although rare) from many pharmaceutical/drug-based therapies (51). Given that 67 million Americans are predicted to have low bone mass by 2020, it is important to continue to identify additional therapeutic approaches/targets for osteoporosis.

One therapeutic target receiving increasing attention is the intestinal microbiome, which is an important regulator of physiologic functions of many organs including bone. The intestinal microbiota accounts for 90% of the cells in our body and amounts to ~100 trillion microbes comprising ~1000 species and 28 different phyla (125). In addition to outnumbering host cell number, the gut microbiota also express 100-fold more genes compared to the human genome. (125). Thus, as the microbiome coevolves with us, changes in that population can have both beneficial and harmful consequences on human health (183). Dysbiosis (a microbial imbalance) is linked to disease and bone loss;

however, more importantly, the reverse is also true: treatment with probiotics can beneficially modulate the gut microbiota to enhance health, including that of bone (184–187). In this review we will focus on 1) probiotics (definition, history, nomenclature, types), 2) the overall effects of probiotics on bone health and 3) mechanisms of probiotic prevention of bone pathologies.

1.5.2 Definition

The word "probiotic" is derived from the Latin word 'pro' and the Greek word 'bios' meaning "for life;" this contrasts with "antibiotic" meaning "against life" (188–197). While "good for life" is a general definition of probiotics, the detailed definition of what constitutes a probiotic has been difficult to achieve and has changed over time. In the 1950s, Werner Kollath, a German scientist, used the word "probiotic" to be inclusive of all organic and inorganic supplements that restored the health of malnourished patients (188,189,196,197). Years later, probiotics were further defined as substances produced by one microorganism to promote growth of another microorganism (188,189,193,195–203). In the 1970s, Fujii and Cook described probiotics as compounds that build resistance to infection in the host but do not inhibit the growth of microorganisms *in vitro* (188,195,204). In the 1980s and 1990s, there was a surge of different probiotic definitions. For example, in 1990 Parker defined probiotics as organisms or substances in feed supplements which contribute to intestinal microbial balance (188,191,195,196,201,203,205). Parker's general definition was unsatisfactory to many since the word "substances" included chemical supplements such as antibiotics (195,205). Most researchers cited the definition of Fuller, who, in 1989, defined probiotics as live microbial feed supplements (188,195,196,198,201,203). Fuller's definition stressed the

importance of live cells as an essential part of the effective probiotic (195). His definition also stated that a probiotic or supplement will benefit the host by improving the intestinal microbial balance (188,199). However, many thought this definition was not as applicable to humans as it was to animals (188). Subsequently, in the early 1990s, the definition was broadened to include viable mono or mixed cultures of live microorganisms which, when given to humans or animals benefits the host by improving the properties of the indigenous microflora (206). Salminen offered the view of incorporating non-viable bacteria in the probiotic definition (188,205). Finally, in 2001, after consultation of international scientists working on behalf of the FAO/WHO (Food and Agricultural Organization/World Health Organization) probiotics were proposed to be defined "as live microorganisms that when administered in adequate amounts will confer a health benefit on the host" (188,192,196,200,203,207,208). Misuse of the probiotic term became a major problem in the ensuing years. For this reason, the International Scientific Association for Probiotics and Prebiotics (ISAPP) organized a meeting of clinical and scientific experts on probiotics in October 2013 to re-examine the concept and definition of probiotics (208). The ISAPP panel recommended that the definition of probiotic as defined by FAO/WHO in 2001 is broad enough to enable a wide range of products to be developed, and at the same time sufficiently narrow to impose some core requirements (203,208). Thus, probiotics remain defined as live microorganisms that when administered in adequate amounts will confer a health benefit on the host.

1.5.3 History of Probiotics

The history of probiotics parallels the evolution of the human race and can be traced back to ancient times (209). During the Neolithic period of the Stone Age, animal

domestication began and animal husbandry was developed (197). Ancient Oriental people, as well as Phrygian, Sarmatian, and Macedonian nomadic shepherds drank milk from cows, sheep, goats, horses, and camels. The ancient Ayurvedic texts, written between 400 and 200 BCE, linked a long and healthy life with the intake of milk and dairy products (197). To store the milk, it was customary to use containers made from animal skins or stomachs (196,197). The containers were a source of bacteria, most likely ancestors of Lactobacillus *acidophilus* and *bulgaricus,* which came into contact with the milk (197). One Turkish legend describes a shepherd, traveling the hot desert, who forgot he had milk in a goatskin bag. When he checked, the milk had transformed into a thick, creamy, and tasty custard and this new product was referred to as yogurt (197). For the Turkish people, yogurt was the elixir of life as they believed that this food gave physical and inner well-being and could prolong life (197). Laban Rayeb and Laban Khad, traditional Egyptian fermented milk products, were consumed as early as 7000 BCE (188,196,209). This tradition of fermenting milk was originally established in Middle and Far East of Asia and spread throughout eastern Europe and Russia by the Tartars, Huns, and Mongols during their land conquests (188). Fermented products other than milk, such as beer, bread, wine, kefir, kumis, and cheese were also consumed (209). Fermentation is one of the oldest methods of long-term storage and can be traced back to the Phoenicians, Egyptians and Eastern cultures (188,196,197).

The modern history of probiotics starts around the late 1880s and early 1900s. Elie Metchnikoff (a Nobel laureate), as well as Theodor Escherich, studied microbial communities in feces and described the need for a complex intestine (microbe-wise) (210). Metchnikoff was a Kharkov/Ukrainian scientist working at the Pasteur Institute (196,197).

Pasteur had identified the microorganisms responsible for fermentation but it was Metchnikoff who investigated the effects these microbes had on human health (197). Metchnikoff associated the longevity of Bulgarian rural people (who had a life-span 87 years) to their regular consumption of fermented dairy products such as yogurt (196,197,203,211). Metchnikoff described two bacteria types: one that leads to putrefying luminal contents and produces unhealthy waste products (NH3, H2S, amines), and another that ferments luminal contents and produces beneficial metabolic products (i.e, lactic acid) (212). This was a key concept because probiotic bacteria secrete enzymes that are not produced by human intestinal cells. These enzymes can ferment non-digestible poly-carbohydrates (mainly dietary fiber) to produce energy for the bacteria as well as other factors such as short chain fatty acids (SCFA) and lactic acid which benefits the intestinal epithelium (213). Metchnikoff theorized that the production of lactic acid would prevent the toxic effects of putrefying microbes. This further lead Metchnikoff to suggest that lactobacilli may benefit gastrointestinal metabolism and counteract illness and aging (188,197,203); thus, he considered lactobacilli a probiotic (197,198,214). Thanks to Metchnikoff the creation of the dairy industry began in France and subsequently spread throughout Europe, due to the use of fermented milk obtained from *Bacillus bulgaricus*, *Streptococcus thermophiles* and *Lactobacillus delbruekii* (196).

About the same time that Metchnikoff was making his discoveries of lactic acid-producing bacteria, French pediatrician Dr. Henry Tissier observed that children with diarrhea had a low number of bacteria shaped like the letter 'Y' in their stools (196,199,203). Healthy children had an abundance of these bacteria. In 1905, he isolated the bacteria, *Bacillus bifidus,* and linked its presence in children to those who were breast-

fed (210). He suggested these bacteria could be administered to patients with diarrhea to help restore their healthy flora (eubiosis) and used it to recolonize the gut of children (14,19–21,27,28,33). As the health benefits of milk-associated bacteria became better known, fermented dairy products were appearing around the world. For example, in 1935 a Japanese Microbiologist, Dr. Shirota, isolated *Lactobacillus casei* and added it to a dairy drink that was ultimately marketed. Today, food products containing probiotics are usually dairy, mainly due to the historical association of lactic acid bacteria with fermented milk (188,197,209).

1.5.4 Probiotic Nomenclature and Types

Probiotics are widely consumed and have a long history of safe use. Bacteria names are derived from descriptors of the bacteria (i.e., Lactobacillus, 'lacto' meaning "milk" and 'bacillus' meaning "rod-shaped"), a scientist's name (i.e., Pasturella, found by Louis Pasteur), the place where found (i.e., Legionella longbeachiae, found in Long Beach California), or an organization (i.e., Legionella and the American Legion). In addition to a general name, the bacteria are described based on a taxonomic/genetic hierarchy (216). Based on this system, bacteria are divided into phylum, class, order, family, genus, species and subspecies and/or strain (Fig 1.6). With more than 23 bacteria phyla, it is easy to see the abundance of specific probiotics and the complexity of their names. Current evidence indicates that the beneficial effect of probiotics are strain-specific (201). It is also important to note that not all bacteria within a species act the same and/or can be regarded as a probiotic. Below, we discuss several of the most notable probiotics including lactic acid bacteria, Bifidobacteria and Enterococcus (also see Table 1.1).

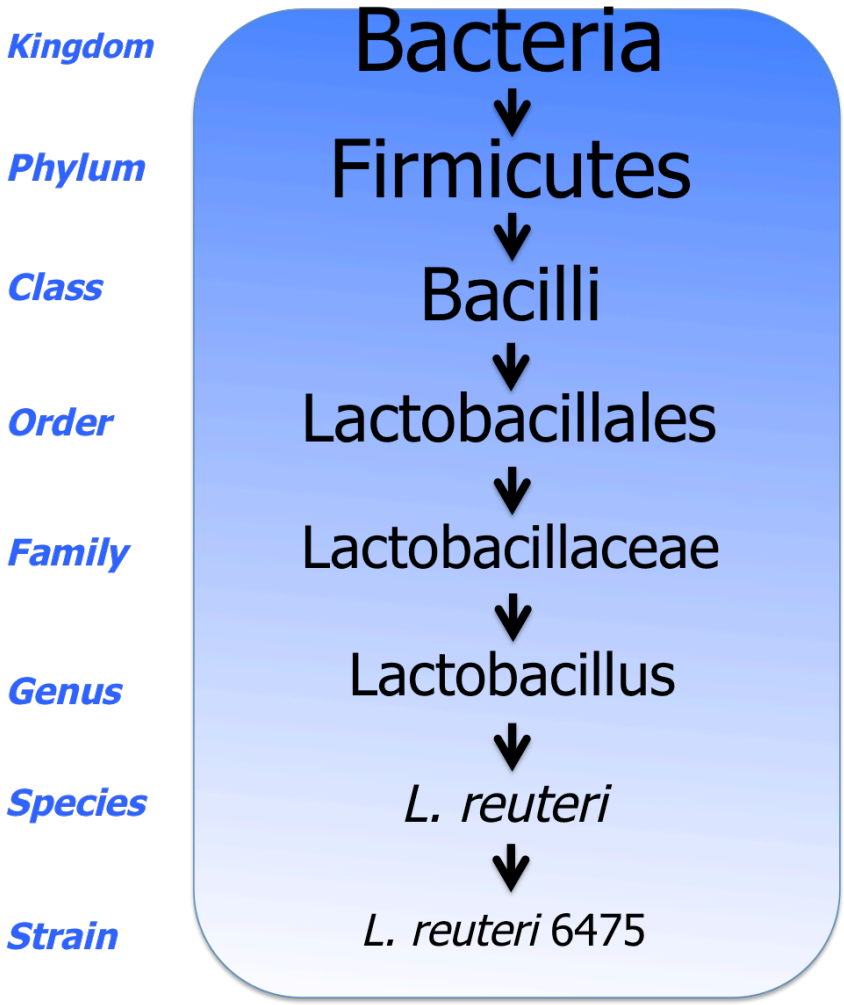

Figure 1.6: Scientific Nomenclature

An example of bacterial scientific nomenclature for the Lactobacillus reuteri 6475 strain.

Genus	Species	Genus	Species	Other
Lactobacillus	*acidophilus*	*Bifidobacterium*	*longum*	*Enterococcus faecalis*
	crispatus		bifidum	*Enterococcus faecium*
	johnsonii		*Infantis*	*Lactococcus lactis*
	gasseri		*animalis*	*Escherichia coli (Nissle 1917)*
	casei		*adolescentis*	*Propionibacterium freudenreichii*
	rhamnosus		*Lactis*	*Saccharomyces cerevisiae*
	reuteri		*breve*	*Streptococcus thermophilus*
	plantarum			*Bacillus cereus*
	fermentum			*Bacillus subtilis*
	salivarius			

Table 1.1: Common Probiotic Bacteria
Adapted from: (217,218)

1.5.4.1 Lactic Acid Bacteria/ Lactobacillales

Lactic acid bacteria (also known as LAB) are one of the most important groups of bacteria/probiotics with health benefits that are thought to result in part from their production of lactic acid, their major fermentation product (188,211,219). In general, they are gram-positive, acid-tolerant, asporogenous rods and cocci which are oxidase, catalase, and benzidine negative; they lack cytochromes, do not reduce nitrates to nitrite, are gelatinase negative, and are unable to utilize lactate (188,215,219). Lactic acid bacteria obtained from fermented milk products have been used for centuries. Traditional fermented milk is a useful source of probiotics because it contains a complex composition of lactic acid bacterial species. In a recent study, 148 lactic acid bacterial strains were isolated from Kurut, a traditional naturally fermented yak milk from China (220). Additional studies are evaluating these traditional fermented products as potential natural sources of probiotic bacteria (220).

Lactic acid bacteria, which consists of a diverse genera, are grouped as either homofermenters or heterofermenters based on the fermentation end product (215,219). Homofermenters produce lactic acid from glucose as a major product and heterofermenters produce a number of products such as carbon dioxide, acetic acid, ethanol as well as lactic acid (215,219). Homofermentive lactics include the genera *Streptococcus* which produces the L(+) lactate isomer and *Pedicoccus* which produces DL lactate (219). Heterofermentive lactics consist of the genus *Leucoostoc* which produce D(-) lactate and a subgroup of the genus *Lactobacillus*, the Betabacteria which produce DL lactate (219).

Lactobacilli are ubiquitous in nature and are usually found in carbohydrate rich environments (188). They also are a part of the normal flora in the intestinal tract of many animals. The genus *Lactobacillus* belongs to the phylum *Firmicutes*, class *Bacilli*, order *Lactobacillales*, family *Lactobacillaceae* (188). The most commonly isolated species are *Lactobacillus acidophilus*, *L. salivarius*, *L. casei*, *L. plantarum*, *L. fermentum*, *L. reuteri*, *L. rhamnousus, L. gasseri, L. reuteri* and *L. brevis* from human intestine (188). Several of these, *Lactobacillus acidophilus, Lactobacillus rhamnosus, Lactobacillus casei*, and *Lactobacillus reuteri* have been extensively studied and well documented (221).

Lactobacillus acidophilus, which was first isolated from children's feces by Ernst Moro in 1900, is capable of colonizing the human colon, has antimicrobial effects, and can be used to treat intestinal infections (199,221). *Lactobacillus rhamnosus GG* or *Lactobacillus GG* (LGG) is commonly used in dairy products marketed for infant and children's consumption. *Lactobacillus GG* was isolated from human feces in 1983 and is indigenous to the human intestinal flora, has a tolerance to low pH environment and adheres to the gastrointestinal tract (221,222). LGG is effective in treating diarrhea (196,223,224). *Lactobacillus gasseri* colonizes the gastrointestinal tract, oral cavity, and vagina in humans and is believed to contribute or potentiate probiotic activity in part by reducing fecal mutagenic enzymes as well as stimulate macrophages (221).

1.5.4.2 Bifidobacteria

Bifidobacteria are the predominant intestinal organism of breast-fed infants. These bacteria are rod-shaped, non-gas producing and anaerobic. Breast milk has been found to contain lactic acid bacteria as well as Bifidobacteria, both now included in formulas and foods targeted to pre-term and full-term infants (220). Bifidobacteria are generally

characterized as gram positive, non-spore forming, non-motile and catalase-negative anaerobes (188). Initially they had been assigned to the genera *Bacillus*, *Bacteroides, Mocardia*, *Lactobacillus* and *Corynebacterium*, before being recognized as a separate genera in 1974 and included in the Actinomycetaceae family (188,221). This family consists of 5 genera: *Bifidobacterium, Propionibacterium, Microbacetium*, *Corynebacterium,* and *Brevibacterium* (188). Currently there are 32 species in the genus *Bifidobacterium*, 12 are isolated from human sources, 15 from animal intestinal tracts or rumen, 3 from honeybees and the other 2 are found in fermented milk and sewage (188,215). Species found in humans are: *Bifidobacteria. adolescentis, B. angulatum, B. bifidum, B. breve, B. catenulatum, B dentium, B. infactis, B. longum, ad B. pseudocatemulatum* (188,221). These probiotic species can induce immunoglobulins, improve food nutritional value by assimilation of substrates not metabolized by the host, have potential anti-carcinogenic activity, and folic acid synthesis (221). Specifically, *Bifidobacterium infantis* has been found to significantly improve symptoms in patients with irritable bowel disease (196).

1.5.4.3 Enterococcus

There are 37 species of *Enterococcus* which have been validated for use as probiotics (225). Enterococci are singular, double or short chained gram positive cocci (221). These bacteria occur in many habitats such as soil, surface water, ocean water, sewage, on plants and in the gastrointestinal tract of animals and humans, with *E. faecalis* being the most predominant (225). Bacteria of the *Enterococcus* genus can also be used to treat diarrhea, irritable bowel syndrome, are considered to be an alternative for antibiotics, and are used for lowering cholesterol and immune regulation (221,225).

1.5.4.4 Other Probiotics

Besides the human gastrointestinal tract, the gastrointestinal tract of other animals such as pigs, rats, and poultry are also good sources of probiotics (220,224). Other probiotic strains have been discovered in marine and freshwater fish such as rainbow trout and shrimp (220), as well as in non-fermented foods such as meat and fruits (220). *Lactobacillus* strains from brine of naturally fermented olives and from pickled juices have also demonstrated probiotic properties (220). Other popular probiotics are *Streptococcus thermophilus, Lactococcus lactis subsp. lactis, Leuconostoc mesenteroides, Propionibacterium freudenreichii, Pediococcus acidilactici, Sporolactobacillus inulinus, Escherichia coli*, other bacteria of the *Bacillus* species, other lactic acid bacteria species, *Saccharomyces cerevisiae* and *Saccharomyces boulardii* yeasts. Many popular probiotics are added to dairy products and can have favorable effects on human health (188,196,200,201,211,221). There is a selection criteria regarding probiotic strains used in such products. There are several components of this criteria: a) the bacterium must be reported in the literature, b) concrete proof of assistance to health must exist, c) the bacterium must be able to colonize the gastrointestinal tract and have a regulatory role in microbial balance in that area, d) must be resistant to low pH values and bile salts in order to be able to sustain their viability, e) must posses natural antibiotic effect in order to prevent pathogen growth with their antimicrobial activity, f) must be safe to consume and show no antibiotic resistance, and g) must be suitable for commercialization (188,201,203,207,220,221).

1.5.4.5 Commensal Bacteria

Through co-evolution, humans not only tolerated the presence of the intestinal microbiota but also evolved to use the colonization of commensal microbes for immune

development and function, intestinal barrier integrity, and overall health (226). Commensal microbes comprise the resident bacteria that live on the human body and in the intestine amount to over 500 different strains including probiotic strains. The composition of intestinal microbes differs depending upon the intestinal region, with gradients existing both vertically and longitudinally (Fig 1.7) (227). Along the longitudinal axis, the number of microbiota increases distally with the greatest level in the colon ($\sim 10^{12}$). Along the vertical axis, certain bacteria are found in the upper mucus layer above the epithelium while others prefer the lumen. Different microbes thrive in different regions because of the local environment, which is influenced by luminal dietary contents, bile, pH, mucus, other bacteria, etc…. Several of the major probiotic strains were originally isolated from humans include: *Lactobacillus acidophilus,* bifidobacteria, several LAB strains, (220), and *Lactobacillus rhamnosus GG* (221,222). In the intestine, the balance of beneficial bacteria with neutral or inflammatory bacteria is critical. Thus, intestinal dysbiosis (microbe imbalance) leads to a reduction in the beneficial commensal microbes and can contribute to disease (226). Probiotic intake can help restore commensal microbe balance.

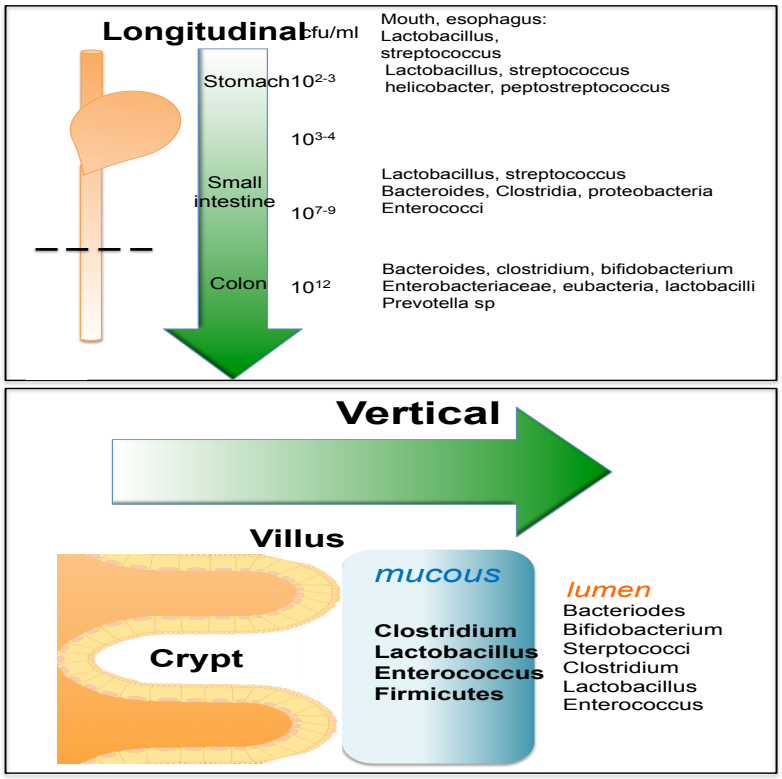

Figure 1.7: Regional bacterial changes of the intestine
The intestine is a major source of commensal microbes containing more than 500 species. Along the longitudinal axis, numbers of bacteria increase going down the intestinal tract from mouth to colon. Additionally the vertical axis contains bacteria in the mucus as well as the lumen. These microbes colonize different environments in which they can use the nutrients and conditions to thrive.

1.5.5 Probiotics Regulate the Gut-Bone Axis

Oral probiotics benefit the intestine as well as extra-intestinal organs including bone (185–187,228,229). Bone is a dynamic organ that depends on a fine balance between the bone forming osteoblasts and bone resorbing osteoclasts. An imbalance in this process can lead to bone disease. Bone homeostasis can be regulated by hormones such as estrogen, parathyroid hormone as well as by immune cells (230–232). The gastrointestinal system also plays a key role in bone health, most notably by regulating absorption of minerals such as calcium, phosphorous and magnesium as well as by being major producers of endocrine factors that signal to bone cells, such as incretins and serotonin. Therefore, agents/conditions that influence intestinal physiology can impact bone health. Recent studies, including some from our lab, indicate that in addition to mineral absorption, the intestinal microbiota can be a critical player in regulating bone physiology (184,185,229,233,234). Thus, we and others have examined the influence of probiotics on gut microbiome and how this modulates bone health. The effect of probiotics on the gut-bone axis is determined by a variety of factors. In this sub-section we will discuss studies examining the effect of probiotics on bone during growth, aging, and menopause. In addition, we will discuss the role of sex in bone responses to probiotics as well as the safety of probiotics.

1.5.6 Probiotic Effects on Bone Growth and Aging Bone

Stability of the intestinal microbiota composition is a critical regulator of intestinal homeostasis throughout life, from new born to adulthood. Increasing evidence also indicates that intestinal homeostasis plays a key role in the development of healthy strong bone during childhood and adolescence, which ultimately leads to a healthy adult skeleton

(235). By comparing microbiota from undernourished and healthy children from a Malawian birth cohort, Blanton et al (236) demonstrated that the microbiota is causally related to childhood nutrition. More importantly, the microbiota effects were functionally transmittable to germ-free mice (mice lacking a microbiome). Specifically, germ-free mice whose intestines were populated with microbiota from the undernourished children displayed reduced growth, altered bone morphology and metabolic dysfunction compared to mice populated with age-matched healthy microbiota (236). Supplementation with two bacterial strains (*Ruminococcus gnavus* and *Clostridium symbiosum*) added to the microbiome from undernourished children ameliorated growth abnormalities in the mice, supporting a role for microbiome composition and by extension probiotics in growth regulation (236). In support of these findings, Schwarzewr et al (237) show that undernourished mice supplemented with the probiotic *Lactobacillus plantarum* are able to maintain normal growth rates. Specifically, under-nutrition suppresses growth and bone growth parameters (femur length, cortical thickness, cortical bone fraction, and trabecular fraction of the femur) and these effects were prevented by *L. plantarum* treatment (237). Importantly, and in agreement with Blanton et al (236), the presence and/or composition of microbiota during development was shown to be important for regulating mouse growth rates. By comparing wild-type and germ-free mice the group found that growth parameters were decreased in the germ-free mice which were 4% shorter and weighed less than the WT mice. This response was shown to be dependent on the IGF-1-IGF-1R axis (Fig 1.8). Analysis of growth hormone (GH), IGF-1, and IGFBP-3 levels indicated a significant decrease in germ free compared to wild type mice 56 days after birth while on under-nourished diet (237). Supplementation with *L. plantarum* brought IGF-1 and IGFBP-3 back

to wild type levels, suggesting *L. plantarum* can recapitulate the beneficial effects of the microbiota on the IGF-1-IGF-1R axis (237). Yan et al. (151) also demonstrate the important role of the gut microbiota in regulating IGF-1 expression, bone formation and growth in mice. These effects cross animal species and are seen in drosophila as well. Specifically, drosophila display growth suppression in response to undernutrition or lack of a microbiome (238). When germ-free flies are repopulated with probiotic lactobacilli strains the flies regain their ability to grow at normal rates (238); and the IGF axis is restored (176). In humans, Steenhout *et al* examined the impact of probiotic-supplemented formulas on growth in both healthy and vulnerable populations (239). They concluded that the probiotic *Bifidobacterium lactis* has a positive effect on growth in infants born to mothers with human immunodeficiency virus (239). Taken together, these studies demonstrate that a healthy gut microbiome is important for bone growth during development.

Aging is associated with many complications including osteoporosis. The use of probiotics to benefit longevity and health dates back to ancient Ayurvedic texts (400 and 200 BCE) (197). Given this, it is surprisingly that only recently research has begun to focus on the critical role and mechanisms of microbiome/probiotic regulation of aging conditions, such as osteoporosis. While there currently are several ongoing studies examining probiotic effects on bone health in the elderly, only a few studies have been published to date. In one study, *Lactobacillus casei Shirota* was given to elderly male and female patients (n=417); after 4 months of treatment these patients showed enhanced fracture healing (distal radius) compared to patients with placebo treatment (Fig 1.8) (58). In a similar study, 50 postmenopausal women with osteopenia (50-72 years of age) were randomly assigned to take either GeriLact (7 probiotic bacteria species) or a placebo for 6

months. The multispecies probiotic GeriLact significantly decreased biomarkers of bone resorption in comparison with the placebo group, though no significant changes in bone mineral density were observed during this period of treatment (240). Interestingly, the probiotic treatment did significantly decreased serum levels of parathyroid hormone and the pro-inflammatory marker TNF-α (240). Another study, that saw an effect on bone density, involved the treatment of osteoporotic males (64-67 years of age) with Kefir fermented milk for 6 months. The group found a 5% increase in femoral neck bone mineral density measured by DEXA (241). This study supports a benefit of probiotics on bone health, but it is important to recognize that only 24 subjects were studied and the contribution of calcium in the Kefir was not separated from the effects of the probiotic bacteria. While not directly examining bone, a recent study by Han et al. screened a library of c. elegan mutants to identify bacterial metabolites that influence lifespan and reduce aging complications (242). The polysaccharide colonic acid was found to be involved in mediating longevity and reducing aging complications, supporting a role for intestinal microbes in regulating lifespan and health. Taken together, ancient texts and recent data indicate the potential for probiotics to maintain bone health throughout life.

1.5.7 Probiotic Effects on Menopausal Osteoporosis

The natural loss of estrogen due to menopause is the most important risk factor for osteoporosis in women. Women, over the course of their lifetime, lose about 50% of their trabecular bone and 30% of their cortical bone; about half of the bone loss occurs during the first 10 years after menopause (243). Recent studies have examined the influence of the microbiota and probiotic treatment during osteoporosis especially under conditions of estrogen deficiency in animal models. For example, while we previously noted that intact

healthy female mice do not display a bone response to L. reuteri, we found that *L. reuteri* treatment can prevent ovariectomy-induced bone loss in mice, suggesting that lack of estrogen may influence responsiveness to *L. reuteri* effects on bone (Fig 1.8) (229). These findings were confirmed by others using similar or distinct probiotics [44,48,63]. In a recent study, Li et al.(169) demonstrated that microbiota is necessary for sex-steroid deficiency-induced bone loss. Female wild type and germ-free mice were given Lupron (ovarian sex steroid antagonist) to block the effect of estrogen in mice. While wild-type mice lost bone as expected, the germ-free mice did not lose bone, demonstrating that the microbiota may be essential for estrogen-deficiency induced bone loss (169). While Lupron increased intestinal permeability in wild type mice, it did not affect permeability in the germ free mice. Supplementation of conventional mice with *Lactobacillus rhamnosus* GG (LGG) or VSL#3 reduced gut permeability, intestinal inflammation and protected mice against bone loss induced by ovariectomy induced estrogen deficiency (169).

Probiotics have been proposed to function in multiple ways under estrogen deficient conditions. One important mechanism is through the suppression of osteoclastogenesis, an event that is upregulated during estrogen deficiency/menopause. Our studies showed that *L. reuteri* can suppress OVX-induced increases in bone marrow CD4+ T-lymphocytes, which are responsible for the overstimulation of osteoclasts (Fig 1.9) (229). In addition, we have also shown that a 3kd fraction of the *L. reuteri* can inhibit osteoclastogenesis *in vitro* (229). Similarly, Ohlson et al showed that the probiotics could affect pro-inflammatory cytokines such as TNFα and IL1β, as well as increase osteoprotegerin levels, all of which will decrease osteoclastogenesis. Similar attenuation of bone loss was also demonstrated with soymilk, that was supplemented with *L. paracasei*

subsp. paracasei NTU101 or *L. plantarum NTU 102* in ovariectomized mice (185). Narva et al has also demonstrated a similar outcome with the use of fermented milk, valyl-prolyl-proline, and *Lactobacillus helveticus LBK-16H* in ovariectomized rats (244). Finally, Rodrigues et.al showed that synbiotics, in this study a combination of prebiotics (Yacon flour) and probiotics (*Bifidobacterium longum)*, increased bone mineral content in rats (228). Together, these studies demonstrate an important role for oral probiotics in reversing estrogen-deficiency-induced bone loss.

1.5.8 Influence of Sex on Probiotic Effectiveness

Sex hormones are known to play a critical role in regulating bone density (245). For example, males have greater bone density than females mainly due to differences in cortical bone expansion and greater trabecular bone volume (246,247). In addition, studies indicate that some mouse models display gender differences in response to hormones, such as PTH, that regulate bone (248). Similarly, in one of the earliest bone studies to identify sex-specific responses to probiotic use, our lab administered *Lactobacillus reuteri* ATCC PTA 6475 (*L. reuteri*) to healthy male and healthy female mice for 4 weeks (186). *L. reuteri* increased bone volume fraction and bone mineral density in healthy male mice and this was associated with a suppression of intestinal inflammation (Fig 1.8) (186). Surprisingly, these effects were not observed in female mice, demonstrating that *L. reuteri* treatment influences bone (and gut) in a sex-specific manner (186). This is also consistent with studies that induce intestinal inflammation by infecting mice with, *H. hepaticus*; in these studies, the pathogenic bacteria caused intestinal inflammation and bone loss in male mice but did not have a significant effect in female mice (249). Taken together the findings suggest that female mice do not respond to either “bad” or “good” bacteria. In later studies,

we identified that intact female mice can respond to probiotic (*L. reuteri*) treatment, but only when they are put into mild inflammatory state through dorsal surgical incision (184), supporting a potential role for inflammatory cells and estrogen in regulating female responses to luminal bacteria.

1.5.9 Probiotic Safety Throughout Life

The above studies indicate that probiotics hold great promise for supporting bone health. While generally regarded as safe (GRAS), there are some situations where probiotics need to be used cautiously. Patients with compromised immune systems, significant intestinal barrier dysfunction or with severe/critical illness may be susceptible to adverse effects such as sepsis, fungemia and intestinal ischemia (250) under these conditions the concern is that the load of intestinal bacteria, even though beneficial, could lead to inflammation and cross-over into the blood system where immune cells may be compromised and unable to remove/kill the bacteria. Recent tolerability studies for one probiotic, lactobacillus rhamnosus GG (LGG), are very positive. Children with Crohn's disease, which involves a barrier break, tolerate orally supplemented LGG and displayed a side effect profile comparable with placebo (251). Similarly, elderly patients (66-80 years old) did not display serious adverse effects in response to probiotic (LGG) treatment (252). Mild symptoms that can occur include bloating, gas and nausea during the adaptation to probiotic ingestion. As with any new therapy, it is important to carry out these safety and tolerability studies.

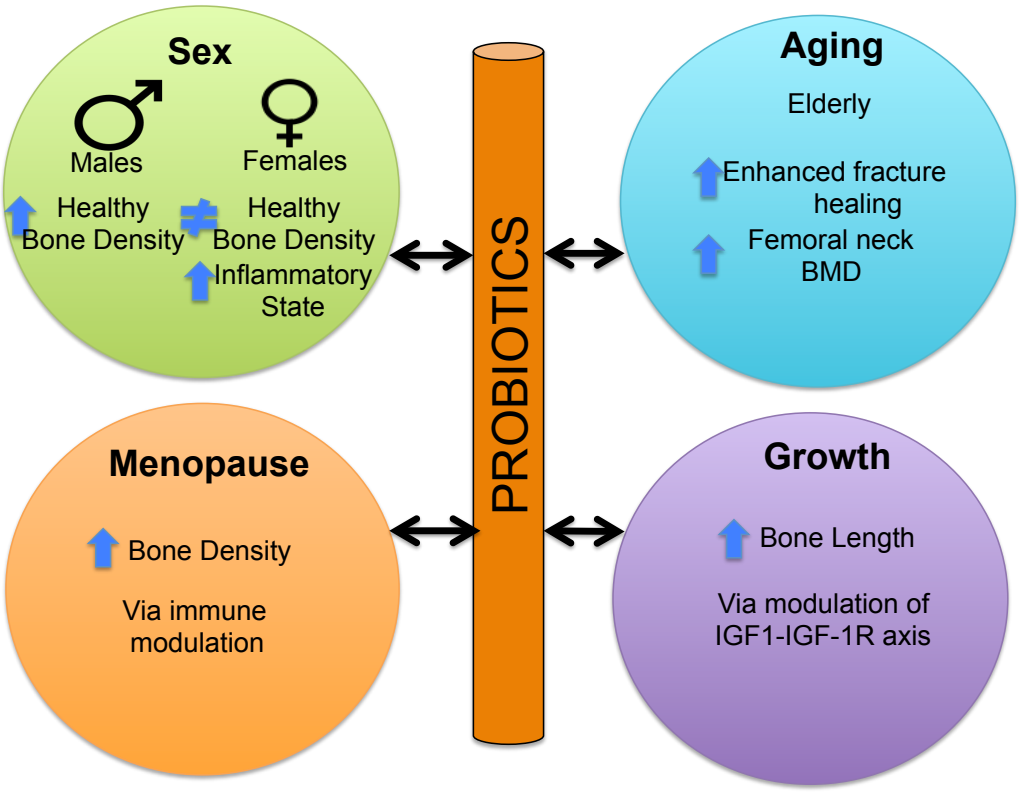

Figure 1.8: Probiotics bone effects in different populations

Probiotic treatment has shown to have beneficial bone effects across differing populations/conditions such as sex, aging, menopause and growth.

1.6 Mechanisms of Probiotic Prevention of Bone Pathophysiology

1.6.1 Effect of Probiotics in Dysbiosis-Induced Bone Loss

Dysbiosis is caused by an imbalance of gut microbiota composition/function (253). While primarily an ailment of the gut, dysbiosis can have systemic effects due to increased permeability of the intestinal mucosa (254). This can result in bacterial products such as lipopolysaccharide to enter systemic circulation resulting in systemic and local tissue inflammation at distant sites including the bone (Fig 1.9) (255,256). Our lab has shown that dysbiosis caused by an infectious *H. hepaticus* bacteria can induce gut inflammation as well as bone loss in male mice (257). Long-term antibiotic treatment can also induce dysbiosis and has been shown to influence bone. Specifically, male mice treated with antibiotics (ampicillin and neomycin) from 4 to 16 weeks of age display decreased bone strength and reduced B and T cell populations (178). In a periodontal model of dysbiosis, bone loss was observed (168). Activation of NOD1 (Nucleotide-binding oligomerization domain containing 1) a receptor for immune function in the gut spared bone-loss in these mice, indicating that it could have important effects in similar cases in humans (168).

Probiotic treatment can benefit dysbiosis and gut health through maintaining intestinal barrier function and thereby preventing toxins from entering systemic circulation (258–262). In a study causing enteropathogenic *E.coli* (EPEC)-induced dysbiosis, administration of probiotic *E.coli Nissle 1917* increased specific claudin expression and prevented increases in intestinal permeability seen after infection with EPEC (Fig 1.9) (263). While pathogenic dysbiosis can damage the intestinal barrier, several studies have shown that this barrier can be rescued through the use of specific probiotics (264–267). These studies suggest that several conditions linked with gut dysbiosis can be

improved through the proper treatment with probiotics. Along with treating the intestinal permeability observed in dysbiosis, probiotics have also been shown to have positive effects on bone health in dysbiosis models. Periodontal disease characterized by dysbiosis of the healthy oral bacterial flora leading to increased inflammation and subsequent bone loss was prevented with probiotic administration. Using this model, mice with periodontitis that were treated with *Lactobacillus brevis* CD2 displayed decreased bone loss and lower expression of pro-inflammatory cytokines such as tumor necrosis factor, interleukin-1β and -17A (Fig 1.9). Similar studies in a rat model of periodontal disease indicate that probiotics (Bacillus *subtilus* and *Saccharomyces cerevisiae)* can decrease bone resorption, increased bone density, and decreased inflammation (268,269); dysbiosis was also prevented by treatment (268).

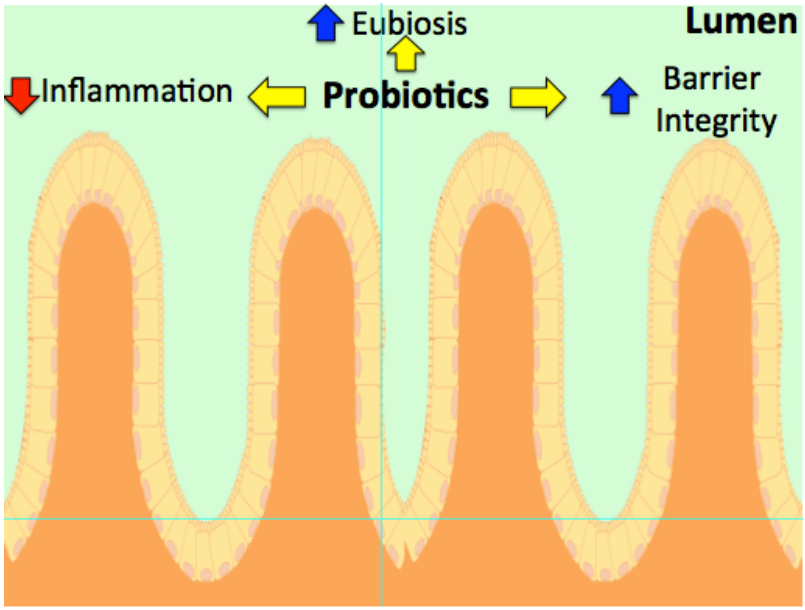

Figure 1.9: Model of Probiotic mechanistic signals regulating bone density
A disruption in homeostasis of the gut microbiota can lead to increased immune inflammation and gut permeability resulting in systemic organ inflammation including bone. Prevention of local gut inflammation and permeability by promoting a healthy gut microflora (Eubiosis) is one of the many ways probiotics can prevent local and systemic responses which can be beneficial to bone health.

1.6.2 Effect of Probiotics in IBD-Induced Bone Loss

Inflammatory Bowel Disease (IBD) can have detrimental effects on bone health by affecting the actions of osteoblasts and osteoclasts and promoting osteoporosis (270). IBD is characterized by gut dysbiosis which generates an inflammatory response both locally and systemically, including within the bone marrow and bone (257). Thus, IBD-induced intestinal inflammation is the primary pathology that leads to IBD-induced osteoporosis (270). When the dysbiosis is recognized by the immune system, an inflammatory response occurs that includes the release of many pro-inflammatory cytokines such as TNF-α, interleukins IL-6, IL-11, IL-17, as well as prostaglandin E_2 (271). Cytokine expression is also elevated in bone (249,257,272). The elevation of pro-inflammatory cytokines promotes osteoclast activity and also suppresses osteoblast activity; the latter occurs by decreasing maturation and increasing cell death. IBD also affects the RANK-RANKL-OPG pathway of bone metabolism and promote excessive bone loss (273). Prostaglandin E_2 promotes RANKL and inhibits OPG, which results in greater osteoclast activation. For a comprehensive review of how IBD affects bone, please refer to the chapter by Dr. Sylvester.

Recent studies have shown the protective effects of probiotics on IBD induced gut inflammation and thereby protective effects on bone. Administration of a commercially available probiotic VSL#3 in a mouse model of ulcerative colitis led to decreased gut permeability and aided in treatment of inflammatory symptoms (Fig 1.10) (260). Using other probiotics such as *L. reuteri* (R2LC) in IL-10 deficient colitis models attenuated disease development, normalizing gut barrier function and reducing pro-inflammatory cytokines and histological disease score (274). Consistent with these studies, DSS induced colitis in female BALB/c mice showed increases in gut permeability which was prevented

with treatment of *Bifidobacterium longum* CCM 7952 (Bl) (275). Additional studies implicate that modulation of toll-like receptor 9 (TLR9) is necessary for the beneficial effects of probiotics in ulcerative colitis treatment (276).

Although these studies did not look at the direct effect of probiotics on bone, they do show the ability for probiotics to have beneficial effects on IBD-induced gut inflammation, which is the one of the main components to IBD-induced bone loss. However, probiotics appear to have differential effects on bone inflammation. Treatment of bone marrow-derived dendritic cells from mice with VSL#3 showed increases in both pro and anti-inflammatory cytokine levels (277). Taken together these studies show that probiotic treatment of IBD patients may be beneficial to correct the dysbiosis and reduce intestinal inflammation but further studies are needed to solidify the beneficial role of probiotics.

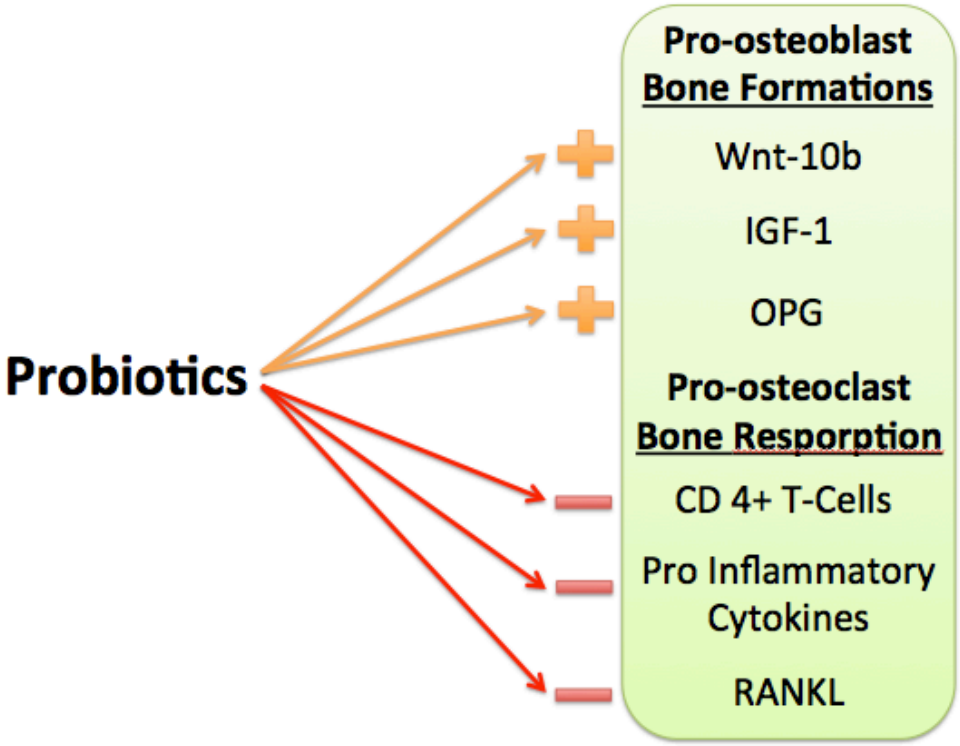

Figure 1.10: Mechanism of probiotics beneficial bone affects
Probiotic treatment can modulate the differentiation and function of osteoblasts through changes in Wnt-10b, insulin like growth factor-1 and OPG as well as osteoclasts through modulation of CD4+ T-cells, pro-inflammatory cytokines and RANKL.

1.6.3 Effect of Probiotics in Type-1 Diabetic-Induced Bone Loss

Type-1 diabetes is a chronic autoimmune disease characterized by destruction of insulin-producing pancreatic β-cells, resulting in the requirement for exogenous insulin to control blood glucose levels. The consequent metabolic dysregulation has many deleterious consequences including bone loss. T1D-induced osteoporosis is thought to result primarily from the dysregulation of osteoblastic activity. Given that probiotics benefit bone health, probiotic treatment in this model has been examined. This is based on early studies indicating a role for the gut microbiome in T1D development. One of the original studies in non-obese diabetic mice (NOD) showed that NOD mice lacking MyD88 protein (adaptor for multiple innate immune receptors that recognize bacterial stimuli) did not develop T1D (278). This protection is dependent on the commensal microbes because germ-free MyD88-negative NOD mice develop severe diabetes, whereas bacterial colonization attenuates T1D (278). Thus, commensal bacteria maybe important to reduce disease susceptibility. Consistent with these findings, another group showed that early life antibiotics alters the gut microbiota and its metabolic capacities, intestinal gene expression and T-cell populations leading to accelerated T1D in NOD mice (279). In addition, our lab has demonstrated that modulation of the gut microbiota with probiotic *L. reuteri 6475* can prevent streptazotocin (STZ) induced T1D-mediated bone loss in mice. In this study, male (C57BL/6 14 weeks old) mice were given an STZ injection to induce type 1 diabetes which displayed a 35% reduction in bone volume fraction 4 weeks post injection (187). Treatment with *L. reuteri 6475* prevented this bone loss. This was further supported by trabecular bone data, which revealed that *L. reuteri 6475* prevented the increase in trabecular spacing and reduction in trabecular number induced by T1D. STZ induced T1D

bone loss comes from reduced osteoblast activity, which was consistent with decreased osteocalcin (bone formation) serum markers and decreases in mineral apposition rate (MAR) compared to controls. *L. reuteri 6475* prevented decreases in both osteocalcin and MAR suggesting that probiotics specifically in this model, can have an anabolic effect on bone (187). Additionally, part of T1D's bone pathology is an increase in bone marrow adiposity, indicating an altered lineage commitment of bone marrow stromal cells toward the adipocyte over osteoblast lineage (72,280). In this study, consistent with benefits on bone health, *L. reuteri 6475*-treated T1D mice did not display increases in adipocyte number (86). Furthermore Wnt10b signaling which in mesenchymal precursor cells stimulates osteoblastogenesis and inhibits adipogenesis was decreased in T1D mouse bone (Fig 1.10). Treatment with probiotic *L. reuteri 6475* fully restored whole bone Wnt10b gene expression back to normal levels (86). Additionally T1D in Wnt10b transgenic mice did not display significant decreases in bone density compared to wild-type mice (86). These findings indicate that treatment with probiotics can prevent trabecular bone loss by modulation of expression of Wnt10b in T1D.

1.7 Conclusion

There are many studies supporting the role for the microbiome in the regulation of bone heath. While direct supplementation of beneficial probiotic bacteria or pathogenic bacteria can affect bone health by regulating aspects of gut, our knowledge in this subject matter remains limited. The overall aim of this study was to investigate and identify the role for the gut microbiota in two mouse models of bone loss, as well as use novel gut therapies to prevent bone loss.

Aim 1: Determine the role of the microbiota in bone loss following antibiotic induced dysbiosis. (Chapter 2)

This study was the first to date to investigate the effects of antibiotic induced dysbiosis on bone health. Using an *in vivo* mouse model I found that 2 weeks of chronic antibiotic treatment followed by a 4-week natural repopulation period lead to significant bone loss. This was accompanied by increased intestinal permeability and gut inflammation. I also treated with a mucus supplement MDY, which prevented post-antibiotic induced barrier break and bone loss. Furthermore, treatment with probiotic *Lactobacillus reuteri* 6475 prevented antibiotic induced changes to the gut microbiota and prevented bone loss.

Aim 2: Determine the role of the microbiota in the pathophysiology of glucocorticoid-induced osteoporosis. (Chapter 3)

Glucocorticoid treatment is the most common cause of secondary osteoporosis. Along with its known effects on osteoblasts decreasing bone formation, it also alters the gut microbiota. Again this study was the first to investigate the role of the gut microbiome in glucocorticoid-induced osteoporosis (GIO). With the use of chronic antibiotics, probiotics

and mucus supplements, I was able to prevent bone loss in GIO suggesting the gut microbiome is playing a major role in the pathophysiology of this disease.

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CHAPTER 2: PROBIOTIC *LACTOBACILLUS REUTERI* PREVENTS POST-ANTIBIOTIC BONE LOSS BY REDUCING INTESTINAL DYSBIOSIS AND PREVENTING BARRIER DISRUPTION

This chapter represents a manuscript that was accepted for publication by the *Journal of Bone and Mineral Research* on January 28th, 2019.

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2.1 Abstract

Antibiotic treatment, commonly prescribed for bacterial infections, depletes and subsequently causes long-term alterations in intestinal microbiota composition. Knowing the importance of the microbiome in the regulation of bone density, we investigated the effect of post-antibiotic treatment on gut and bone health. Intestinal microbiome repopulation at 4-weeks post-antibiotic treatment, resulted in an increase in the *Firmicutes:Bacteroidetes* ratio, increased intestinal permeability and notably reduced femoral trabecular bone volume (~30%, $p<0.01$). Treatment with a mucus supplement (MDY) prevented the post-antibiotic induced barrier break as well as bone loss, indicating a mechanistic link between increased intestinal permeability and bone loss. A link between the microbiome composition and bone density was demonstrated by supplementing the mice with probiotic bacteria. Specifically, *Lactobacillus reuteri*, but not *Lactobacillus rhamnosus* GG or non-pathogenic *Escherichia coli*, reduced the post-antibiotic elevation of the *Firmicutes:Bacteroidetes* ratio and prevented femoral and vertebral trabecular bone loss. Consistent with causing bone loss, post-antibiotic induced dysbiosis decreased osteoblast and increased osteoclast activities, changes that were prevented by both *Lactobacillus reuteri* and MDY. These data underscore the importance of microbial dysbiosis in the regulation of intestinal permeability and bone health as well as identify *Lactobacillus reuteri* and MDY as novel therapies for preventing these adverse effects.

2.2 Introduction

The discovery in 1928 of the first antibiotic, penicillin, is one of the most important medical advances in the history of human health. Since that time, new classes of antibiotics have been developed and used to treat human bacterial infections (1). For this reason, antibiotic use is widespread resulting in more than 260 million prescriptions issued in the US in 2011 (2). Although antibiotics can be lifesaving, they also deplete commensal flora causing long-term changes in intestinal microbiota composition. This can impact overall health (3–9) and increase the risk for developing diseases such as inflammatory bowel disease (IBD)(10,11), obesity (12–14) and diabetes (15).

The intestinal microbiota includes ~100 trillion bacteria as well as fungi and viruses. Research has focused predominantly on gut bacteria composition which is thought to comprise of ~1000 bacterial species and 28 different phyla (7,16). *Firmicutes* and *Bacteroidetes* are the predominant phylum in the human intestine (17). The environment, diet, drugs and disease can affect microbiota composition (18–20) and lead to dysbiosis, an altered microbial community that contributes to disease. Dysbiosis has been linked to increased intestinal permeability/leaky gut (10,21) and is thought to be a key contributor to the pathogenesis of several diseases including IBD, obesity and diabetes (11,14,15).

Previous studies support a role for the gut microbiota in the regulation of bone health. For example, intestinal infection with pathogenic bacteria induces bone loss in male mice (22). In contrast, treatment with beneficial bacteria (probiotics) enhances bone density in healthy male mice (23), ovariectomized female mice (24–26), inflamed intact female mice (27), and type 1 diabetic male mice (28). Although earlier studies in germ free mice suggested a negative role for normal microbiota in bone health (29), subsequent

findings have been inconsistent (24,30,31). This may be due to several factors including differences in the composition of the microbiota used to conventionalize germ free mice. For example, the presence of segmented filamentous bacteria can influence immune responses and osteoarthritis (32,33). In addition, past germ free studies have used various mouse strains (C57BL/6, BALB/c and Swiss Webster (29–31,34,35) which can further contribute to disparate responses. Finally, another confounding factor is that germ free mice have significantly altered immune systems (29,34) which can affect intestinal and bone signaling and responses.

Antibiotic treated and post-treated mice serve as additional models for investigating the roles of a depleted microbiome and dysbiosis, respectively, on organ health (36–39). Only a few studies to date have examined the effect of antibiotic treatment on bone health and most focused on the impact of chronic treatment. The results have been inconsistent, likely due to different treatment lengths, differences in age at the initiation of treatment and possibly differences in starting microbiome composition (40–43). Acute oral antibiotics deplete the intestinal microbiome and subsequently alter the microbiota composition long after cessation of treatment (44,45). Very few studies have investigated the effects of a repopulated microbiome following antibiotic treatment, and none have investigated the effects on bone health in skeletally mature mice. Based on previous studies demonstrating the beneficial and harmful effects of probiotic and pathogenic bacteria respectively on bone health (22,23,25,28), we hypothesized that dysbiosis following antibiotic treatment would cause bone loss. To test this, we treated skeletally mature mice with oral antibiotics for 2 weeks to deplete the microbiome and allowed the treated mice to naturally repopulate the gut microbiome for 4 weeks. Our results demonstrate that

although microbiota depletion per se has no effect on bone health, post-antibiotic dysbiosis markedly decreased bone density. Furthermore, we present evidence that the dysbiosis-induced bone loss is preventable by treatment with *Lactobacillus reuteri* and by enhancement of intestinal barrier function. Our studies underscore a key role for dysbiosis, following antibiotic treatment, in the pathogenic regulation of the gut-bone axis.

2.3 Materials and Methods

2.3.1 Animals and Experimental Design

Eleven-week old male BALB/c mice were obtained from Jackson Laboratories (Bar Harbor, ME). Mice were allowed to acclimate to the animal facility for 1-week prior to start of experiment. To deplete the commensal microbiota (15,46), at 12-weeks of age mice were treated with broad-spectrum antibiotics; ampicillin 1.0 g/L (Sigma, St. Louis, MO) and neomycin 0.5 g/L (Sigma, St. Louis, MO)(15,46), which are poorly absorbed by the intestine (47–49). Fresh antibiotics were given weekly into sterile drinking water; activity of the antibiotic-water was confirmed by its prevention of bacterial growth on agar plates. After 2-weeks, antibiotic treatment was stopped and mice were treated for 4-weeks with sterile drinking water (Aquavive water; Innovive, Dan Diego, CA) alone or containing 3.3 x 10^8 cfu/ml of either *Lactobacillus reuteri* 6475 (LR), *Lactobacillus rhamnosus* (LGG), non-pathogenic *E. coli* (EC, ATCC O6:B1) or 1.25% MDY (a high molecular weight polymer used as a mucus supplement)(50,51). In addition, to assure adequate intake of bacteria the mice were gavaged 3 times a week with 300 ul of either broth (used for bacterial cultures) or 1x10^9 cfu/ml of the corresponding bacteria (LR, LGG, or EC). Experiments were repeated at least twice and the control and post-ABX mouse conditions were used in 5 separate experiments and gave similar responses in all runs. Mice were maintained on sterilized Teklad 2019 chow (Madison, WI) ad libitum and a 12 hr light/dark cycle in specific pathogen free conditions and housed in groups of 4-5 per cage. All animal procedures were approved by Michigan State University Institutional Animal Care and use Committee and conformed to NIH guidelines.

2.3.2 Bacterial Culture

LR and LGG were cultured under anaerobic conditions on a de Man, Rogosa, Sharpe media (MRS, Difco) plates, while EC was cultured on Luria broth plates (LB, Invitrogen). Plates were kept at 37˚C for a maximum of 1 week. For gavaging, single bacterial colonies were cultured in 10 ml of their respective broths. After 16-18 hours at 37° C, mice were gavaged with 300µl of bacteria ($1x10^9$ cfu/ml). When adding the bacteria to the drinking water, the 10 ml overnight culture was centrifuged at 5,000 RCF, re-suspended and cultured in fresh MRS/LB and grown until log phase (OD600 =0.4). Bacteria were pelleted, re-suspended in 60 mls of sterile PBS, stored in 1 ml aliquots at -80°C. Samples are thawed and subsequently re-suspended in sterile drinking water at a final concentration of $3.3x10^8$ cfu/ml. Drinking water is changed 3X/week.

2.3.3 RNA Analysis

Tibias were cleaned of muscle and connective tissue, flash frozen in liquid nitrogen and stored at -80°C. Frozen tibias were crushed under liquid nitrogen conditions with a Bessman Tissue Pulverizer (Spectrum Laboratories, Rancho Dominguez, CA). RNA was isolated using TriReagent (Molecular Research Center, Cincinnati, OH) and checked for integrity by agarose-gel electrophoresis. cDNA was produced by reverse transcription using Superscript II reverse transcriptase kit and oligo dT primers (Invitrogen, Carlsbad, CA). Intestines were flushed of their contents with 1X PBS and RNA isolated from mid colon sections. Gene expression levels were amplified by real-time PCR with iQ SYBR Green supermix (BioRad, Hercules, CA) and specific gene primers. Hypoxanthine guanine phosphoribosyltransferase (HPRT) mRNA levels, which do not change between treatment groups, were used as a house-keeping gene. PCR was carried out to 40 cycles using the

iCycler (Bio-Rad) and data evaluated using the iCycler software. The cycle protocol consisted of 15 seconds at 95°C, 30 seconds at 60°C and 30 seconds at 72°C. Negative controls included primers without cDNA. Bacterial primers (targeted to bacterial specific 16S RNA (52)) were as follows: *Eubacteria* (Forward, **5'-** ACTCCTACGGGAGGCAGCAG- **3',** Reverse, **5'-** ATTACCGCGGCTGCTGG- **3'**). Primers for mouse genes were as follows: *HPRT* (Forward, **5'-** AAGCCTAAGATGAGCGCAAG- **3',** Reverse, **5'-** TTACTAGGCAGATGGCCACA), Osteocalcin (Forward, **5'-** AAGCAGGAGGGCAATAAGGT- **3'**, Reverse, **5'-** TAGGCGGTCTTCAAGCCAT- **3'**), TNF-α (Forward, **5'-** AGGCTGCCCCGACTACGT- **3'**, Reverse, **5'-** GACTTTCTCCTGGTATGAGATAGCAA- **3'**) and IL-10 (Forward, **5'-** TTGGAATCCCGAATTAACG- **3',** Reverse, **5'-** GGTCACAGTGAAATACTGCTC- **3'**).

2.3.4 μCT Bone Imaging

Femurs, tibias and vertebrae were scanned using a GE Explore Locus micro-computed tomography at a resolution of 20 μm obtained from 720 views. Each scan had control and antibiotic treated bones and was phantom calibrated to maintain consistency. A fixed threshold of 980 (determined based on automated and isosurface analyses) was used for all bones. The distal femur trabecular bone region was defined as beginning proximal (a distance of 1% of the total bone length) to the growth plate and then extending 10% of bone length toward the diaphysis and excluding the cortical bone. Trabecular bone was also analyzed within the lumbar (L3) vertebrae. Trabecular bone parameters including volume, thickness, spacing and number values were obtained using GE Healthcare MicroView software version 2.2. Cortical measurements were performed in a 2x2x2 mm cube centered midway down the length of the bone. All bone analysis was performed blinded to the mouse condition.

2.3.5 Femoral Static and Dynamic Measures

For histomorphometric measures of bone formation, mice were intraperitoneally injected at 7 and 2 days prior to harvest with 200µl of 10-mg/ml sterile calcein (Sigma, St. Louis, MO) dissolved in sterile saline. Femurs were embedded in paraffin blocks and sectioned in 5 micron sections, as previously described (53). Distal femur metaphyseal sections were viewed with a fluorescent Nikon Eclipse E800 microscope (Nikon Instruments Inc, Melville, NY) and five digital images per section were taken. The distance between the calcein lines (mineral apposition rate, MAR) and the length of the calcein lines (single + double labeled surfaces; mineralized surface, MS) along the total bone surface (BS) were measured to calculate the bone formation rate (BFR) using Image Pro-Plus 7.0 (Media Cybernetics, Rockville, MD). Additionally, slides were stained for tartrate-resistant acid phosphatase (TRAP) activity and counterstained with hematoxylin according to manufacturer's protocol (387A-1KT; Sigma-Aldrich). TRAP-positive osteoclast number (Oc.N/BS) and surface (Oc.S/BS) were quantitated and expressed relative to the total bone surface. Histomorphometric measures were made blinded to the mouse condition. Standard nomenclature for JBMR was used for all bone histomorphometry measures (54).

2.3.6 Serum Measurements

Sterile blood was collected at the time of harvest, allowed to clot at room temperature for 5 minutes and then centrifuged at 5000g for 10 minutes. Serum was removed, aliquoted and snap frozen and in liquid nitrogen, and stored at -80°C. Serum tartrate resistant acid phosphatase 5b (TRAP5b) and osteocalcin (OC) were measured using mouse TRAP (Immunodiagnostic Systems Inc., Fountain Hills, AZ) and OC assay kits

(Biomedical Technologies Inc., Stoughton, MA), respectively, according to manufacturer's protocol.

2.3.7 Mechanical Testing

Before testing, the $I_{A/P}$ and c were determined at the site of fracturing by microCT imaging as described above. Mechanical properties of the mouse tibias were then determined under four-point bending using an EnduraTech ELF 3200 Series (Bose®, MA)(55). The base support span was 9mm with a load span of 3mm. The tibia was positioned in the loading device so the medial surface was in tension by placing the most distal portion of the tibia and fibula junction directly over the left-most support. Each tibia was loaded at 0.01 mm/s until failure, while the load and displacement were recorded. The force-deflection curve then used to calculate the structural-level properties, while tissue-level properties were estimated using the following beam-bending equations: Stress = $\sigma = f \cdot a \cdot c / 2 \cdot I_{A/P}$; Strain = $\varepsilon = 6 \cdot c \cdot d / a\,(3 \cdot L - 4 \cdot a)$. In each equation, f is the applied force, d is the resulting displacement, a is the distance between the inner spans (3mm), L is the distance of the outer spans (9mm), $I_{A/P}$ is the moment of inertia about the anterior/posterior axis, and c is the distance from the neutral axis to the medial surface under tension. The yield point was determined from the stress-strain relationship using a 20% offset method (56). Mechanical testing was done blinded to conditions.

2.3.8 *In vivo* Intestinal Permeability

For whole intestinal permeability, mice were gavaged with 300 mg/kg of 4kD fluorescein isothiocyanate dextran (FITC) in sterile PBS 4 hours prior to time of death. Sterile serum was collected via cardiac puncture. Serum fluorescence was analyzed using a Tecan Infinite M1000 fluorescent plate reader at an excitation/emission wavelength of

485/530 nm. The rate of 4kD FITC-dextran transfer into the serum was calculated and normalized to control mouse measures for each day of the experiment.

2.3.9 *Ex Vivo* Using Chamber Intestinal Permeability

Mice were harvested and segments of the mid-distal colon removed. Sections were mounted in Lucite chambers and placed in Ussing chambers (physiologic Instruments, San Diego, CA, USA) exposing mucosal and serosal surfaces to oxygenated (95% O_2, 5% CO_2) Krebs bicarbonate ringer buffer (Sigma, St, Louis, MO, USA). Buffer was maintained at 37°C by a heated water jacket and samples allowed to equilibrate for 20 minutes. For measurements of tissue flux, 4 kDa FITC-dextran (2.2 mg/ml final concentration) was added to the mucosal side of the chamber. 10 kDa rhodamine B isothiocyanate (RITC)-dextran (0.55mg/ml final concentration) was also added to the mucosal chamber to control for tissue integrity. Serosal chamber samples were taken at 0 and 60 minutes and fluorescence intensity determined (FITC excitation, 485 nm; emission, 530nm; RITC excitation, 595nm; emission, 615nm; Tecan). FITC-dextran/RITC dextran concentrations were determined using a standard curve and FITC-dextran flux calculated.

2.3.10 DNA Preparation of Fecal Samples

Fecal samples were transferred to Mo Bio Ultra Clean Fecal DNA bead Tubes (MoBio) containing 360µl of buffer ATL (Qiagen) and homogenized for one minute in a BioSpec Mini-Beadbeater. 40µL Proteinase K (Qiagen) was added and samples were incubated for 30 minutes at 55°C, then homogenized again for one minute and incubated at 55°C for additional 30 minutes. DNA was extracted with Qiagen DNeasy Blood and Tissue kit.

2.3.11 DNA Extraction from Mouse Fecal Samples, 16S rRNA gene amplification, and sequencing

DNA for microbial sequence analysis was extracted from mouse fecal samples by bead-beating and modified extraction with Qiagen DNeasy Blood and Tissue kits as described previously (25,30). Bacterial 16S sequences spanning variable region V4 were amplified by PCR with primers F515/R806 with a dual indexing approach and sequenced by Illumina MiSeq described previously(57). 20µl PCR reactions were prepared in duplicate and contained 40ng DNA template, 1X Phusion High-Fidelity Buffer (New England Biolabs), 200 µMdNTPs (Promega or Invitrogen), 10 nM primers, 0.2 units of Phusion DNA Polymerase (New England Biolabs), and PCR grade. Reactions were performed in an Eppendorf Pro thermal cycler with an initial denaturation at 98 °C for 30 s, followed by 30 cycles of 10 s at 98 °C, 20 s at 51 °C, and 1 min at 72 °C. Replicates were pooled and purified with Agencourt AMPure XP magnetic beads (Beckman Coulter). DNA samples were quantified using the QuantIt High Sensitivity DNA assay kit (Invitrogen) and pooled at equilmolar ratios. The quality of the pooled sample was evaluated with the Bioanalyzer High Sensitivity DNA Kit (Agilent).

2.3.12 Microbial Community Analysis

Sequence data was processed using the MiSeq pipeline for mothur using software version 1.38.1 (58) as described previously(30). In brief, forward and reverse reads were aligned, sequences were quality trimmed and aligned to the Silva 16S rRNA gene reference database formatted for mother, and chimeric sequences were identified and removed using the mothur implementation of UCHIME. Sequences were classified according to the mothur-formatted Ribosomal Database Project (version 16, February 2016) using the

Bayesian classifier in mothur, and those sequences classified as Eukarya, Archaea, chloroplast, mitochondria, or unknown were removed. The sequence data were then filtered to remove any sequences present only once in the data set. After building a distance matrix from the remaining sequences with the default parameters in mothur, sequences were clustered into operational taxonomic units (OTUs) with 97% similarity using the average-neighbor algorithm in mothur. 871 OTUs were identified across all samples with an average rarefaction depth of 54,791 reads per sample. Alpha and beta diversity analyses and visualization of microbiome communities were performed with R, utilizing the phyloseq package (59,60). The Bray-Curtis dissimilarity matrix was used to describe differences in microbial community structure. Analysis of similarity (ANOSIM) was performed in mothur.

2.3.13 Statistical Analysis

All measurements are presented as the mean ± SE. Significant outliers were removed using the ROUT test (5 total outliers were found with 1 maximum outlier detected per treatment group). All outliers excluded from data analysis shown in red. Student's t-test and 1-way ANOVA with Tukey post-test were performed using GraphPad Prism software version 7 (GraphPad, San Diego, CA, USA). A p-value ≤ 0.05 was considered significant and <0.01 highly significant.

2.4 Results

2.4.1 Natural Repopulation Following Antibiotic Treatment causes Dysbiosis

To deplete the intestinal microbiota, we treated 12-week old male BALB/c mice with broad-spectrum antibiotics, ampicillin (1 g/L) and neomycin (0.5 g/L), for 2 weeks. Microbiota depletion in antibiotic treated (ABX) mice was confirmed by a significant decrease in fecal bacterial colonies observed on agar dishes 24 hours after plating ($p<0.0001$; Fig 1A) and decreased colonic bacterial 16S rRNA DNA levels ($p<0.001$; Fig 1A). ABX-treated mice were allowed to naturally repopulate their microbiota during the next 4 weeks after which the gut microbiome was analyzed to assess if communities had recovered to their initial composition (Fig 1B). Analysis of colonic 16S rRNA bacterial levels indicated that there were no differences in bacterial load between 4-week post-ABX mice and controls (Fig 1C). However, the composition of the repopulated microbiome in post-ABX-treated mice was different compared to control mice. Post-ABX mice displayed changes in major phyla, specifically *Firmicutes* levels were increased and *Bacteroidetes* levels were decreased (Fig 1D). Diversity metrics that utilize species richness and evenness (Bray-Curtis) also showed a statistically significant separation between the ABX and control groups ($R=0.392$ $p<0.002$; Fig 1E). These data indicate that post-ABX mice develop an altered, potentially dysbiotic gut microbiota.

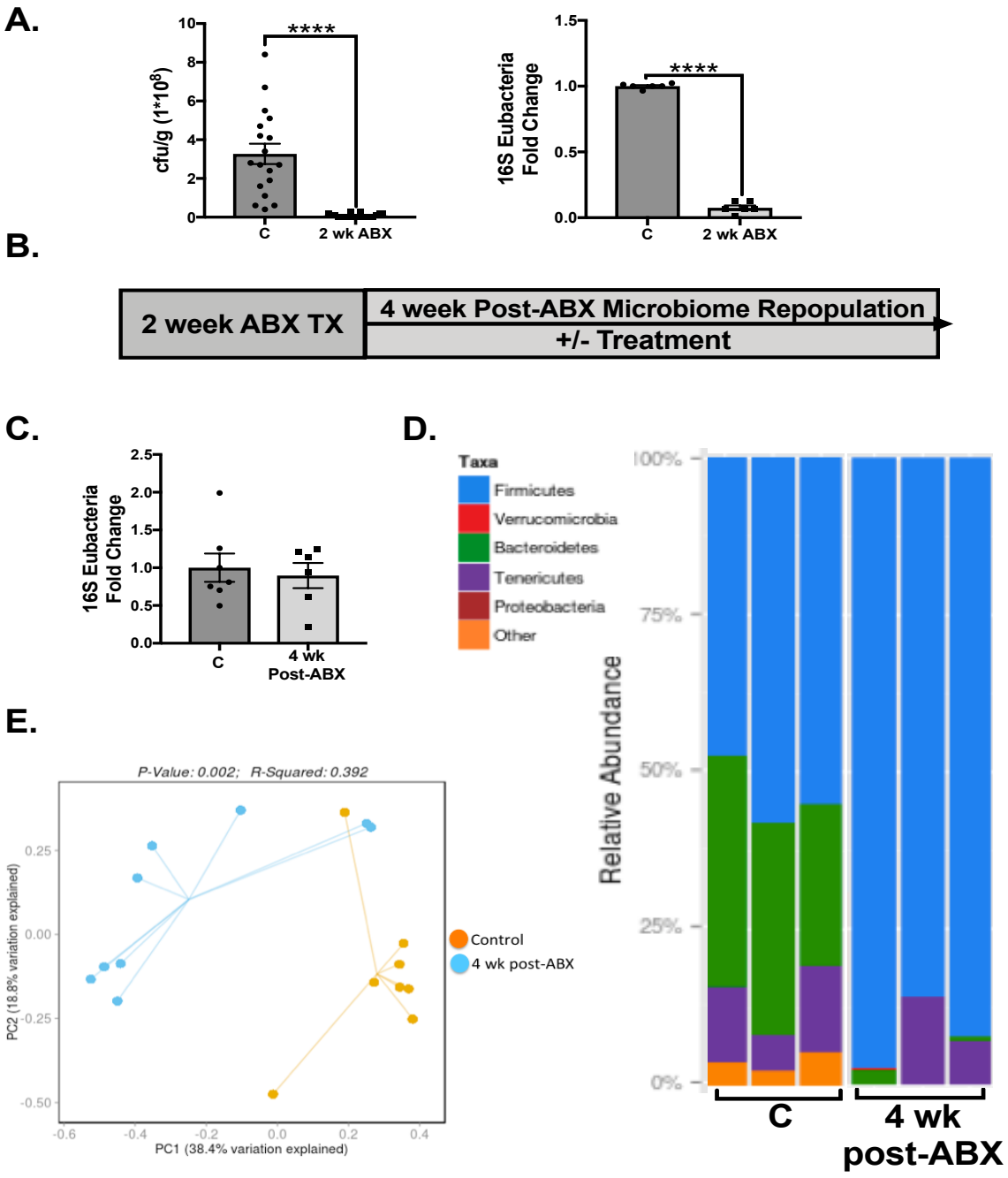

Figure 2.1: Impact of 2-week ABX treatment on the intestinal microbiota immediately and 4-weeks post-ABX treatment

12-week-old male BALB/c mice were given sterile water or ABX (ampicillin and neomycin) for 2-weeks to deplete the intestinal microbiota. A) Fecal samples, from control and 2-week

Figure 2.1. (cont'd)
ABX treated mice, were plated on agar dishes to determine number of colony forming units (cfu)(n=18-20). Additionally, bacterial 16S rRNA was analyzed to assess levels of total eubacteria (n=6). B) The experimental design used to induce post-ABX dysbiosis. C) Total eubacteria levels were assed from control and 4-week post-ABX treated mice in first experimental run (n=6-7). D) Relative abundance of bacterial taxa in control and 4-week post-ABX fecal samples (n=3). Statistical analysis performed by Student's t-test E) PCoA plot of fecal microbiome data, Bray-Curtis analysis performed to determine significance (n=8-9). Values are average ± SEM. **** $p<0.0001$.

2.4.2 Antibiotic-Induced Dysbiosis Decreases Trabecular Bone Density

To understand the effects of post-ABX dysbiosis on bone health parameters, we assessed femur trabecular bone density (Fig 2A). While ABX-depletion of the microbiota (at 2 weeks of ABX treatment) had no significant effect on trabecular bone volume fraction ($p= 0.1466$, Fig 2B), the 4-week post-ABX mice displayed a significant decrease in bone volume (~30%, Fig 2B). Analyses of femoral bone architecture indicated a decrease in trabecular thickness (Tb.Th; $p<0.01$) and increase in trabecular spacing (Tb.Sp; $p<0.05$) in the post-ABX compared to control mice (Fig 2C). No significant differences were seen in cortical bone parameters following microbiota depletion or dysbiosis (data not shown). These data indicate that while microbiome depletion per se does not affect bone volume, post-ABX dysbiosis was associated with bone loss.

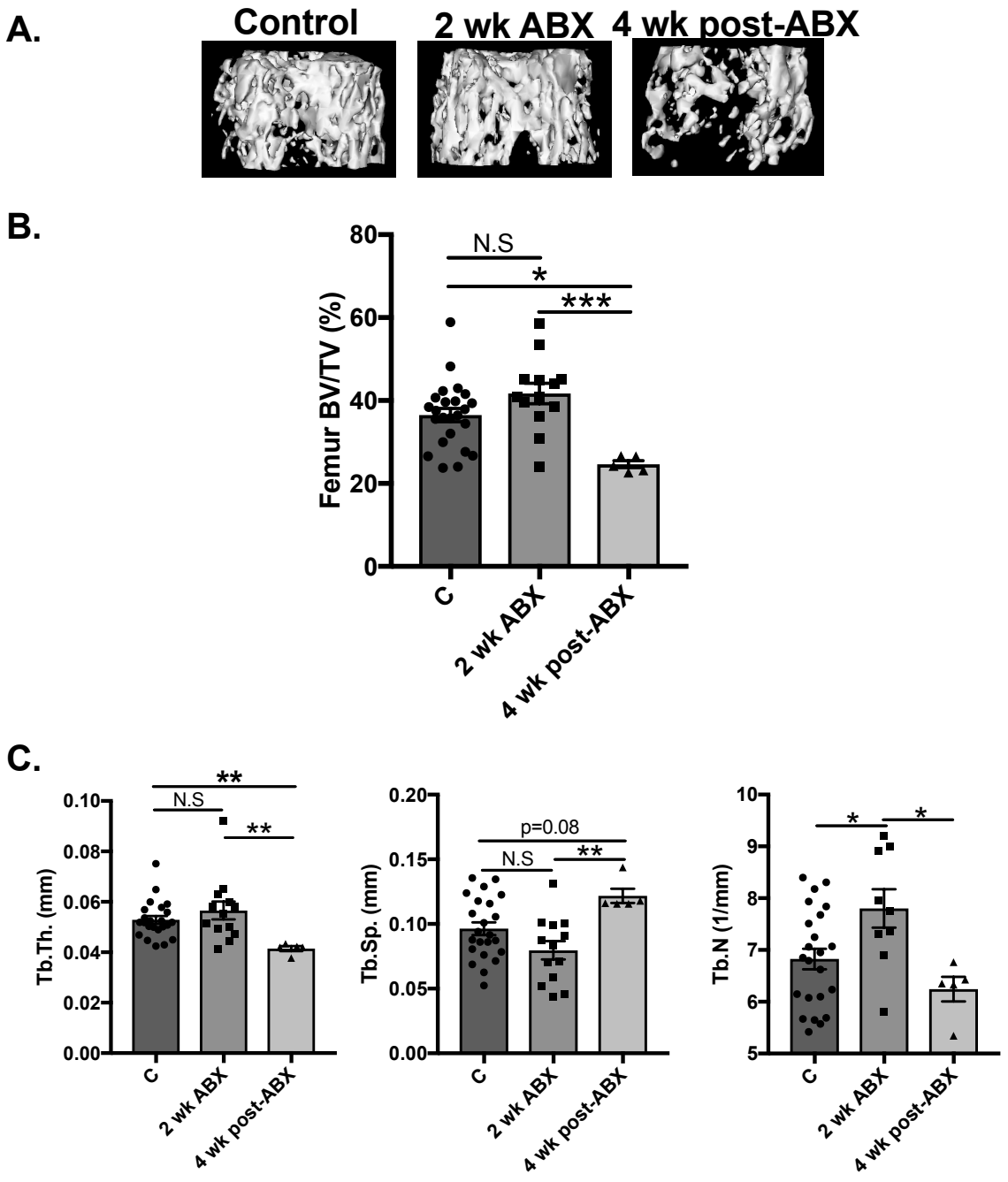

Figure 2.2: Effect of 2-week ABX treatment and subsequent 4-week natural microbiota repopulation on distal femur trabecular bone volume and architecture
12-week-old male BALB/c mice were given sterile water or antibiotics for 2-weeks followed by sterile water for 4-weeks. A-B) Distal femoral metaphyseal trabecular bone volume was investigated at 2 weeks of antibiotic treatment and 4-weeks post-ABX

Figure 2.2. (cont'd)
treatment (n= 24,13,5 respectably). C) Femoral bone architecture measures in control versus 2- weeks ABX and 4-weeks post-ABX mice (n= 24,13,5 respectably). Values are average ± SEM. Statistical analysis performed with 1-way ANOVA with Tukey post-test. *** $p<0.001$;** $p<0.01$; *$p<0.05$.

2.4.3 Antibiotic-Induced Dysbiosis causes Bone Loss via Gut Barrier Dysfunction

Previous studies have shown that increased intestinal permeability observed in diseases, such as IBD, is correlated with bone loss (50). Studies have also shown that ABX-induced dysbiosis can affect intestinal permeability (61,62). Thus we posited that ABX-induced dysbiosis decreases bone health through altering intestinal permeability (24). To test this directly, 2 week-ABX mice were given a high molecular weight polymer, MDY-1001 (MDY), or vehicle for 4 weeks after cessation of ABX treatment. MDY is a non-absorbed mucus supplement that has previously been used to protect the intestinal epithelial layer during radiation injury (63). *In vivo* intestinal permeability was determined by gavaging mice with 4-kDa FITC-dextran 4 hours prior to harvest and then measuring serum FITC levels at harvest. Examination of permeability at the 2-week ABX treatment time point demonstrated no significant effect on intestinal flux compared to controls (Figure 3A). As expected, ABX-induced dysbiosis significantly increased permeability as demonstrated by elevated serum FITC levels, and MDY treatment was effective in preventing the increase (Fig 3A). Consistent with *in vivo* permeability, *ex vivo* colon flux was not affected by the 2-week ABX treatment but was increased in the post-ABX-induced dysbiosis mice ($p<0.05$) and this trended to be reduced by MDY treatment ($p=0.07$, Fig 3B).

Next, we examined if MDY prevention of post-ABX-dysbiosis induced intestinal barrier dysfunction also prevents bone loss. As postulated, MDY treatment following ABX treatment prevented post-ABX induced femoral and vertebral bone loss ($p<0.05$, Fig 3C-D) as well as changes in femoral bone architecture (Fig 3E). Together, these data suggest that

dysbiosis following ABX treatment causes intestinal barrier dysfunction leading to bone loss that is prevented by enhancing barrier function.

Body Weight Parameters (n)	Control (18)	ABX (18)	ABX+LR (16)	ABX+LGG (10)	ABX+E.C. (10)	ABX+MDY (16)
Body weight (g)	29.1 ± 0.5	28.4 ± 0.5	28.3 ± 0.4	29.05 ± 0.8	29.0 ± 0.5	28.6 ± 0.4
Liver (g)	1.5 ± 0.03	1.40 ± 0.05	1.43 ± 0.06	1.39 ± 0.05	1.39 ± 0.5	1.35 ± 0.06
Inguinal Fat (g)	0.11 ± 0.01	0.13 ± 0.02	0.15 ± 0.01	0.11 ± 0.02	0.11 ± 0.01	0.12 ± 0.01
Retroperitoneal Fat (mg)	49.7 ± 4.2	49.7 ± 5.4	56.3 ± 9.1	55.5 ± 9.0	58 ± 5.0	48.8 ± 8.9
Kidney (g)	0.25 ± 0.01	0.24 ± 0.01	0.25 ± 0.01	0.25 ± 0.01	0.25 ± 0.01	0.24 ± 0.01
Spleen (mg)	87.9 ± 2.1	91.1 ± 4.4	88.3 ± 3.0	91.4 ± 4.6	89.2 ± 3.3	92.8 ± 3.0

Table 2.1: Antibiotic treatment does not change general body parameters
Body weight, inguinal fat, retroperitoneal fat, kidney, spleen and liver were weighed after 2-weeks of ABX and 4-weeks of treatment. Values are averages ± SE. Statistical analysis was performed with 1-way ANOVA with Tukey post-test.

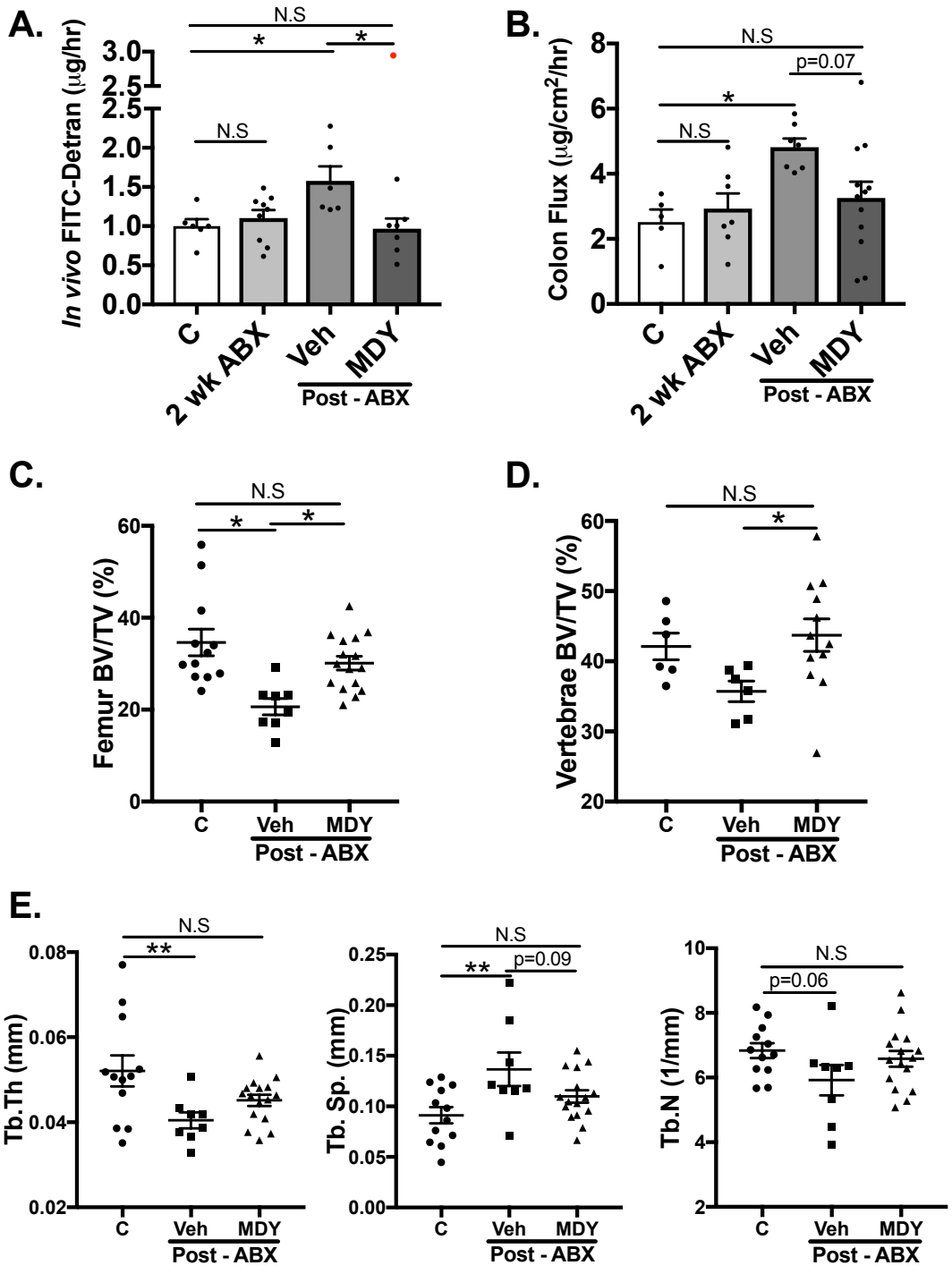

Figure 2.3: MDY supplementation prevents increased intestinal permeability and bone loss

12-week-old male BALB/c mice were untreated (controls) or treated with ABX for 2 weeks. Post-ABX mice were given sterile water (Veh) or supplemented a high molecular weight

Figure 2.3. (cont'd)
polymer (MDY). A) Whole intestinal in vivo flux was measured by FITC dextran gavage (n=6-8). B) Colon sections were analyzed ex vivo. 4-kDa FITC flux was measured in ussing chambers (n=5-12). C-D) MicroCT analysis of femoral and vertebral trabecular bone volume fraction (n=6-16). E) Femoral bone microarchitecture μCT analyses (n=8-16) Values are average ± SEM. Statistical analysis was performed with 1-way ANOVA with Tukey post-test. ** $p<0.01$; *$p<0.05$. Outlier excluded from data analysis shown in red.

2.4.4 Post-ABX-Induced Bone Loss is Prevented by Probiotic *Lactobacillus reuteri* Administration.

To examine if treatment with probiotic bacteria could alter microbial repopulation and potentially overcome the effects of post-ABX dysbiosis, mice were supplemented with *L. reuteri* ATCC PTA 6475 (LR) bacteria immediately following antibiotic cessation. LR was selected based on previous studies demonstrating oral LR benefits bone health in different models of bone loss (23,27,28). LR or broth (vehicle) control were administered for 4-weeks continuously to post-ABX mice. In addition, another probiotic, *Lactobacillus rhamnosus* GG (LGG) and a non-pathogenic bacterium E*scherichia coli* (EC, ATCC O6:B1) were included as treatment groups to determine response specificity. The relative abundance and composition of the intestinal microbiota was examined at the end of the study (Fig 4A). Analyses of diversity metrics, utilizing species richness and evenness (Bray-Curtis), showed significant separation between the control group (non-ABX) and all treated groups (Bray-Curtis $R= 0.272$, $p<0.001$, Fig 4B). However, no significant differences in diversity metrics were detected between post-ABX mice treated with broth versus any of the bacterial treatment groups (Fig 4B). In contrast, analysis of OTUs identified differences in major bacterial phyla between groups. Treatment with LR did not significantly alter the abundance of most phyla examined but was the only treatment to decrease the post-ABX increase in *Firmicutes:Bacteriodetes* ratio (by 63%, Fig 4C). At the specific strain level, the post-ABX induced dysbiosis was characterized by an increase in *Firmicutes* and significantly decreased levels of *Bacteroidetes* ($p<0.0001$; Fig 4D). LGG and EC, on the other hand, significantly increased OTUs classified to the phylum *Verrucomicrobia* compared to control and post-ABX cohorts (by 20-fold and 3.9-fold, respectively; Fig 4D) and decreased

levels of *Firmicutes* (2-fold) compared to post-ABX cohorts. Together, these results suggest that supplementation with different bacteria following ABX treatment can affect the repopulation of major phyla.

Knowing that bacterial supplementation altered the gut microbiota, we examined effects on bone density. As expected, post-ABX mice displayed a decrease in bone volume fraction (femur trabecular region) compared to untreated controls (Fig 5A, $p<0.001$). Interestingly, only LR treatment, not LGG or EC, prevented this bone loss (Fig 5B). Similarly, the post-ABX decrease in vertebral trabecular volume was prevented by LR supplementation ($p<0.01$; Fig 5B), but not by LGG or EC. Measures of femur trabecular microarchitecture were correspondingly modulated by post-ABX, changes only prevented by LR (Fig 5C). Pearson's correlation analyses demonstrated that femoral trabecular BV/TV negatively correlated with the *Firmicutes*:*Bacteroidetes* ratio ($r = -0.4256$, $p=0.0097$**; Fig 5D). Cortical bone parameters were not affected by bacterial supplementation (Table 2).

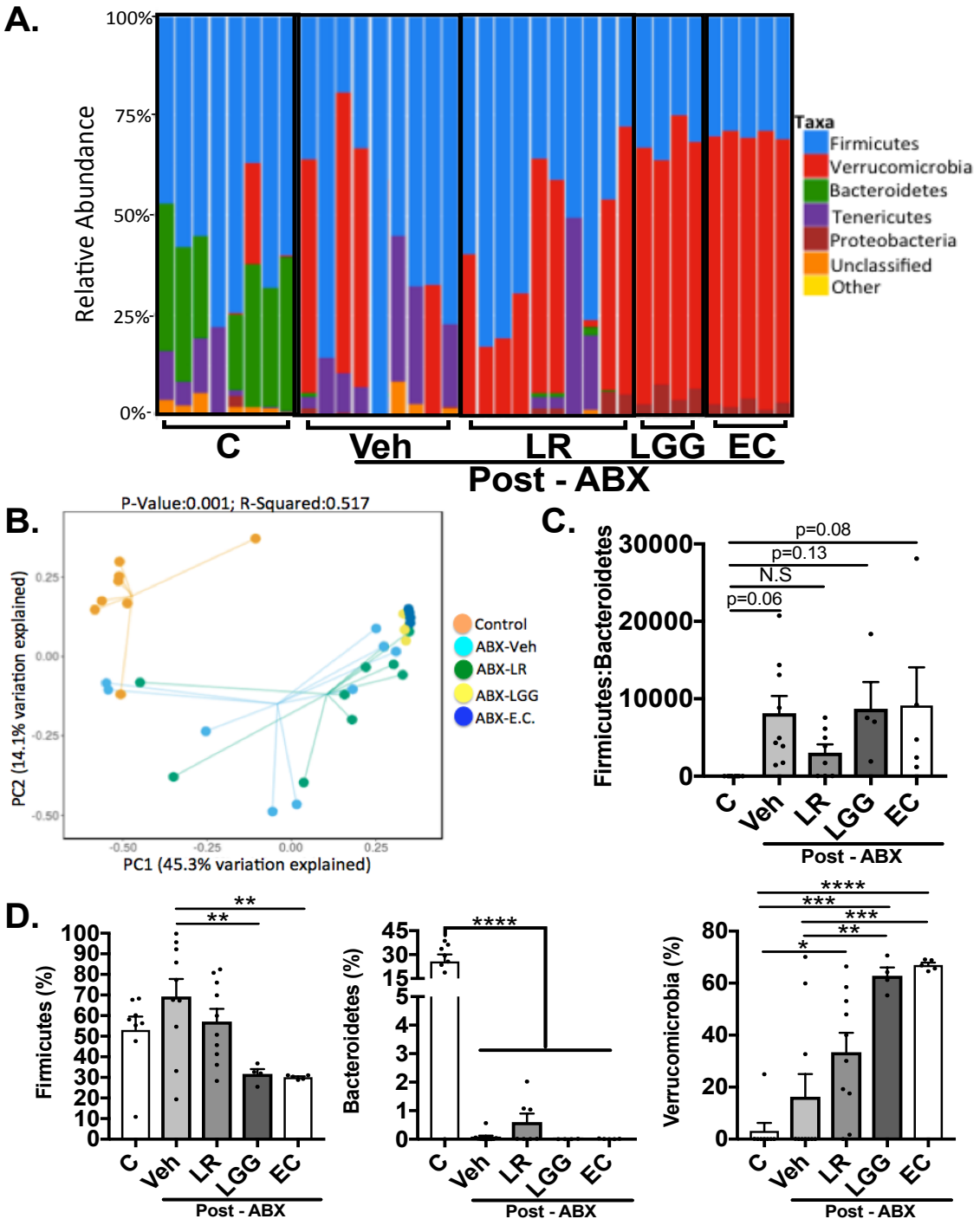

Figure 2.4: Gut microbiome supplementation following antibiotics affects intestinal microbial composition

12-week-old BALB/c male mice were untreated (controls) or treated with ABX for 2 weeks. Post-ABX mice were either given sterile water (Veh) or supplemented with *L. reuteri* (LR),

Figure 2.4. (cont'd)
Lactobacillus rhamnosus GG (LGG) or non-pathogenic *Escherichia coli* (EC) for 4 weeks. A) Relative abundances of bacterial communities following gut supplementation. B) PCoA plot of fecal microbiome, Bray-Curtis analysis performed. Analysis of operational taxonomic units (OTUs) classified to the phylum's C) *Firmicutes* to *Bacteroidetes* ratio D) *Firmicutes*, *Bacteroidetes* and *Verrucomicrobia*. n = 8,9,10,4,5 respectively per group, Values are average ± SEM. Statistical analysis of operational taxonomic units were performed by 1-way ANOVA with Tukey post-test. **** $p<0.0001$; *** $p<0.001$; ** $p<0.01$.

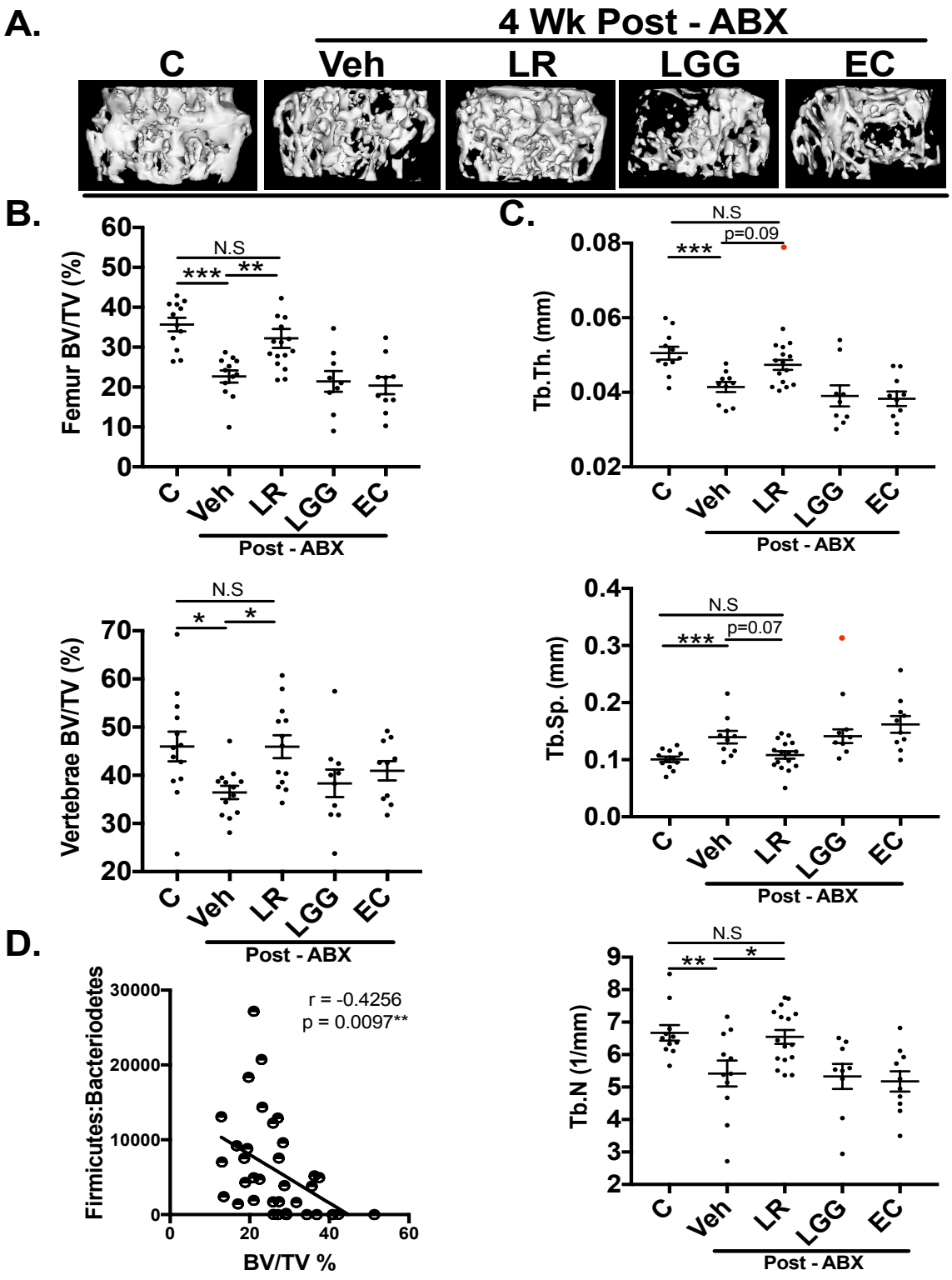

Figure 2.5: *L. reuteri* prevents bone loss following ABX treatment

Figure 2.5. (cont'd)
A) Representative μCT isosurface images of control, antibiotic treated ± *L. reuteri,* LGG or EC. B) MicroCT analysis of femoral and vertebral trabecular bone volume fraction (n=9-16). C) Bone femur microarchitecture μCT analyses. (n=9-16) D) Correlation between bone volume fraction and *Firmicutes* to *Bacteroidetes* ratio. Both LGG and EC cohorts were statistically significant compared to controls in femoral BV/TV (%) and femur microarchitecture ($p<0.05$). Values are average ± SEM. Statistical analysis was performed with 1-way ANOVA with Tukey post-test. *** $p<0.001$; ** $p<0.01$; *$p<0.05$. Outliers excluded from data analysis shown in red.

Cortical Parameters (n)	Control (18)	ABX (18)	ABX+LR (16)	ABX+LGG (10)	ABX+E.C. (10)	ABX+MDY (16)
Ct.Ar (mm^2)	1.08 ± 0.05	1.02 ± 0.03	1.03 ± 0.04	1.01 ± 0.02	1.05 ± 0.03	1.05 ± 0.02
Ct.Th (mm)	0.29 ± 0.01	0.28 ± 0.003	0.29 ± 0.003	0.29 ± 0.003	0.28 ± 0.005	0.28 ± 0.005
Ma.Ar (mm^2)	0.65 ± 0.04	0.62 ± 0.04	0.64 ± 0.03	0.61 ± 0.05	0.65 ± 0.02	0.60 ± 0.02
Tt.Ar (mm^2)	1.39 ± 0.10	1.42 ± 0.08	1.38 ± 0.09	1.5 ± 0.05	1.53 ± 0.05	1.4 ± 0.06
BMD (mg/cc)	1019 ± 24.34	1024 ± 18.5	1045 ± 39.05	1044 ± 55.7	1043 ± 27.23	1021 ± 27.02
BMC (mg)	0.022 ± 0.001	0.021 ± 0.0005	0.022 ± 0.001	0.022 ± 0.001	0.023 ± 0.0007	0.021 ± 0.001
Inner Perimeter (mm)	3.12 ± 0.09	3.03 ± 0.09	3.06 ± 0.08	3.05 ± 0.12	3.14 ± 0.05	3.00 ± 0.05
Outer Perimeter (mm)	4.91 ± 0.13	4.75 ± 0.11	4.8 ± 0.11	4.79 ± 0.14	4.75 ± 0.08	4.74 ± 0.05

Table 2.2: Analyses of femoral cortical bone parameters

Cortical area (Ct.Ar); cortical thickness (Ct.Th); marrow area (Ma.Ar); total area (Tt.Ar); bone mineral density (BMD); bone mineral content (BMC); inner perimeter and outer perimeter. Values are averages ± SE. n = 10-16 per group. Nothing significant compared to control. Statistical analysis was performed with 1-way ANOVA with Tukey post-test.

2.4.5 *L. reuteri* Administration Reverses ABX-Dysbiosis-Induced Barrier Leak

In Figure 3, we demonstrated that ABX-induced dysbiosis causes intestinal barrier disruption and identified that MDY treatment prevents barrier disruption and bone loss. To test if LR administration also prevents barrier disruption, we measured *in vivo* and *ex vivo* intestinal barrier function in control and post-ABX mice supplementation with broth, LR, LGG and EC. As shown in Figure 6A, ABX-dysbiosis-induced *in vivo* permeability was inhibited by LR treatment but not by LGG or EC (Fig 6A). Consistent with the *in vivo* intestinal flux data, post-ABX mice displayed a trend toward increased colon permeability, which was prevented with LR but not LGG or EC treatment ($p<0.05$, Fig 6B). Furthermore, all colon flux measures negatively correlated with femoral trabecular bone density ($r= -0.4235$, $p=0.0027$; Fig 5C). A compromised intestinal barrier in diseases such as IBD has been shown to skew gut inflammation towards a more pro-inflammatory state(64–66). Analyses of colon pro- and anti-inflammatory cytokine expression revealed that increased permeability in the post-ABX, dysbiotic cohort was accompanied by a trending increase in the TNF-α/IL-10 ratio (Fig 6D). Additionally, LR and MDY, which prevented barrier dysfunction, reduced the TNF-α/IL-10 ratio to a level that was not significantly different from control mice (Fig 6D). Together, the data suggest that LR and MDY enhancement of intestinal barrier function prevents post-ABX gut inflammation and bone loss.

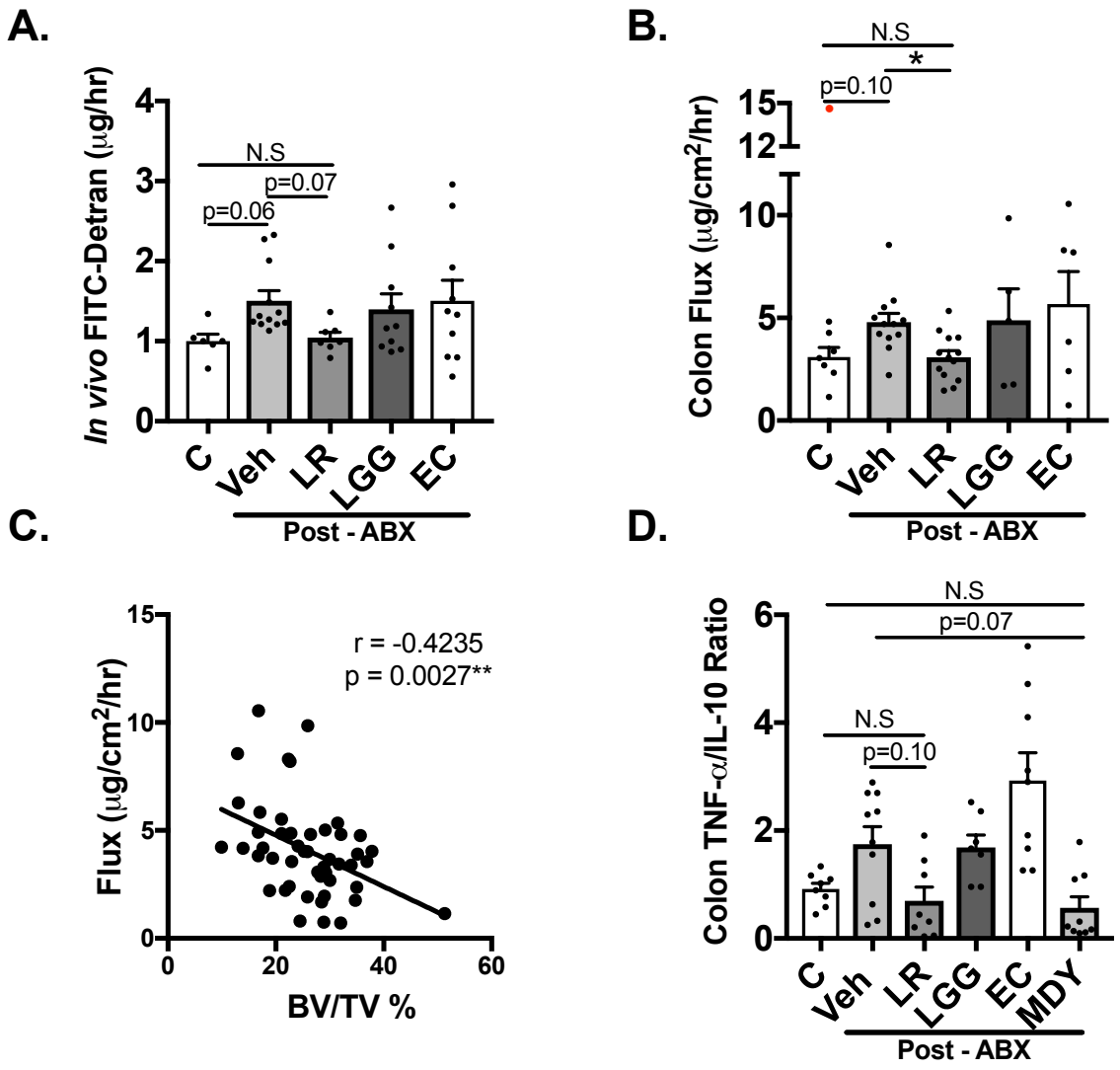

Figure 2.6: *L. reuteri* prevents increased gut permeability caused by antibiotic induced dysbiosis

To examine how the post-ABX treatments altered intestinal permeability. A) Whole in vivo intestinal flux was measured by 4-kDa FITC dextran gavage (n=6-12). B) Colon sections were analyzed ex vivo. 4-kDa FITC flux was measured in ussing chambers (n=5-13. C) Correlation between Colon flux measures and femoral trabecular BV/TV % in all mice that underwent 4-week post ABX +/- treatments. Number of XY pairs observed is 49. D) Colon RNA gene expression of TNF-α/IL-10 ratio (n=8-10). Both LGG and EC cohorts were not statistically significant compared to controls. Values are average ± SEM. Statistical analysis was performed with 1-way ANOVA with Tukey post-test. ** $p<0.01$, *$p<0.05$. Outlier excluded from data analysis shown in red.

2.4.6 Analysis of Osteoblast/Osteoclast Bone Remodeling Markers

To determine whether the gut microbiota manipulations affect catabolic bone parameters, we measured markers of osteoclast activity. Osteoclast number, surface and serum levels of tartrate resistant alkaline phosphatase (TRAP) were increased in the post-ABX induced dysbiosis group (Fig 7A-C) and decreased by LR and MDY treatment (Fig 7A-C). LGG and EC treatment did not significantly affect osteoclast markers relative to post-ABX mice (Fig 7A-C).

Markers of bone formation were also affected in post-ABX and treated mice. Serum osteocalcin was decreased in the post-ABX cohort (Fig 8A; $p<0.05$); both LR and MDY treatments prevented the suppression (Fig 8A; $p<0.05$) while LGG and EC did not. Consistent with serum osteocalcin levels, mineral apposition rate (MAR) and bone formation rate (BFR) were decreased in the post-ABX mice, and LR and MDY (but not LGG or EC treatment) prevented the suppression (Fig 8B, C). These results suggest that gut microbiome repopulation and/or manipulations following ABX treatment affect both anabolic and catabolic processes in bone.

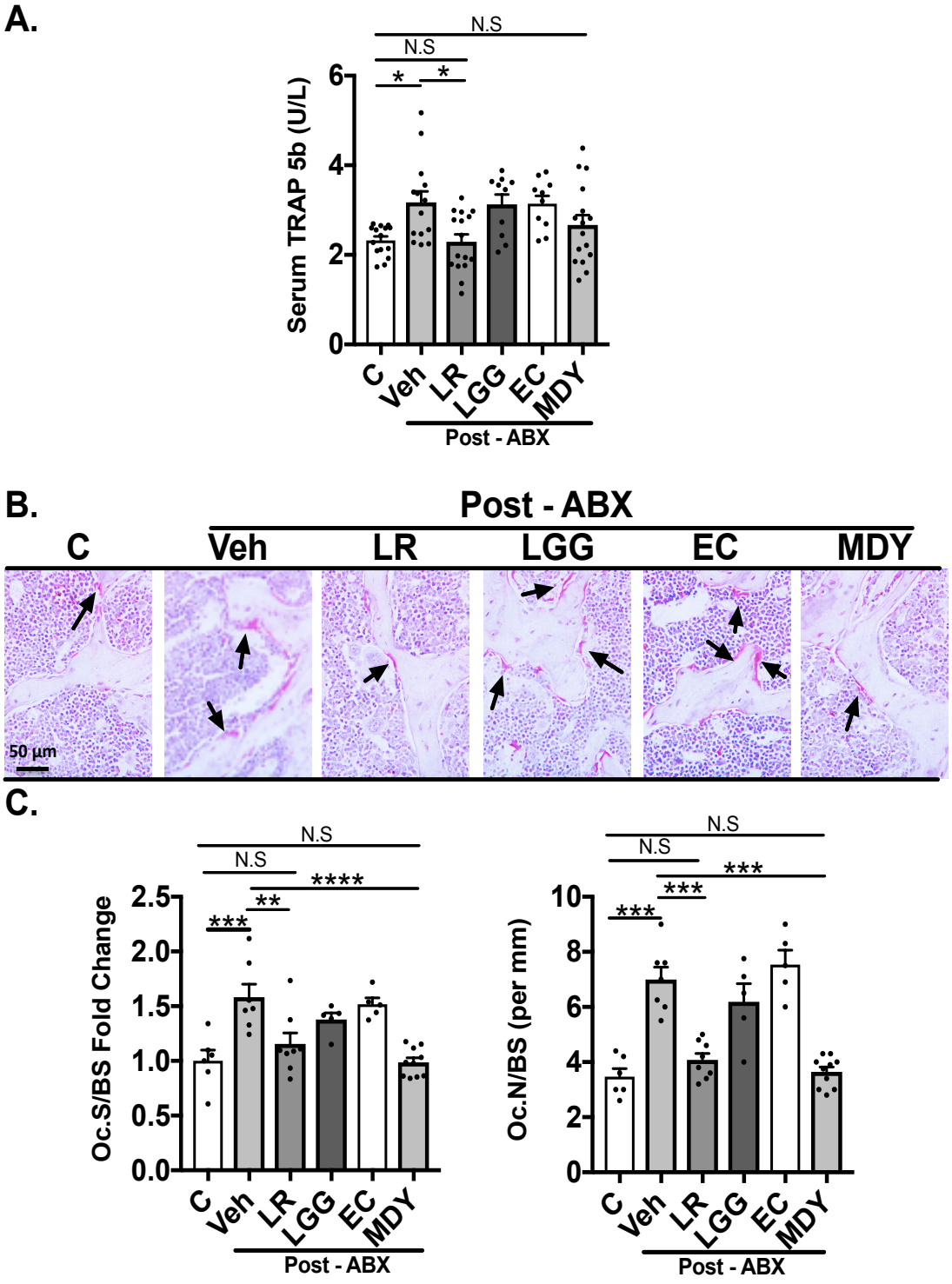

Figure 2.7: *L. reuteri* and MDY prevent increase in bone resorption markers induced by antibiotic microbial repopulation

Figure 2.7. (cont'd)
A) Serum TRAP 5b levels (n=10-15). B) Representative images of TRAP staining on section of the distal femoral trabecular region. C) Quantification of TRAP stain, osteoclast number and osteoclast surface/total bone surface in distal femur trabecular bone region (n=5-8). Both LGG and EC cohorts were statistically significant to controls in all resorption analyses ($p<0.05$). Values are average ± SEM. Statistical analysis was performed with 1-way ANOVA with Tukey post-test. **** $p<0.0001$; *** $p<0.001$; ** $p<0.01$; * $p<0.05$.

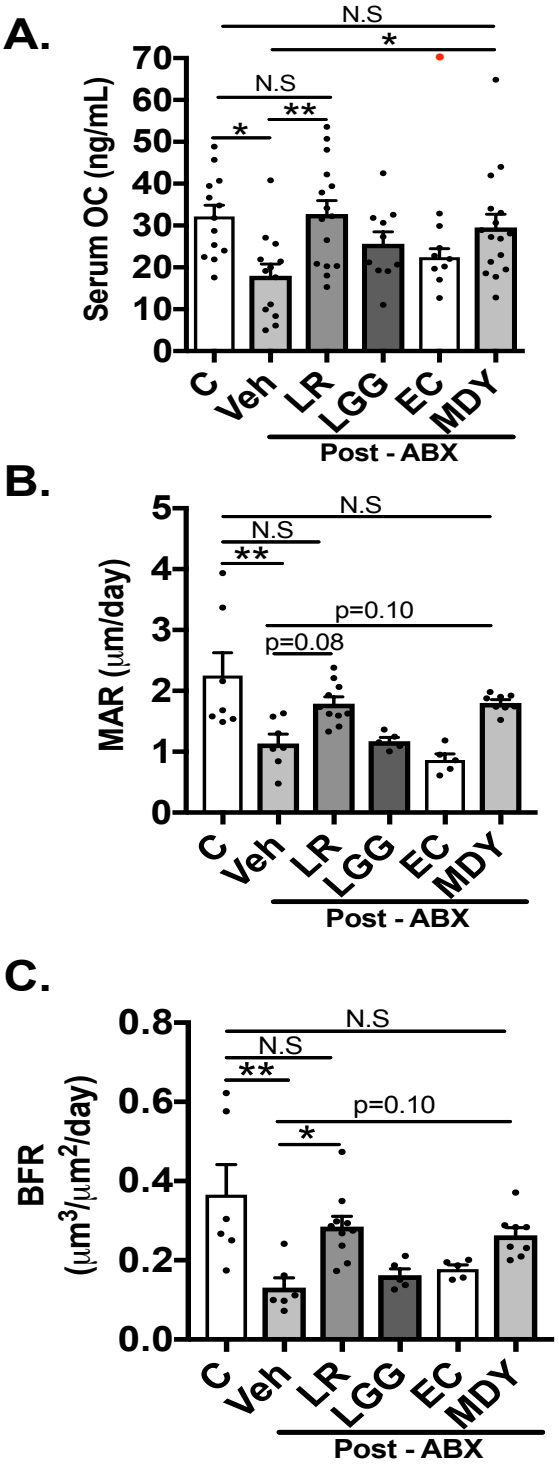

Figure 2.8: *L. reuteri* and MDY prevent imbalance of bone formation markers induced by antibiotic microbial repopulation

Figure 2.8. (cont'd)
A) Serum levels of bone formation marker osteocalcin (OC) (n=10-13). B) Quantitation of trabecular bone mineral apposition rate (MAR)(n=5-10). C) Quantitation of trabecular bone formation rate (BFR)(n=5-10). Both LGG and EC cohorts were statistically significantly compared to controls in MAR/BFR analysis ($p<0.05$). Values are average ± SEM. Statistical analysis was performed with 1-way ANOVA with Tukey post-test. *** $p<0.001$; ** $p<0.01$; *$p<0.05$. Outlier excluded from data analysis shown in red.

2.4.7 Analysis of Mechanical Strength Testing Properties

Gut microbiome alterations under chronic antibiotic treatment have been shown to impair whole-bone mechanical properties (43). Therefore, we investigated whether acute antibiotic-induced gut dysbiosis affected structural or tissue level properties. Comparisons across all treatment groups revealed no significant change in structural-level mechanical properties (Fig 9A, B). Analysis of tissue-level mechanical properties, which estimate material properties of bone, did not reveal differences between groups (Fig 9C, D). These results suggest that post-ABX microbial repopulation does not affect the overall strength and tissue properties of cortical bone in skeletally mature mice.

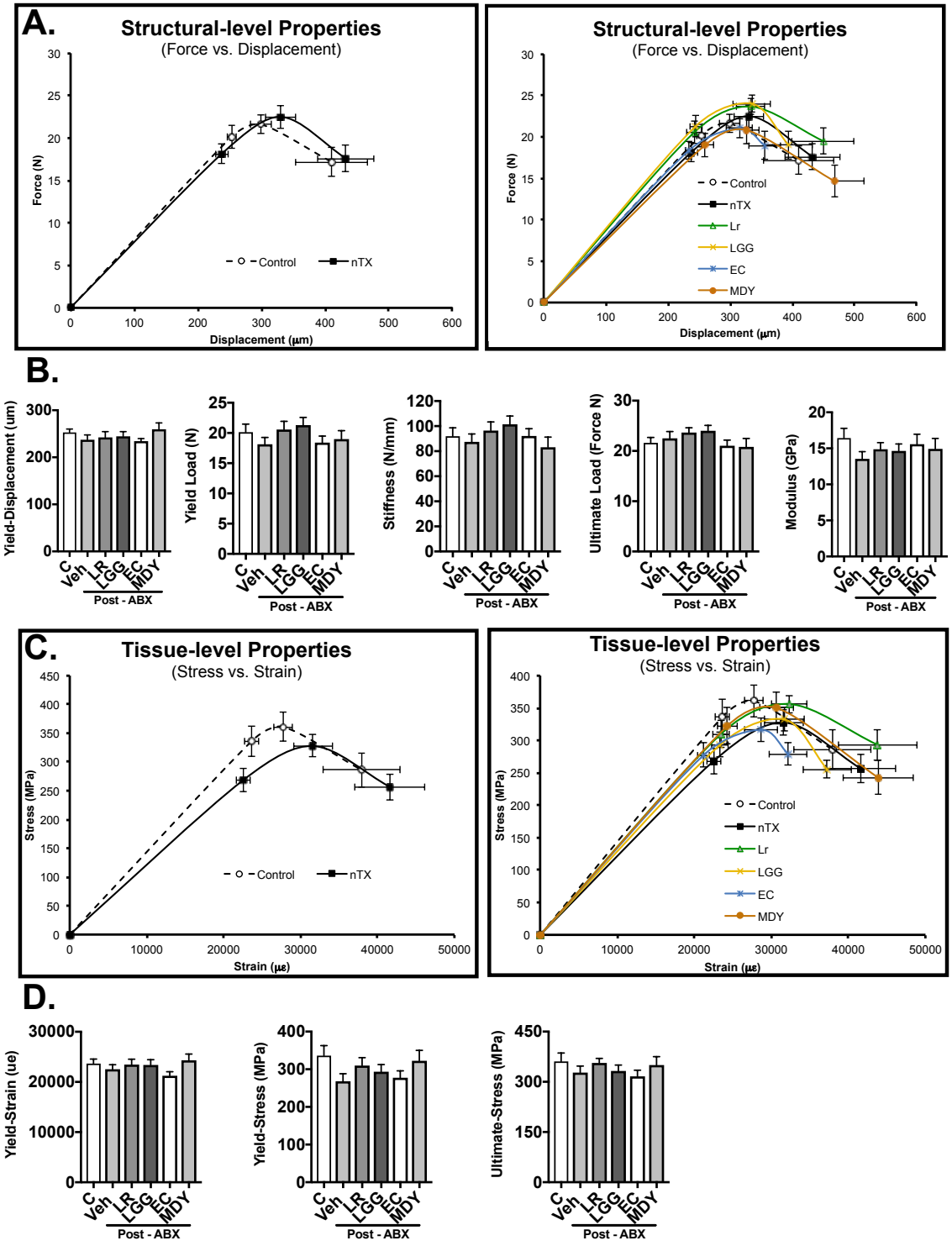

Figure 2.9: Microbial Manipulation does not Alter Mechanical Bone Properties
Analysis of tibia (A and B) structural and (C and D) tissue level properties. Values are average ± SEM. n = 8-12 per group Statistical analysis was performed with 1 way ANOVA with Tukey post-test.

2.5 Discussion

Antibiotics save countless lives and can prevent the spread of pathogenic bacteria, however, a significant side-effect of antibiotics is that they also deplete the commensal microbiota (9,15,46). Increasing numbers of studies demonstrate a role for the intestinal bacteria, as well as its composition in the regulation bone health (11,23–25,27,30,67–69). In the present study we identify that repopulation of the microbiota following acute antibiotic treatment results in dysbiosis, increases intestinal permeability and has a detrimental effect on trabecular bone health. We further reveal that oral supplementation with the probiotic *L. reuteri* 6475 or direct inhibition of gut barrier leak significantly prevents trabecular bone loss and strengthens the role of the gut as a therapeutic target for bone health.

Antibiotics provide an effective way to deplete the microbiota (70–72) and are a means to understand 1) the requirement of the microbiota for bone health and 2) the consequence of subsequent microbial dysbiotic repopulation (37,44,45) on bone health. Our studies utilized a 2-week treatment of ampicillin and neomycin to deplete the microbiome. Ampicillin and neomycin are poorly absorbed in the rodent intestine (47–49) and, when administered together, effectively kill a broad-spectrum of bacteria and deplete the intestinal microbiota (15,46). The depletion of bacteria, particularly the beneficial commensal bacteria, has been demonstrated to provide an environment conducive to dysbiotic bacterial repopulation (44,45,71). This doesn't occur under microbiota replete conditions, in part because commensal bacteria produce anti-bacterial factors that decrease invasion by new bacterial competitors (73). Interestingly, acute antibiotic use in humans has been demonstrated to cause long-term (in some cases 4-years) alterations to

microbiota composition (ie: dysbiosis)(37,44,45,74). Thus, our model gave us the opportunity to study the consequences of dysbiosis on bone health.

In the current study, antibiotic treatment caused significant changes in microbial composition as evidenced by principle coordinate analyses (PCoA) and manifested in a substantial decrease in *Bacteroidetes,* a predominant phylum in healthy mice and humans (17,74–76). A decrease in *Bacteroidetes* abundance has been associated with pathologies such as IBD, IBS, and type 1 diabetes (10,17,68,69,77–79). While our treatment approaches did not shift the overall PCA fingerprint of the post-antibiotic treated mice, LR was able to reduce the *Firmicutes:Bacteroidetes* ratio and prevent bone loss, while LGG and EC did not affect the ratio or prevent bone loss. Interestingly, only mice treated with LGG or non-pathogenic EC displayed an increase in the phylum *Verrucomicrobia* that has recently been shown to be increased by pro-inflammatory high fat-high sugar diets (80). The responsiveness observed in *Bacteroidetes* level and the link of the *Firmicutes:Bacteroidetes* ratio to bone health is most likely due to the abundance of these phyla and therefore reduced variability in comparisons. Deeper analyses of specific strains are variable across repeated experiments performed at different times of the year and in different animal rooms. We hypothesize that it is not the specific microbiota composition that regulates bone health; rather it is the balance of "disease promoting" versus "health promoting" bacteria. Our data support this but further studies are needed to understand which specific groups of bacterial strains are "healthy" in this context.

How does post-ABX induced dysbiosis promote bone loss? Dysbiosis has been linked to increased intestinal permeability/leaky gut (10,21,62) which has been linked to bone loss in diseases such as IBD (11,22,24,69,81) Our analyses indicate that colon flux

significantly negatively correlates with trabecular bone density. By directly inhibiting barrier permeability with MDY treatment, our studies demonstrate that dysbiosis-induced intestinal barrier break contributes to post-ABX bone loss. This is consistent with our previous report demonstrating that chickens infected with intestinal salmonella benefit from MDY treatment and do not lose trabecular bone compared to untreated birds (82). Because MDY is not absorbed, its benefits to bone health are a consequence of its effects on the intestine, thereby underscoring the importance of the gut-bone signaling axis as a therapeutic target for osteoporosis. Under conditions of decreased barrier function (increased permeability), bacteria and their factors can translocate across the intestinal epithelium and move throughout the blood stream (50,83). Factors that promote intestinal permeability such as detergents (ie: DSS) skews the gut towards a more pro-inflammatory state (81) that is often characterized by increased levels of pro-inflammatory factors such as TNF-α and/or decreased levels of anti-inflammatory factors such as IL-10 (81,84). We used the colon TNF/IL-10 ratio as a marker of the balance between a pro- versus anti-inflammatory state. Post-ABX trended to increase TNF/IL-10 levels while MDY and LR trended to reduce the TNF/IL-10 ratio, suggesting a reduction in colon inflammation compared to post-ABX (dysbiotic) mice.

While previous studies have shown that probiotics can promote intestinal barrier function and reduce intestinal inflammation (24,62,85–88), in our studies only LR had this beneficial effect. Based on our current results, LR had the ability to alter the *Firmicutes:Bacteriodetes* ratio, strengthen intestinal barrier function and reduce intestinal inflammation while LGG did not. As expected, the non-pathogenic EC also did not influence these parameters. The ability of LR but not LGG to benefit gut and bone health was

unexpected given that LGG has been elegantly shown to benefit bone health in ovariectomized mice and in male and female mice (24,89). It is possible that an effective response is dependent upon the specific dysbiosis/microbiota composition as well as the disease model, inflammatory status, sex, age and strain (24,27,28,90,91). LGG is known to differ from LR because of its requirement for epithelial cell interaction to mediate it effects(92). In addition, specific probiotic strains produce unique and varying combinations of biologically active metabolites and proteins that can benefit intestinal microbiome, inflammation and permeability (25,93,94).

Our studies demonstrate that post-ABX induced dysbiosis had a marked effect on trabecular bone parameters of both long bone and vertebral sites. This was observed in repeated experiments. On the other hand, cortical bone microarchitecture and strength were not affected. A response likely due to the length of the study which could be too short to see changes in the slow remodeling cortical bone, as we have seen in other mouse models (95). Additional analyses of bone anabolic and catabolic processes indicated that both are affected by dysbiosis. Specifically, dysbiosis significantly increased osteoclast markers and decreased osteoblast markers. Past studies examining dysbiosis and enhanced intestinal permeability-induced bone loss have reported a predominant suppression of anabolic markers and some enhancement of resorption markers (22,81,83,96). Our treatment groups also affected these parameters but only MDY and LR were able to significantly reverse the effect of post-ABX dysbiosis. This is consistent with previous reports showing that LR can increase mineral apposition, osteoblast number, serum osteocalcin and decrease osteoclast markers in other mouse models such as type 1 diabetes and estrogen deficiency (23,25,28).

Our studies show that the microbiota depletion seen at the 2-week time point of antibiotic treatment did not affect bone density measures. Our results are similar to some germ free mouse studies (24,30). Studies in germ free mice, however, are inconsistent in terms of bone growth and density (increase, decrease, no change)(24,25,29,31,97). Caveats to the germ-free model are that the mice have intestinal and immunological abnormalities (including fewer T cells and T cells skewed toward a Th2 phenotype)(21,29,98,99). While differences in mouse genetic strain, age, and sex can contribute to study variation, the differences in the microbiota composition used to conventionalize the germ-free mice could be a major factor in directing the final outcome. While both the germ-free and antibiotic models of microbiota depletion are useful, the antibiotic treatment model overcomes the issue of long-term absence of microbiota and its associated developmental abnormalities.

Previous chronic antibiotic treatment studies examining bone health predominantly focused on longer antibiotic treatment times and used young mice (1-month of age) that are undergoing bone growth rather than remodeling. For example, a study treating young C57BL/6J mice at weaning with penicillin, chlortetracycline, or vancomycin for 4-months found a decrease in bone mineral content in males but not females (100). Chronic antibiotic treatment of 4 week old C57BL/6J mice with ampicillin and neomycin for 16 weeks decreased femoral bone bending (43). Studies in female mice have been more variable. For instance, 1-month old female C57BL/6J mice treated for ~2 months with penicillin, vancomycin and a combination displayed an early increase in bone mineral content at 3 weeks, but by the end of the study (7 weeks) bone mineral content (BMC) did not differ from controls (40). Whereas, another study treating 2-month old female BALB/c mice with

an antibiotic cocktail (ampicillin, vancomycin, metronidazole and neomycin) for 6 weeks showed a decrease in bone formation that was related to reduced IGF-1 (31) which is critical for postnatal bone growth (101,102). In our studies, we did not observe changes in serum IGF-1 levels (data not shown) or bone length, which suggests that IGF-1 does not play a role in microbiome-mediated bone remodeling in skeletally mature male mice. Together, these studies suggest that chronic antibiotic treatments can affect bone health in a sex- and age-dependent manner. Direct comparison between studies is complicated due to differences in treatment duration, sex, genetic strain, age and antibiotics used.

There has yet to be any clinical studies examining the effects of ABX and post-ABX treatment on bone health. However, there are clinical studies looking into antibiotics/probiotics and their effects on the gut microbiome. Past studies have shown that a common one-week antibiotic treatment regimen with clarithromycin and metronidazole resulted in marked compositional disturbances in the throat and the gut microbiota, which persisted for up to 4 years post treatment (44). In a recent study examining antibiotic perturbation in mice and humans (103), probiotics were shown to induce a different stool and mucosal microbiome repopulation that was maintained and therefore delayed recovery of the original pre-ABX microbiota. This study did not look at functional outcomes to systemic health following antibiotic induced alterations to the gut microbiota or probiotic treatment, so it is unclear which microbiota composition is most beneficial to the host.

In summary, it is now recognized that a healthy intestinal microbiota benefits overall host health. Our studies highlight the importance of microbiota composition and intestinal barrier function in regulating bone health and suggest that the period following antibiotic

treatment could be important not only for intestinal health but also for bone health. Correcting dysbiosis, increasing barrier function, and decreasing intestinal inflammation can enhance gut and bone health. The mouse model serves as a straight forward way to study dysbiosis effects on bone and has implications for the treatment of bone loss linked to other conditions of dysbiosis such as inflammatory bowel disease, diabetes, menopause and poor dietary intake (high fat diets). Discovering the connection between the microbiome and bone health can speed identification of novel therapeutic targets for osteoporosis.

2.6 Acknowledgments

The authors thank the Investigative Histology Laboratory in the Department of Physiology, Division of Human Pathology and the Biomedical Imaging Center at Michigan State University for their assistance with histology and imaging, respectively. The authors are also grateful to the staff of Campus Animal Resources for the excellent care of our animals. These studies were supported by funding from the National Institute of Health, grants RO1 DK101050 and RO1 AT007695. Authors' roles: Study design: JS, NP and LRM. Study conduct: JS. Data collection/analysis: JS, FC, NDA, SR, LS and JDD. Data interpretation: JS, LS, JDD, RB, NP and LRM. Drafting/Revising manuscript content: JS, FC, NDA, RB, NP and LRM. Approving final version of manuscript: All authors. Integrity of data analysis: JS, NP and LRM.

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CHAPTER 3: INVOLVEMENT OF THE GUT MICROBIOTA AND BARRIER FUNCTION IN GLUCORTICID INDUCED OSTEOPOROSIS

This chapter is an edited version of a manuscript that is being submitted for publication.

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3.1 Abstract

Glucocorticoids (GCs) are potent immune-modulating drugs that have significant side effects including glucocorticoid-induced osteoporosis (GIO). GCs directly induce osteoblast and osteocyte apoptosis but can affect other organs including the intestine. Our lab and others identified that the microbiota contributes to the regulation of bone density; however, the role of the gut in mediating GIO has never been examined. We report that GC treatment alters the microbiota composition therefore to determine the contribution of the microbiota to GIO pathogenesis, we treated adult male mice for 8-weeks with GC (prednisolone, 2.5 mg/kg/day via subcutaneous pellet) in the presence or absence of broad-spectrum antibiotic treatment (ABX) to deplete the microbiota. Strikingly, depletion of the microbiota prevented GC-induced bone loss and establishes the requirement of a microbiota for GIO. We next altered the GC-induced microbiota changes by chronic probiotic supplementation (*Lactobacillus reuteri,* LR; 10^8 cfu/day) and found that LR also prevented GIO. Interestingly, we identified that GC treatment causes intestinal barrier leaks and raises serum endotoxin levels, a response that was prevented by both LR and ABX treatments. Accordingly, enhancement of barrier function, with a mucus supplement, prevented both GC-induced barrier dysfunction and GIO, indicating that intestinal barrier function is mechanistically important for GIO. Taken together, these data highlight the unappreciated role of the gut microbiota and intestinal barrier function in GIO pathogenesis and identify the gut as a novel therapeutic target for preventing GIO.

3.2 Introduction

Glucocorticoids are potent immune-modulating drugs that are widely prescribed to more than 1.2% of the US population (1). They are used for alleviating inflammatory processes in diseases such as inflammatory bowel disease (IBD), rheumatoid arthritis and bronchial asthma as well as for relieving allergic conditions. Chronic glucocorticoid treatment however, is associated with detrimental side effects including glucocorticoid-induced osteoporosis (GIO) (2–5). GIO is the most common form of secondary osteoporosis, resulting in fractures in 30-50% of patients undergoing chronic glucocorticoid treatment (1,6). Though some osteoporosis treatments can help reduce GIO, the number of women under 50 taking glucocorticoids and being monitored and treated for bone loss is less than 6% (6). This is in part due to a lack of GIO risk awareness and unwillingness to take additional medications to prevent the bone loss.

Glucocorticoids (GC) promote bone loss by causing rapid bone resorption, followed by prolonged and profound suppression of bone formation (4,7,8). The physiologic glucocorticoid hormone cortisol is released from the adrenal cortex and elevated in response to psychological or psychosocial stress. While physiologic concentrations of GC can stimulate osteoblast differentiation (9), abnormal increases in cortisol levels associated with aging (10,11) and pharmacologic doses of GC can decrease osteoblast differentiation and viability (9,12). GC treatment has been shown to induce apoptosis of osteoblasts as well as osteocytes in both mouse (13–17) and human (18–23) bone samples. In addition, GCs have been shown to skew mesenchymal stem cell differentiation towards adipocytes and thus impair osteoblast lineage selection and differentiation (9)(24–26). Consistent with suppressed osteoblast lineage selection, GC treatment reduces Wnt10b expression (a

positive regulator of osteoblastogenesis), cell viability and bone density (9,27–32). GC signaling in osteoblasts involves binding of GC to the cytosolic glucocorticoid receptor (alpha) which then translocates to the nucleus, binds to glucocorticoid response elements and regulates gene expression (4,12). In addition to the direct effects of GC on bone, GCs also affect other organ systems that can further influence bone health. Especially relevant to the present study, GCs can alter the intestinal microbiota composition in animal models (33,34).

The intestinal microbiota comprises approximately 100 trillion bacteria as well as fungi and viruses (35). The gut bacterial community is composed of more than 1000 species from 28 phylum. The microbiota can benefit host health by producing essential nutrients, digesting otherwise indigestible food components and enhancing maturation of the immune system (36). An unhealthy imbalance in the microbiota community composition, called dysbiosis, is linked to a variety of metabolic, inflammatory and immunologic diseases (37–39). Dysbiosis is also linked to increased intestinal permeability/leaky gut (36,40). A leaky intestinal barrier cannot prevent translocation of bacteria and their products into the lamina propria where they can activate immune cells, cause inflammation and enter the systemic circulation. Thus, barrier leaks, like dysbiosis, are associated with disease (41).

It is now clear that changes in the gut microbiota can impact bone density and health (42–46). For example, intestinal infection with pathogenic bacteria can induce bone loss in male mice (47). In contrast, treatment with beneficial bacteria (probiotics) alters gut microbiota composition (45,46,48) and enhances bone density in healthy male mice (49), post-antibiotic treated male mice (44), ovariectomized mice (43,45,50), inflamed intact

female mice (42) and type 1 diabetic male mice (51). Given the powerful role of the intestinal microbiota in regulating bone health (42–46,49–52), we hypothesized that the gut microbiota and barrier function may play a role in the regulation of GIO pathogenesis. Here we report that the presence of a microbiota is necessary for GIO. We further show that GC treatment causes intestinal barrier leaks and raises serum endotoxin levels. Treatments that target the gut to alter the microbiota (*Lactobacillus reuteri*) or enhance intestinal barrier function (MDY) prevented GC-mediated osteoblast and osteocyte apoptosis, Wnt10b suppression and GIO. Correspondingly, we demonstrate that GIO is prevented by up regulation of Wnt10b. Taken together; we identify the gut microbiome and intestinal barrier as key components in GIO pathology that can serve as therapeutic targets for GIO prevention.

3.3 Materials and Methods

3.3.1 Animals and Experimental Design

3.3.1.1 Mice

Adult C57BL/6 male mice (15-week old) were purchased from Jackson Laboratory (Bar Harbor, Maine) and allowed to acclimatize to the animal facility for 1-week prior to start of experiment. Mice were housed in shoebox cages (5 mice/cage), given sterile Teklad 2019 chow (Madison, WI), sterile water ad libitum and were maintained on a 12-hour light/dark cycle at 24°C in specific pathogen free facilities. In addition, C57BL/6 osteocalcin promoter-driven Wnt10b transgenic mice (generously provided by Dr. Ormond MacDougald, University of Michigan (53)) were used in studies at 16 weeks of age. Mice were genotyped with genomic DNA isolated from ear tissue samples (DNeasy Blood and Tissue Kit; Qiagen). DNA was amplified by RT-PCR with iQ SYBR Green Supermix (Bio-Rad Laboratories) and primers specific to the transgene (53). All animal procedures were approved by the Michigan State University Institutional Animal Care and Use Committee and complied with NIH guidelines. Experiments were repeated twice to ensure reproducibility.

3.3.1.2 Mouse Treatments

Mice at 16-weeks of age were anesthetized (isoflurane inhalation) and implanted subcutaneously with either a placebo pellet (control) or a pellet containing 5mg of prednisolone (60-day slow release pellet; Innovative Research of America, Sarasota)(54). Briefly, a small (10mm) skin incision was made on the upper back and using a trochar the pellet was inserted into the interscapular region. This treatment corresponds to a daily dose of 0.08 mg prednisolone per day or on average 2.5 mg/kg/day (54).

For probiotic and MDY treatment experiments, mice were randomly split into five experimental groups (9-10 mice per group): 1) Control, 2) prednisolone treatment (GC; 2.5 mg/kg/day via subcutaneous implant) and sterile drinking water (Veh), 3) GC treatment and sterile drinking water containing $3.3x10^8$ cfu/ml *L. reuteri* 6475 (LR), 4) GC treatment and sterile drinking water containing $3.3x10^8$ cfu/ml *Lactobacillus rhamnosus* GG (LGG) or 5) GC treatment and sterile drinking water containing 1.25% MDY, a high molecular weight polymer used as a mucus supplement (55,56). These oral treatments continued for the duration of the 56-day (8 week) glucocorticoid experiment.

3.3.1.3 Microbiota Depletion Experiment

Male 16-week old mice were split into four experimental treatment groups: a) control (C), b) prednisolone treated (GC; 2.5 mg/kg/day via subcutaneous implant), c) continuous broad-spectrum antibiotics (ABX) or d) continuous broad-spectrum antibiotics with prednisolone treatment (ABX+GC). One week prior to the start of GC treatment, the ABX mouse groups were treated with broad-spectrum antibiotics ampicillin (1.0 g/L Sigma, St. Louis, MO) and neomycin (0.5 g/L Sigma, St. Louis, MO) at a dose of 160 and 80 mg/kg/day respectively, (39,57). These antibiotics are poorly absorbed by the intestine (or unabsorbed in the case of neomycin)(58–60) to allow the successful targeting and depletion of intestinal commensal microbes (39,44,61). Water intake was measured to account for any increase in consumption due to glucocorticoid treatment (62) and antibiotic dose altered to keep dose the same among antibiotic treatment groups.

3.3.2 Bacterial Culture for Oral Treatment

L. reuteri ATCC PTA 6475 (LR) and *Lactobacillus rhamnosus* GG (LGG) were initially cultured and kept under anaerobic conditions on a deMan, Rgosa, Sharpe media (MRS) plates at 37˚C. To prepare bacteria for oral treatments (via drinking water), LR and LGG were anaerobically cultured in 10 mls of MRS media overnight at 37˚C. Bacteria were then sub-cultured into 250 mls of fresh MRS broth and grown until log phase (OD_{600} = 0.4), spun down, washed with sterile PBS, then re-suspended in 60 ml sterile PBS, and stored at -80˚C. Aliquots were taken prior to freezing to determine cfu/ml. Bacteria were re-suspended in drinking water at a final concentration of $3x10^8$ cfu/ml. Bacteria viability in the drinking water was confirmed by plating aliquots on MRS plates and growing anaerobically overnight at 37˚C. Both LR and LGG bacterial identity was confirmed using strain-specific primers via quantitative real-time polymerase chain reaction (PCR) (63,64).

3.3.3 Bone Marrow CD4+ FACS

Total bone marrow (BM), both nucleated and non-nucleated cells were isolated from the mouse femur. Specifically, femurs were isolated and extraneous tissue/muscle detached. The femoral head was severed and the femur placed cut side down into a 0.5 ml micro-centrifuge tube with a small hole in the base. The 0.5 ml micro-centrifuge tube was placed inside a 1.5 ml micro-centrifuge tube and centrifuged at 5000 rcf for 20 seconds. Bone marrow was collected in the 1.5 ml micro-centrifuge tube and re-suspended in 1 ml of alpha-MEM. Cells (@ $2x10^6$) were incubated with Fc block (BD Pharmingen, CA, USA) for 15 min. Cells were stained with anti-mouse CD4-V500 (Clone RM 4–5, BD Bioscience, CA, USA) for 30 minutes at 4˚C. Cells were washed three times in assay buffer (PBS, 0.5% bovine

serum albumin (BSA), 5mM EDTA) followed by permeabilization in cytofix / cytoperm per manufacturer's instructions (BD Biosci-ences).

3.3.4 Serum Measurements

Sterile blood was collected at the time of harvest via cardiac puncture, allowed to clot at room temperature for 5 minutes and then centrifuged at 4,000 rpm (2500 rcf) for 10 minutes. Serum was removed and stored at -80˚C. Serum went through no more than two freeze/thaw cycles. Serum bacterial endotoxin levels were detected using the HEK-Blue™ LPS Detection Kit (inVivoGen, California) according to manufacturer's instructions. Standard curves where used for each individual endotoxin assay.

3.3.5 µCT Bone Analysis

Fixed femurs and vertebrae were scanned using GE Explore Locus microcomputed tomography system using a voxel resolution at 20µm obtained from 720 views. Angle of increment was 0.5, and beam strength was set at 80 peak kV and 450 uA. Each run consisted of controls, glucocorticoid (+/- treatments), and a calibration phantom to standardize gray scale values and maintain consistency. A fixed threshold of 905 (determined based on automated and isosurface analyses) was used for all bones. Femoral bone analyses were performed on trabecular bone defined as beginning proximal (a distance of 1% of total bone length) to the growth plate and then extending 10% of total bone length toward the diaphysis, excluding cortical bone. Trabecular bone was also analyzed within the lumbar (L3) vertebrae. Trabecular bone volume fraction, thickness, spacing and number values were calculated by a GE Healthcare MicroView software application for visualization and analysis of volumetric image data. Cortical measurements

were performed in 2x2x2 mm cube midway down the length of the femur. All bone measurements were performed blind.

3.3.6 Mechanical Testing

Before testing, the $I_{A/P}$ and c were determined at the site of fracturing by microCT imaging as described above. Mechanical properties of the mouse tibias were then determined under four-point bending using an EnduraTech ELF 3200 Series (Bose®, MA) (65). The base support span was 9mm with a load span of 3mm. The tibia was positioned in the loading device so the medial surface was in tension by placing the most distal portion of the tibia and fibula junction directly over the left-most support. Each tibia was loaded at 0.01 mm/s until failure, while the load and displacement were recorded. The force-deflection curve then used to calculate the structural-level properties, while tissue-level properties were estimated using the following beam-bending equations: Stress = $\sigma = f \cdot a \cdot c / 2 \cdot I_{A/P}$; Strain = $\varepsilon = 6 \cdot c \cdot d / a (3 \cdot L - 4 \cdot a)$. In each equation, f is the applied force, d is the resulting displacement, a is the distance between the inner spans (3mm), L is the distance of the outer spans (9mm), $I_{A/P}$ is the moment of inertia about the anterior/posterior axis, and c is the distance from the neutral axis to the medial surface under tension. The yield point was determined from the stress-strain relationship using a 20% offset method (66).

3.3.7 Bone Histology and Histomorphometry

For dynamic histomorphometric measures of bone formation, mice were injected intraperitoneally with 200 µl of 10mg/ml calcein (Sigma, St. Louis, MO) dissolved in sterile saline at 7 and 2 days prior to harvest. Femurs were embedded in paraffin blocks and sectioned in 5 micron slices (67). Distal femur metaphyseal sections were viewed under a fluorescent Nikon Eclipse E800 microscope (Nikon Instruments Inc, Melville, NY) and 4-5

digital images taken. The distance between the calcein lines (mineral apposition rate, MAR) and the length of the calcein lines (single + double labeled surfaces; mineralized surface, MS) along with the total bone surface (BS) were measured to calculate the bone formation rate (BFR) using Image Pro-Plus 7.0 (Media Cybernetics, Rockville, MD). Slides were stained for tartrate-resistant acid phosphatase (TRAP) activity and counterstained with hematoxylin according to manufacturer protocol (387A-1KT, Sigma, St. Louis, MO). Slides were photographed 5 images per slide at 25X magnification for osteoclast and osteoblast counts and at 10X magnification for adipocytes. Image Pro-plus software was used in analysis of slide images. In the distal femoral trabecular region, ranging from the growth plate to 2mm towards the diaphysis, osteoblast surface area was measured and expressed as a percentage of total bone surface. Adipocytes >30 μm in size were counted in the same trabecular area and expressed as number per μm of marrow area. The identity of sections was not revealed until all measures were obtained. Cell death of osteoblasts and osteocytes was determined using a TACS-XL Basic In Situ Apoptosis Detection Kit (TUNEL, Trevigen Inc., Gaithersburg, MD) in same trabecular and cortical region. Osteoblasts and osteocytes with positive nuclei were counted and expressed as a percentage of total osteoblasts/osteocytes counted per bone. Five trabecular and cortical regions were examined for each mouse.

3.3.8 RNA Analysis

Tibias were collected and cleaned of muscle and connective tissue and immediately flash frozen in liquid nitrogen and stored at -80°C. Frozen tibias were crushed under liquid nitrogen conditions with a Bessman tissue pulverizer (Spectrum Labratories, Rancho Dominguez, CA). RNA was isolated using TriReagent (Molecular Research Center,

Cincinnati, OH) and checked for integrity by agarose-gel electrophoresis. cDNA was produced by reverse transcription using Superscript II reverse transcriptase kit and oligo dT primers (Invitrogen, Carlsbad, CA). Intestines were flushed with 1X PBS and RNA isolated from mid colon sections. Gene expression levels were amplified by real-time PCR with iQ SYBR Green supermix (BioRad Hercules, CA) and specific gene primers. House keeping gene, hypoxanthine guanine phosphoribosyltransferase (HPRT) mRNA levels, which is not effected by treatment groups, was used as house-keeping gene. The PCR protocol included 40 cycles using the iCycler (Bio-Rad) and resulting data was evaluated using iCycler software. Each cycle consists of 95°C for 15 seconds, 60°C for 30 seconds and 72°C for 30 seconds. Negative controls included primers without cDNA. Primers for mouse genes were as follows: *HPRT* (Forward, **5'-** AAGCCTAAGATGAGCGCAAG- **3',** Reverse, **5'-** TTACTAGGCAGATGGCCACA), *TRAP5* (Forward **5'**- ACTTCCCCAGCCCTTACTACCG- **3'**, Reverse, **5'-** TCAGCACATAGCCCACACCG- **3'**), *Bax* (Forward **5'** GACAGGGGGCTTTTTGCTA **3'**, Reverse, **5'-** TGTCCACGTCAGCAATCATC- **3'**), *Bcl-2* (Forward **5'-** GACAGAAGATCATGCCGTCC- **3',** Reverse, **5'-** GGTACCAATGGCACTTCAAG- **3')**, *Wnt10b* (Forward **5'-** AATGCGGATCCACAACAACA- **3',** Reverse, **5'-** TTCCATGGCATTTGCACTTC- **3')**.

3.3.9 DNA Preparation of Fecal Samples

Fecal samples were transferred to Mo Bio Ultra Clean Fecal DNA bead Tubes (MoBio) containing 360µl of buffer ATL (Qiagen) and homogenized for one minute in a BioSpec Mini-Beadbeater. 40µL Proteinase K (Qiagen) was added and samples were incubated for 30 minutes at 55°C, then homogenized again for one minute and incubated at 55°C for additional 30 minutes. DNA was extracted with Qiagen DNeasy Blood and Tissue kit.

3.3.10 DNA Extraction from Mouse Fecal Samples, 16S rRNA gene amplification, and sequencing

DNA for microbial sequence analysis was extracted from mouse fecal samples by bead-beating and modified extraction with Qiagen DNeasy Blood and Tissue kits as described previously (45,68). Bacterial 16S sequences spanning variable region V4 were amplified by PCR with primers F515/R806 with a dual indexing approach and sequenced by Illumina MiSeq described previously (69). 20µl PCR reactions were prepared in duplicate and contained 40ng DNA template, 1X Phusion High-Fidelity Buffer (New England Biolabs), 200 µMdNTPs (Promega or Invitrogen), 10 nM primers, 0.2 units of Phusion DNA Polymerase (New England Biolabs), and PCR grade. Reactions were performed in an Eppendorf Pro thermal cycler with an initial denaturation at 98 °C for 30 s, followed by 30 cycles of 10 s at 98 °C, 20 s at 51 °C, and 1 min at 72 °C. Replicates were pooled and purified with Agencourt AMPure XP magnetic beads (Beckman Coulter). DNA samples were quantified using the QuantIt High Sensitivity DNA assay kit (Invitrogen) and pooled at equilmolar ratios. The quality of the pooled sample was evaluated with the Bioanalyzer High Sensitivity DNA Kit (Agilent).

3.3.11 Microbial Community Analysis

Sequence data was processed using the MiSeq pipeline for mothur using software version 1.38.1 (70) as described previously (68). In brief, forward and reverse reads were aligned, sequences were quality trimmed and aligned to the Silva 16S rRNA gene reference database formatted for mother, and chimeric sequences were identified and removed using the mothur implementation of UCHIME. Sequences were classified according to the mothur-formatted Ribosomal Database Project (version 16, February 2016) using the

Bayesian classifier in mothur, and those sequences classified as Eukarya, Archaea, chloroplast, mitochondria, or unknown were removed. The sequence data were then filtered to remove any sequences present only once in the data set. After building a distance matrix from the remaining sequences with the default parameters in mothur, sequences were clustered into operational taxonomic units (OTUs) with 97% similarity using the average-neighbor algorithm in mothur. 871 OTUs were identified across all samples with an average rarefaction depth of 54,791 reads per sample. Alpha and beta diversity analyses and visualization of microbiome communities were performed with R, utilizing the phyloseq package (71,72). The Bray-Curtis dissimilarity matrix was used to describe differences in microbial community structure. Analysis of similarity (ANOSIM) was performed in mothur.

3.3.12 Statistical Analysis

All measurements are presented as mean ± SEM. Significant outliers were removed using the ROUT test. Power analysis conducted with data from pilot studies, determined using an F-test that at a 5% level of significance and 80% power to detect differences among the means, that 8 mice are needed per group for ANOVA analyses of femur bone outcomes(73). Each study was repeated 2 times to rule out single environmental effect and to enhance study findings and power. Student's t-test and 1-way ANOVA with Tukey post-test were performed using GraphPad Prism software version 7 (GraphPad, San Diego, CA, USA). A p-value ≤ 0.05 was considered significant and <0.01 highly significant.

3.4 Results

3.4.1 Depletion of the Gut Microbiota Prevents Glucocorticoid-Induced Bone Loss.

Previous studies demonstrate glucocorticoids and gut microbiota as important regulators of bone health (2,5,44,46,74–76). However, whether gut microbiota is necessary for the glucocorticoid effects on bone is not known. To first assess whether GC treatment alters microbiota, we treated sixteen-week-old male C57BL/6 mice with prednisolone (subcutaneous pellet, 2.5 mg/kg/day) or a placebo for 8 weeks (54). Stool samples were used for 16S rRNA sequencing which revealed shifts in the relative abundance of bacteria phylum in GC treated compared to control mice (Fig 1A). Statistical analyses of community structure differences (using ANOSIM) determined that GC treatment was significantly different from the control ($R = 0.807$, $p<0.0001$). Knowing that GC treatment significantly alters the mouse gut microbiome, we next tested whether the gut microbiota is necessary for glucocorticoid-induced osteoporosis (GIO). For this, we treated mice with prednisolone or sham pellet (as above) with or without continuous oral broad-spectrum antibiotics (ampicillin (1g/L) and neomycin (0.5g/L)) for the duration of the experiment. Control groups included mice that were not treated with prednisolone but treated with antibiotics and mice that did not undergo any treatment. Microbiota depletion in the antibiotic treated groups was confirmed by a significant decrease in fecal bacterial cfu at 1 and 8 weeks (Fig 1B). To investigate whether microbiota depletion affected GIO, distal femur metaphyseal and L3 vertebral trabecular bone volume fraction were examined at the 8-week time point using microcomputed tomography (Fig 1C). As expected, GC treatment of control mice caused more than a 50% loss of trabecular bone volume in the distal femur (Fig 1D).

Remarkably, GC treatment of microbiota-depleted mice (antibiotic treated) did not cause femoral bone loss (Fig 1D). Vertebral trabecular bone showed similar trends (Fig 1E). Microbial depletion, on its own, had no effect on the bone volume of untreated mice (Fig 1 C-E, control vs ABX). Detailed analyses of trabecular architectural parameters corresponded with changes in femoral bone volume fraction (Fig 1F). Interestingly, GC induced changes in cortical bone parameters were not prevented by microbial depletion (Table1). These results indicate that the gut microbiota mediates glucocorticoid-induced trabecular bone loss.

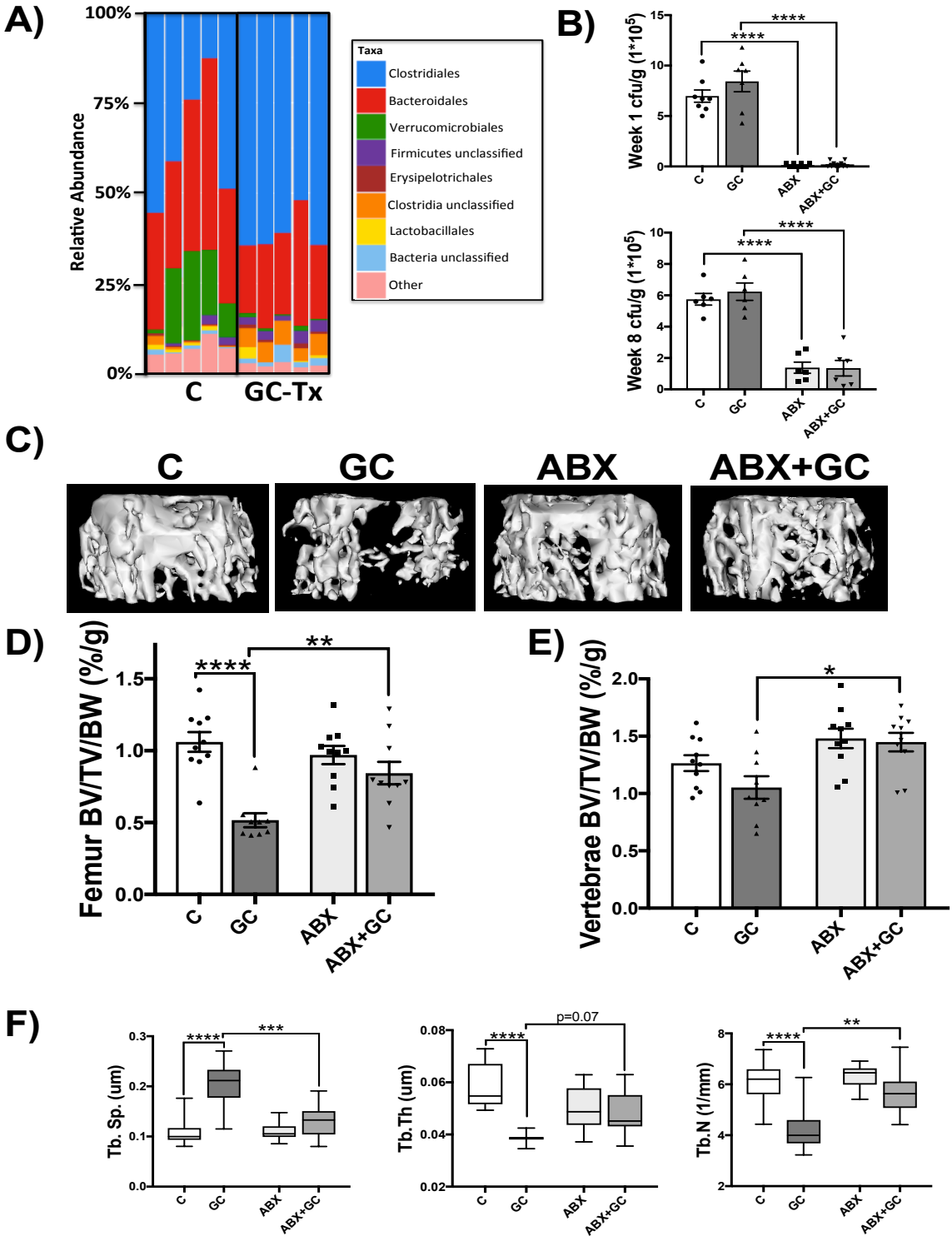

Figure 3.1: Depletion of the gut microbiota prevents glucocorticoid induced trabecular bone loss

Figure 3.1. (cont'd)
15-week old C57BL/6J male mice were split into four experimental groups: sterile drinking water (C), glucocorticoid treatment (GC), continuous broad-spectrum antibiotics (ABX) or glucocorticoid treatment with continuous broad-spectrum antibiotics (ABX+GC). A) Relative abundances of bacterial communities following gut treatments. B) Fecal samples from mice were taken at week 1 and week 8 and colony forming units (CFU/g) determined. C) Representative μCT iso-surface images of distal femur. D-E) MicroCT analysis of femoral and vertebral trabecular bone volume fraction corrected for body weight. F) Bone femur microarchitecture μCT analyses. n = 5-10 per group. Values are average ± SE. Statistical analysis was performed with 1 way ANOVA with Tukey post-test. **** $p<0.0001$; *** $p<0.001$; ** $p<0.01$; *$p<0.05$.

	Controls	GC-Tx	ABX	ABX+GC
(n)	**(10)**	**(9)**	**(10)**	**(10)**
General Parameters				
Body Weight (g)	33.9 ± 0.59	32.8 ± 0.90	32.9 ± 0.78	32.8 ± 0.40
Femur BV/TV (%)	35.9 ± 2.28	**15.5 ± 0.85***	32.8 ±3.04	27.7 ± 2.63
Vertebrae BV/TV (%)	42.7 ± 2.21	34.2 ± 2.44	46.3 ± 2.43	47.4 ± 2.39
Bone length (mm)	15.0 ± 0.21	15.2 ± 0.32	14.9 ± 0.16	15.1 ± 0.12
Cortical Parameters				
Ct.Th (mm)	0.22 ± 0.004	**0.20 ± 0.005***	0.22 ± 0.005	**0.19 ± 0.002#**
Ct.Ar (mm^2)	0.98 ± 0.009	0.98 ± 0.02	0.98 ± 0.08	0.97 ± 0.02
Ma.Ar (mm^2)	0.77 ± 0.04	**0.83 ± 0.02***	0.78 ± 0.07	**0.84 ± 0.03#**
Tt.Ar (mm^2)	1.57 ± 0.04	1.55 ± 0.03	1.50 ± 0.10	1.51 ± 0.04
BMD (mg/cc)	963.4 ± 8.44	926.0 ± 23.5	978.2 ± 41.96	946.2 ± 26.03
BMC (mg)	0.020 ± 0.0008	0.020 ± 0.0006	0.020 ± 0.001	0.019 ± 0.0006
Inner Perimeter (mm)	2.64 ± 0.10	**2.91 ± 0.05***	2.69 ± 0.10	**2.92 ± 0.03#**
Outer Perimeter (mm)	4.63 ± 0.07	**4.91 ± 0.04***	4.65 ± 0.09	**4.85 ± 0.05#**
MOI (J)	0.11 ± 0.003	0.11 ± 0.005	0.11 ± 0.010	0.12 ± 0.005

Table 3.1: Femoral general and cortical bone parameters
Values are averages ± SE. n = 9-10 per group. *Significant bolded compared to controls, # Significant compared to antibiotic controls (ABX). Statistical analysis was performed with 1 way ANOVA with Tukey posttest.
GC-Tx = glucocorticoid-treated; ABX = antibiotic controls; ABX+GC = Antibiotic + glucocorticoid treated; Ct.Th = cortical thickness; Ct.Ar = cortical area; Ma.Ar = marrow area; Tt.Ar = total area; BMD = bone mineral density; BMC = bone mineral content; MOI = moment of inertia.

3.4.2 Probiotic *Lactobacillus reuteri* 6475 Supplementation Prevents Glucocorticoid-Induced Bone Loss.

Having established that GC alters gut microbiome and that gut microbiota mediates GIO, we next examined if supplementation of gut microbiota with beneficial probiotic bacteria will prevent GIO. For this we chose LR and LGG, which have proven to benefit bone health in humans and mouse models of osteoporosis. Mice were treated with prednisolone or placebo with or without LR or LGG supplementation ($3x10^8$ cfu/ml drinking water) for the duration of the experiment. Statistical analyses of community structure differences (using ANOSIM) indicated that treatment with the probiotics further shifted the GC mouse microbiota to unique compositions ($R=0.944$, $p<0.001$; Fig 2A). As we found before, microcomputed tomography of femurs from GC treated mice demonstrated a significant decrease in trabecular bone volume fraction compared to untreated controls (Fig 2B, C). More importantly, supplementation with LR, but not LGG, prevented GC-induced bone loss (Fig 2B,C) suggesting that the beneficial bone response is specific to the species of probiotic bacteria. Vertebral trabecular bone changes showed similar trends (Fig 2D). Like femoral bone volume fraction, femoral trabecular measure (including thickness, number and spacing) were modulated by GC treatment and importantly were prevented with LR but not LGG supplementation (Fig 3E). Control mice (placebo pellets) that were treated with the probiotics did not exhibit any significant bone response (Table 2).

To be effective in preventing GIO, a treatment must block GC induced bone loss while maintaining the beneficial immunosuppressive effects of GCs. To examine this in our model, we isolated bone marrow cells from the mice and quantitated the number of CD4+ T-lymphocytes. Figure 3F demonstrates that GC suppression of CD4+ cells is maintained in

all treatment groups. Taken together these data suggest that LR supplementation prevents GC-induced bone loss without preventing GC suppression of CD4+ T-lymphocytes.

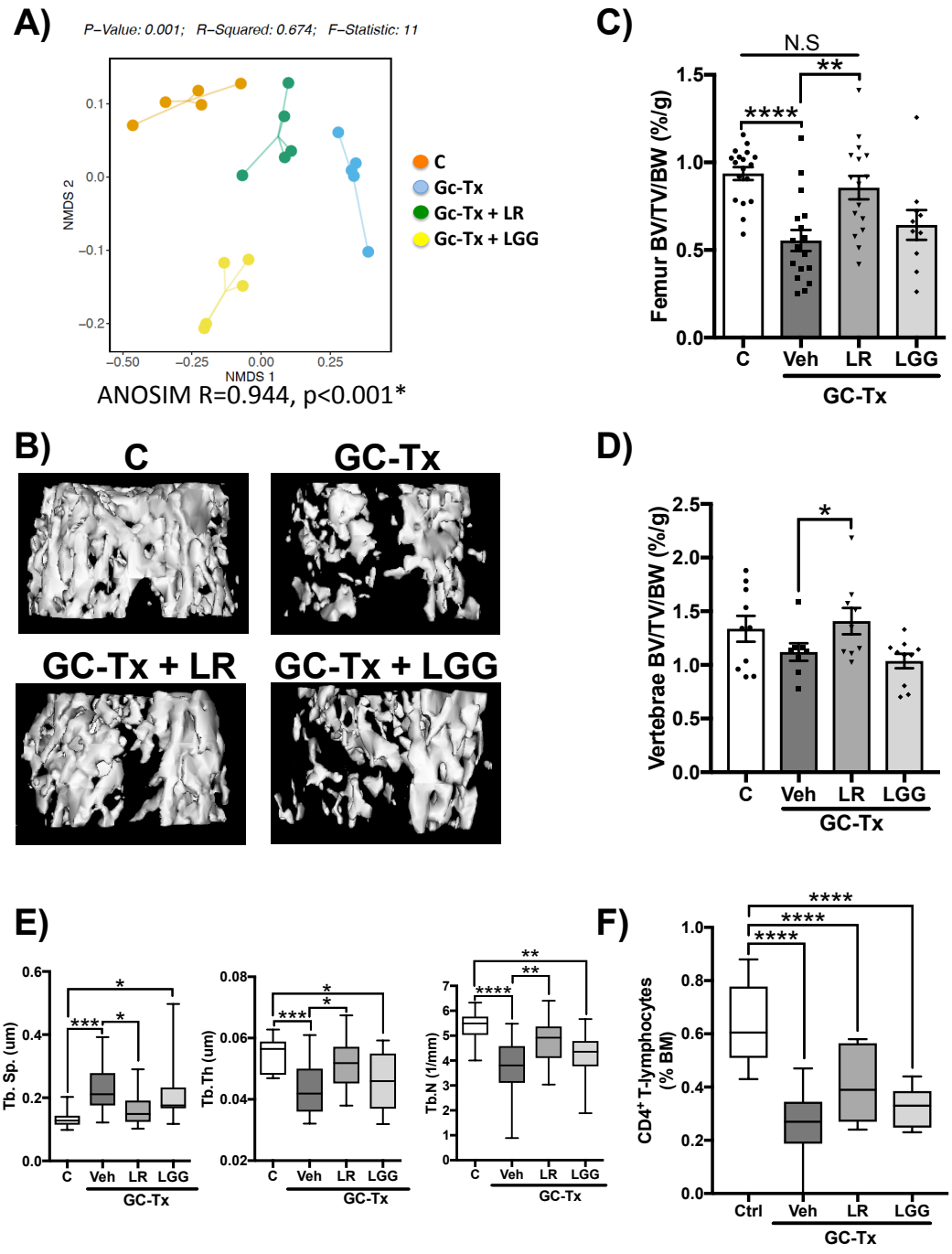

Figure 3.2: Probiotic *Lactobacillus reuteri* 6475 supplementation prevents glucocorticoid-induced bone loss

16-week old male C57BL/6J mice were treated with glucocorticoids (prednisolone) for 8 weeks. Mice were either given sterile water (Veh) or supplemented with *L. reuteri* (LR) or *Lactobacillus rhamnosus* GG in water for duration of experiment. A) NMDS plot of fecal microbiome, Bray-Curtis analysis performed (n=5). B) Representative µCT iso-surface images of distal femur. C-D) MicroCT analysis of femoral and vertebral trabecular bone volume fraction corrected for body weight. E) Bone Femur microarchitecture µCT analyses.

Figure 3.2. (cont'd)
F) Percentage of CD4+ T lymphocytes within femoral bone marrow. n = 8-10 per group. Values are average ± SE. Statistical analysis was performed with 1 way ANOVA with Tukey post-test. **** $p<0.0001$; *** $p<0.001$; ** $p<0.01$; *$p<0.05$.

	Controls	GC-Tx	ABX	ABX+GC
(n)	**(10)**	**(9)**	**(10)**	**(10)**
General Parameters				
Body Weight (g)	33.9 ± 0.59	32.8 ± 0.90	32.9 ± 0.78	32.8 ± 0.40
Femur BV/TV (%)	35.9 ± 2.28	**15.5 ± 0.85***	32.8 ±3.04	27.7 ± 2.63
Vertebrae BV/TV (%)	42.7 ± 2.21	34.2 ± 2.44	46.3 ± 2.43	47.4 ± 2.39
Bone length (mm)	15.0 ± 0.21	15.2 ± 0.32	14.9 ± 0.16	15.1 ± 0.12
Cortical Parameters				
Ct.Th (mm)	0.22 ± 0.004	**0.20 ± 0.005***	0.22 ± 0.005	**0.19 ± 0.002#**
Ct.Ar (mm^2)	0.98 ± 0.009	0.98 ± 0.02	0.98 ± 0.08	0.97 ± 0.02
Ma.Ar (mm^2)	0.77 ± 0.04	**0.83 ± 0.02***	0.78 ± 0.07	**0.84 ± 0.03#**
Tt.Ar (mm^2)	1.57 ± 0.04	1.55 ± 0.03	1.50 ± 0.10	1.51 ± 0.04
BMD (mg/cc)	963.4 ± 8.44	926.0 ± 23.5	978.2 ± 41.96	946.2 ± 26.03
BMC (mg)	0.020 ± 0.0008	0.020 ± 0.0006	0.020 ± 0.001	0.019 ± 0.0006
Inner Perimeter (mm)	2.64 ± 0.10	**2.91 ± 0.05***	2.69 ± 0.10	**2.92 ± 0.03#**
Outer Perimeter (mm)	4.63 ± 0.07	**4.91 ± 0.04***	4.65 ± 0.09	**4.85 ± 0.05#**
MOI (J)	0.11 ± 0.003	0.11 ± 0.005	0.11 ± 0.010	0.12 ± 0.005

Table 3.2: Femoral general and cortical bone parameters
Values are averages ± SE. n = 9-10 per group. Nothing significant compared to controls. Statistical analysis was performed with 1 way ANOVA with Tukey posttest.
LR = *Lactobacillus reuteri*; LGG = *Lactobacillus rhamnosus* GG; Tb.Sp = trabecular Spacing; Tb.Th = trabecular thickness; Tb.N = trabecular number; Ct.Th = cortical thickness; Ct.Ar = cortical area; Ma.Ar = marrow area; Tt.Ar = total area; BMD = bone mineral density; BMC = bone mineral content; MOI = moment of inertia.

3.4.3 Probiotics do not affect Cortical and Mechanical Strength Properties following GC Treatment

Because long-term GC treatment alters whole-bone cortical and mechanical strength properties in humans and animal models (13,30,77–79), we investigated whether LR or LGG affected GC-induced cortical, structural or tissue level properties of mouse bones. While GC treatment significantly decreased cortical thickness and bone mineral density (BMD) compared to control (Table 3), this decrease was not prevented with probiotic supplementation (Fig 3A). Likewise, all GC treated mouse groups showed an increase in marrow area, total area, inner perimeter and outer perimeter and no change in moment of inertia (Fig 3A; Table 3). Analysis of structural-level mechanical properties indicated no significant changes across all treatment groups (Fig 3B). Analysis of tissue-level mechanical properties, which estimate material properties of bone, again revealed no significant change among treatment groups (Fig 3C). These results suggest that in our model, neither glucocorticoid treatment nor probiotic treatments alter bone mechanical properties.

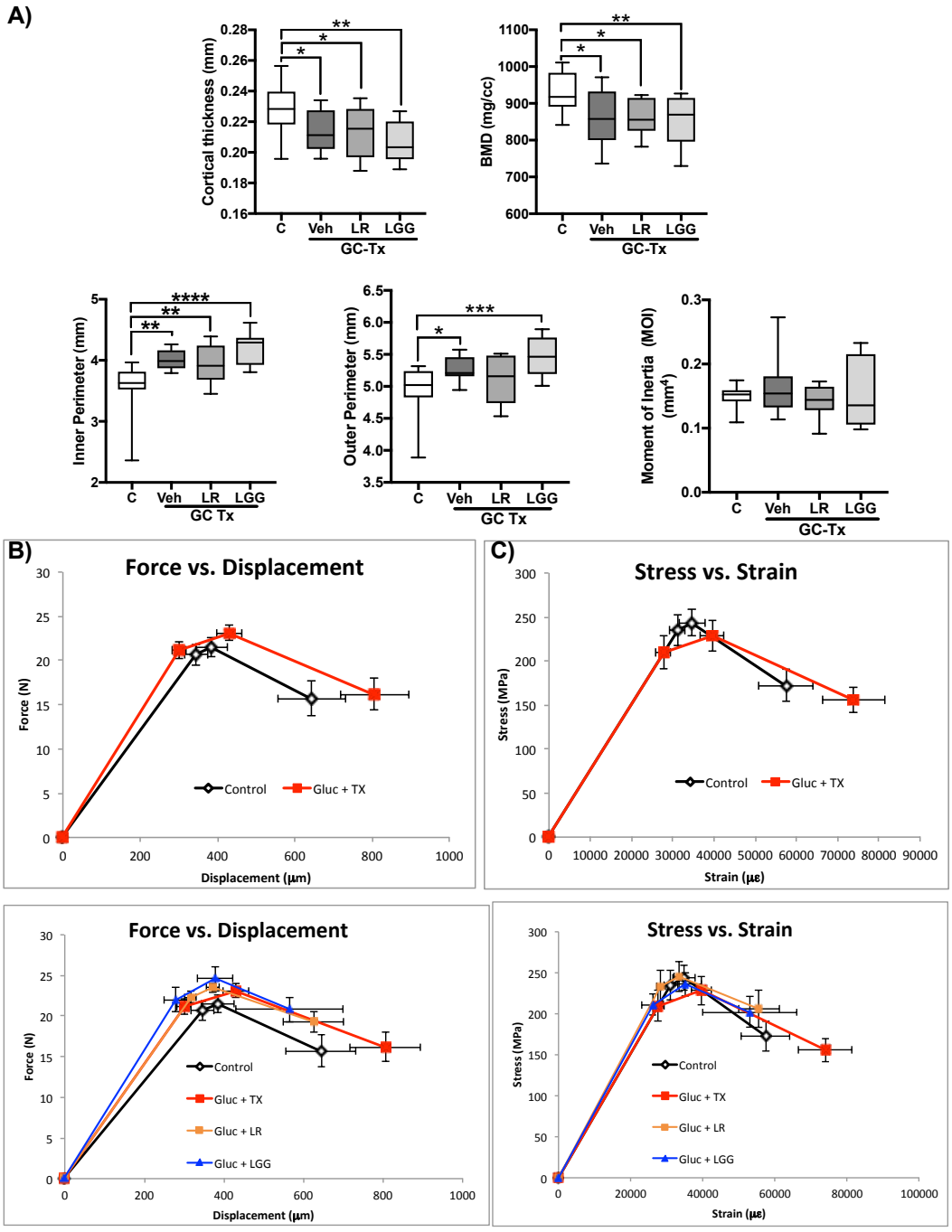

Figure 3.3: Probiotics do not affect Cortical and Mechanical Strength Properties following GC treatment

A) Analysis of femoral cortical bone parameters. Analysis of B) structural and C) tissue level properties. Values are average ± SE. n = 8-10 per group Statistical analysis was performed with 1 way ANOVA. **** p<0.0001; *** p<0.001; ** p<0.01; *p<0.05.

	Control	GC-Tx	GC+LR	GC+LGG	GC+MDY
(n)	**(10)**	**(9)**	**(9)**	**(10)**	**(10)**
General Parameters					
Body Weight (g)	31.6 ± 0.86	30.3 ± 0.57	30.2 ± 0.59	32.5 ± 0.57	31.4 ± 1.08
Femur BV/TV (%)	30.1 ± 1.29	**16.3 ± 1.67***	25.9 ± 1.88	**19.5 ± 2.50***	24.5 ± 2.17
Vertebrae BV/TV (%)	44.7 ± 4.15	**33.7 ± 2.34***	42.4 ± 3.61	33.6 ± 2.3	40.0 ± 1.23
Bone Length (mm)	15.2 ± 0.12	14.9 ± 0.18	15.0 ± 0.16	14.9 ± 0.25	15.1 ± 0.20
Cortical parameters					
Ct.Th (mm)	0.23 ± 0.005	**0.21 ± 0.005***	**0.21 ± 0.006***	**0.21 ± 0.004****	**0.21 ± 0.004***
Ct.Ar (mm^2)	0.88 ± 0.03	0.88 ± 0.02	0.84 ± 0.04	0.83 ± 0.05	0.86 ± 0.03
Ma.Ar (mm^2)	0.89 ± 0.03	**1.06 ± 0.03****	**1.03 ± 0.06***	**1.13 ± 0.05*****	1.00 ± 0.04
Tt.Ar (mm^2)	1.72 ± 0.08	**1.96 ± 0.05***	1.87 ± 0.10	**1.96 ± 0.09***	1.87 ± 0.05
BMD (mg/cc)	929.5 ± 17.7	**863.1 ± 25.8***	**862.4 ± 17.4***	**853.2 ± 22.1****	**871.3 ± 15.0***
BMC (mg)	0.016 ± 0.0007	0.015 ± 0.0006	0.014 ± 0.0008	**0.013 ± 0.001****	0.015 ± 0.0006
Inner Perimeter (mm)	3.55 ± 0.14	**4.01 ± 0.05****	**3.94 ± 0.11****	**4.19 ± 0.08*****	**3.93 ± 0.07****
Outer Perimeter (mm)	4.94 ± 0.13	**5.28 ± 0.07***	5.13 ± 0.13	**5.47 ± 0.10*****	5.19 ± 0.07
MOI (J)	0.14 ± 0.01	0.16 ± 0.01	0.14 ± 0.014	0.15 ± 0.016	0.14 ± 0.008

Table 3.3: Femoral general and cortical bone parameters
Values are averages ± SE. n = 9-10 per group. Significant bolded compared to controls. Statistical analysis was performed with 1 way ANOVA with Tukey posttest.
GC-Tx = glucocorticoid-treated; LR = *Lactobacillus reuteri*; LGG = *Lactobacillus rhamnosus* GG; MDY = high molecular weight polymer; Ct.Th = cortical thickness; Ct.Ar = cortical area; Ma.Ar = marrow area; Tt.Ar = total area; BMD = bone mineral density; BMC = bone mineral content; MOI = moment of inertia.

3.4.4 Barrier Dysfunction Mediates Glucocorticoid-Induced Bone Loss

Intestinal barrier disruption and endotoxin leakage into the blood stream are now recognized as important pathogenic events in a number of chronic diseases (80–82). Importantly, we recently identified intestinal barrier dysfunction as an key mediator of dysbiosis-induced bone loss (44). Since our results so far indicate that gut microbiota is altered and necessary for GIO, we tested whether GC treatment affects barrier function and if strengthening the intestinal barrier can protect mice from GIO. Specifically, mice were treated with or without prednisolone in the presence or absence of MDY-1001 (MDY, (44,55)), a high molecular weight barrier enhancing polymer, for the duration of the experiment. As shown in Fig 4A, GC treatment caused an increase in intestinal permeability (barrier leaks) as evidenced by increased serum endotoxin levels, however MDY treatment effectively prevented the GC-induced elevation of serum endotoxin. More important is that MDY treatment prevented femoral trabecular bone loss induced by GC treatment (Fig 4B, C). Similar trends were seen for vertebral bone volume measures (Fig 4C). Trabecular architectural parameters corresponded with the femoral bone volume fraction (Fig 4D). Pearson's correlation analyses identified a negative correlation between serum endotoxin levels and femoral BV/TV% ($r = -0.4995$, $p=0.0001$)(Data not shown). These results suggest that GC-induced barrier leaks are an important mediator of bone loss in the GIO model.

To identify if LR and chronic antibiotic treatments from the experiments described earlier also enhanced intestinal barrier function, we measured serum endotoxin levels. Consistent with the role of barrier leaks in regulating bone health in the GIO model, LR and chronic antibiotic treatments prevented GC-induced barrier leaks (Fig 4A). Together, these

data demonstrate that GC altered microbiota composition causes barrier dysfunction (and therefore barrier leaks), which are a key pathogenic event in GIO.

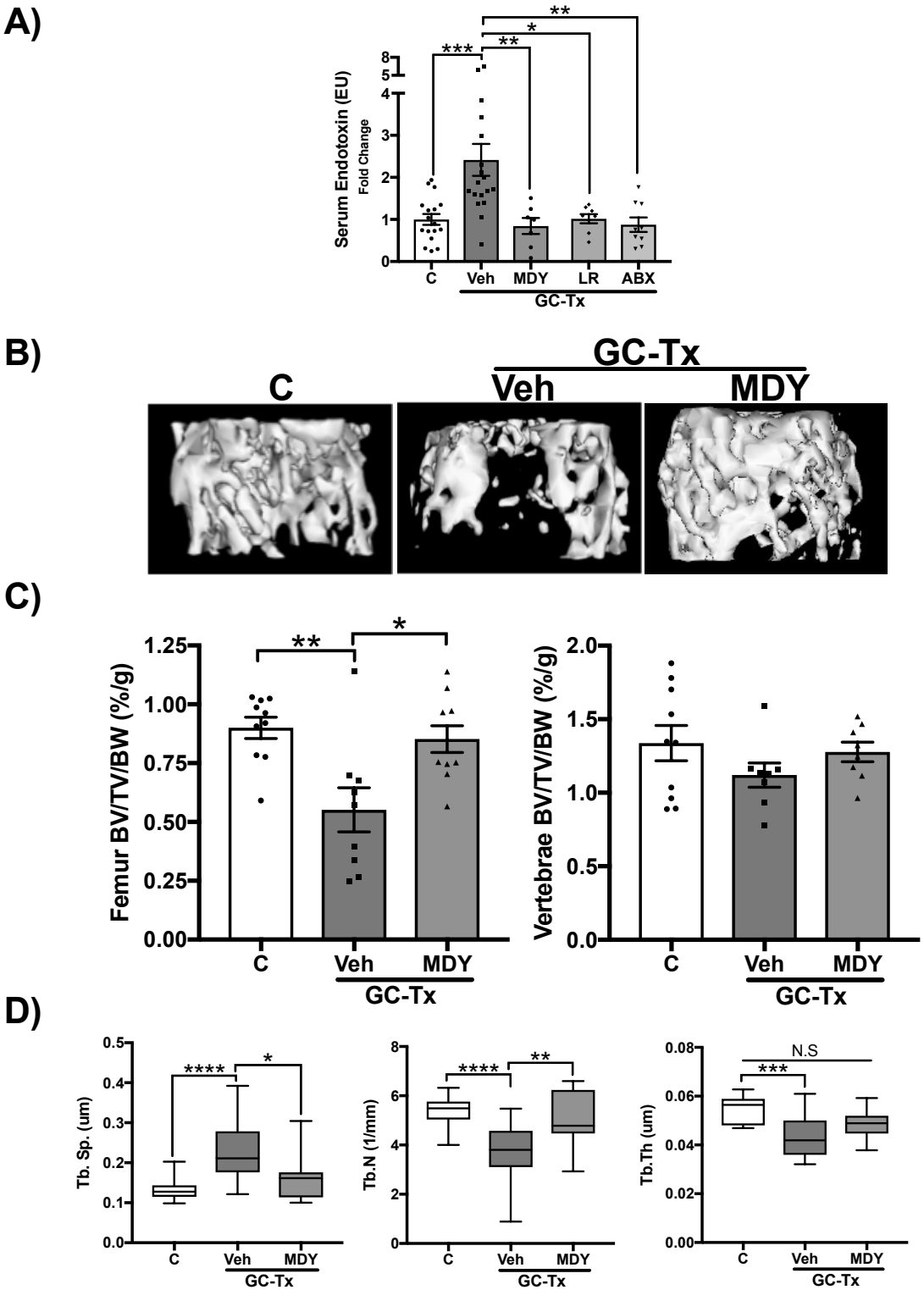

Figure 3.4: Barrier dysfunction mediates glucocorticoid-induced bone loss

Figure 3.4. (cont'd)
16-week old male C57Bl/6J mice were treated with glucocorticoids (prednisolone) for 8 weeks. Mice were either given sterile water (Veh) or supplemented with high molecular weight polymer (MDY). A) Intestinal flux measured by serum endotoxin (Fold-Change) of control, glucocorticoid treated (Veh) ± MDY, LR or ABX. n=7-17. B) Distal femur representative μCT iso-surface images. C) MicroCT analysis of femoral and vertebral trabecular bone volume fraction corrected for body weight. n = 9-10 per group. Values are average ± SE. Statistical analysis was performed with 1 way ANOVA with Tukey post-test. **** $p<0.0001$ *** $p<0.001$; ** $p<0.01$; *$p<0.05$.

3.4.5 Lactobacillus reuteri and MDY Prevent Glucocorticoid Suppression of Osteoblast Activity.

To determine whether the probiotic and barrier enhancement treatments affected anabolic and/or catabolic bone parameters, markers of osteoblast and osteoclast activity were measured. Dynamic anabolic bone measures, mineral apposition rate (MAR) and bone formation rate (BFR), were significantly decreased in GC treated mice, while both LR and MDY treatments reduced the suppression (Fig 5A, B). Analysis of distal trabecular osteoblast surface, showed that LR trended towards preventing the GC reduction of osteoblasts, (Fig 5C). Given that osteoblast and adipocyte numbers are often reciprocally related, due to sharing a common mesenchymal stem cell (83), we analyzed marrow adiposity in the bone metaphyseal region. Consistent with our osteoblast data, GC increased the number of bone marrow adipocytes and both LR and MDY treatments prevented the adiposity (Fig 5D). Analyses of catabolic bone parameters, such as osteoclast surface, indicated that GC trended to increase osteoclast surface and LR significantly prevented this change (Fig 5E). Together, these results suggest that prevention of GIO by LR and MDY is the result of retaining anabolic bone activity and reducing catabolic activity under GC treatment conditions.

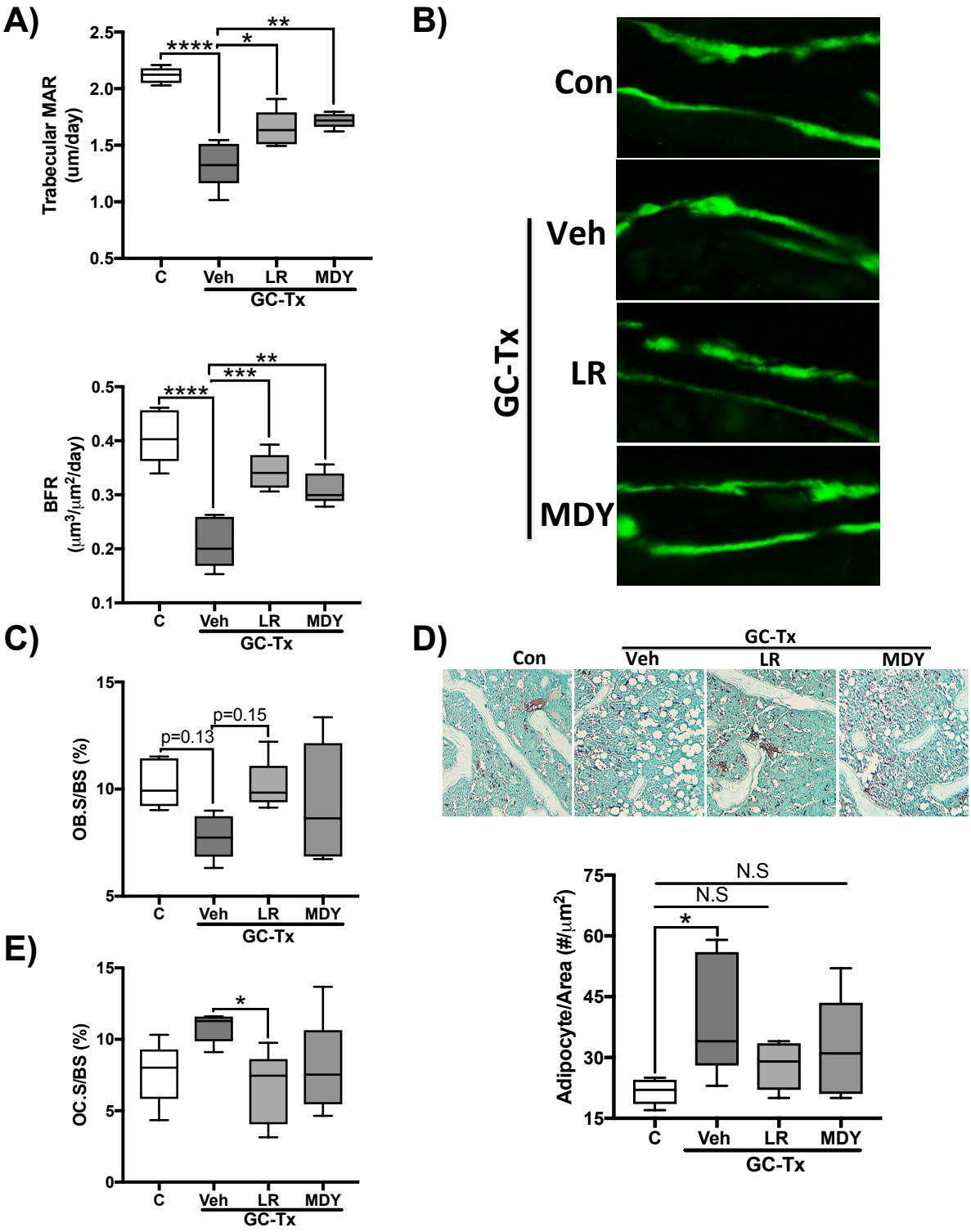

Figure 3.5: Lactobacillus reuteri and MDY prevent glucocorticoid suppression of osteoblast activity

Figure 3.5. (cont'd)
A) Quantitation of trabecular bone mineral apposition rate (MAR) and bone formation rate (BFR). B) Representative images showing the mineral apposition rate (MAR) in distal femur trabecular area. C) Quantification of osteoblast surface/ total bone surface in distal trabecular bone region. D) Representative histological adipocyte images of distal femur at a magnification of 10X; number of adipocytes in the marrow area of the distal femur. E) Quantification of osteoclast surface/ total bone surface in distal trabecular bone region. n = 5 per group. Values are average ± SE. Statistical analysis was performed with 1 way ANOVA with Tukey post-test. **** $p<0.0001$; *** $p<0.001$; ** $p<0.01$; *$p<0.05$.

3.4.6 Microbiota Mediates GC-Induced Osteoblast and Osteocyte Apoptosis

Our data thus far support the role of gut microbiota and barrier dysfunction in mediating GC- suppression of osteoblast activity and GIO. Next, we wanted to further understand the cellular mechanisms of intestinal microbiota prevention of GIO. We focused on examining osteoblast and osteocyte apoptosis, which is known to be increased by GC treatment (4,12,14,21,84,85), and tested if chronic antibiotic treatment of GC treated mice (described earlier) could affect bone cell death. Tibial RNA analyses demonstrated that the GC-induction of the BAX/BCL-2 expression ratio (pro-apoptotic/anti-apoptotic; elevation is an indicator of apoptosis) is prevented by microbiota depletion (Fig 6A). To identify specific responses of osteoblast and osteocyte death, femur sections were TUNEL stained. Figure 6B-D shows that GC-treatment increases both osteoblast and osteocyte (trabecular and cortical) death. Importantly, under microbiota-depleted conditions GC-treatment does not increase apoptosis compared to the placebo treated control groups. These findings support the role of the microbiota in mediating GC-induced osteoblast and osteocyte death and ultimately GIO.

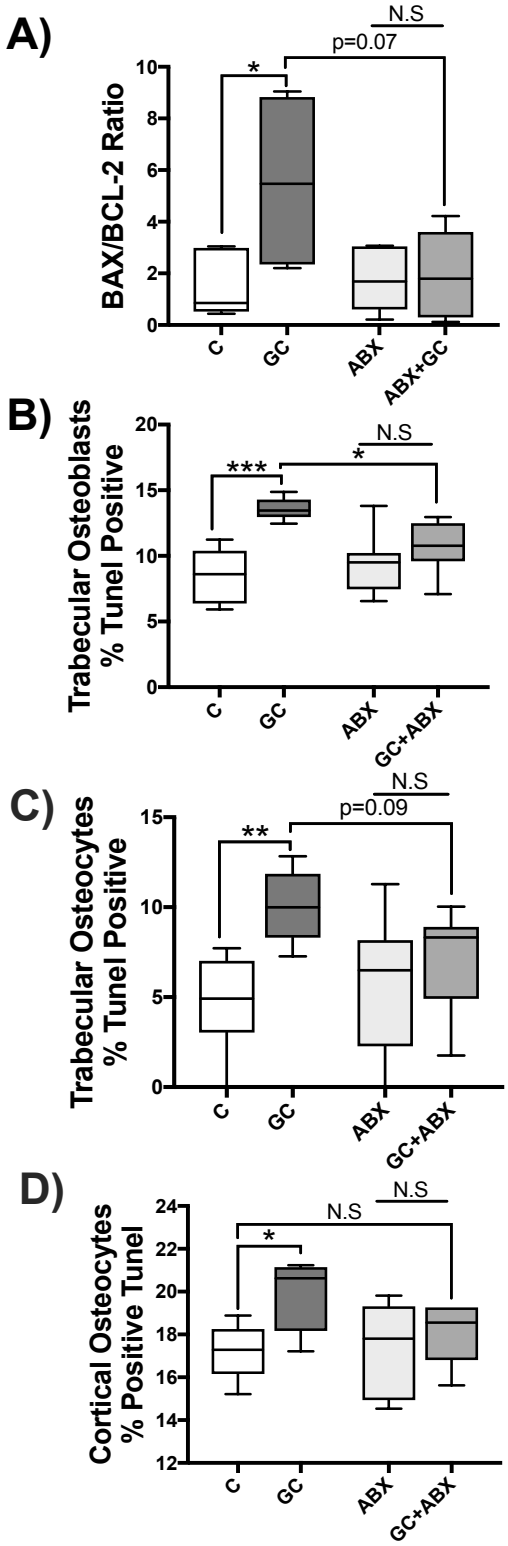

Figure 3.6: Microbiota mediates GC-induced osteoblast and osteocyte apoptosis

Figure 3.6. (cont'd)
A) Gene expression levels and ratio of pro-apoptotic (BAX) and anti-apoptotic (BCL-2) in whole bone tibia. B-D) Percentage of TUNEL positive stained osteoblast and osteocytes in femoral trabecular bone, as well as femoral cortical osteocytes. Values are average ± SE. n = 5-9 per group Statistical analysis was performed with 1 way ANOVA with Tukey post test. *** $p<0.001$; ** $p<0.01$; *$p<0.05$.

3.4.7 LR and MDY Prevent GC-Induced Osteoblast and Osteocyte Apoptosis

Next, we examined if LR (probiotic) and MDY (barrier enhancer) treatments also prevented GC-induced osteoblast and osteocyte apoptosis. Consistent with inhibiting bone loss, both LR and MDY prevented GC-induction of BAX/BCL-2 expression (Fig 7A). Similarly, both treatments reduced the number of TUNEL positive osteoblasts and osteocytes (Fig 7B-D). Pearson's correlational analyses revealed that TUNEL positive trabecular osteoblasts ($R=0.4456$, $p=0.004$) and osteocytes ($R=0.4022$, $p=0.01$) correlated with serum endotoxin levels (data not shown). Together, these results indicate that enhanced barrier function as well as supplementation with oral LR reduces osteoblast and osteocyte apoptosis, which likely contributes to preventing GC suppression of bone formation and GIO.

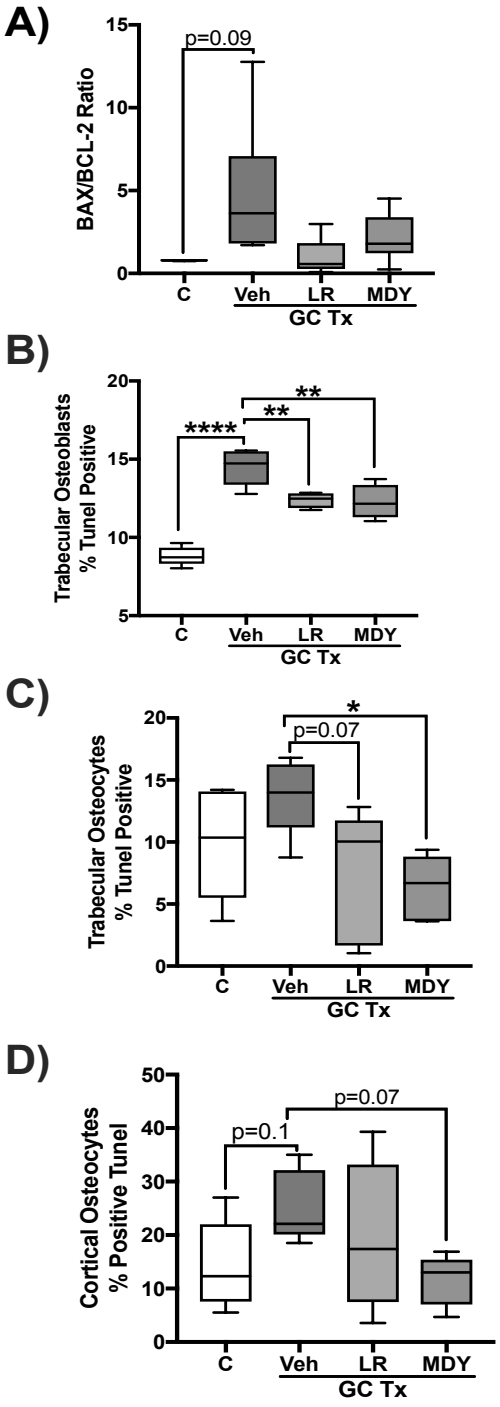

Figure 3.7: LR and MDY prevent GC-induced osteoblast and osteocyte apoptosis
A) Gene expression levels and ratio of pro-apoptotic (BAX) and anti-apoptotic (BCL-2) in whole bone tibia. B-D) Percentage of TUNEL positive stained osteoblast and osteocytes in femoral trabecular bone, as well as femoral cortical osteocytes. Values are average ± SE. n = 5-9 per group Statistical analysis was performed with 1 way ANOVA with Tukey post test. **** $p<0.0001$; ** $p<0.01$; *$p<0.05$.

3.4.8 LR and MDY Prevent GC Induced Wnt10b Suppression

A key regulator of anabolic bone activity is the Wnt/β-catenin signaling pathway. Elevated Wnt10b expression promotes osteogenesis as well as osteoblast differentiation and viability (5,9,25–27,29,86). Given that GC treatment is known to suppress Wnt10b expression (29,87), we examined if LR and MDY treatments prevent the negative effects of GC on osteoblasts through regulation of Wnt10b. Indeed, Figure 8A shows that both LR and MDY treatment prevent the marked suppression of Wnt10b expression by GC treatment. These data suggest that the suppression of Wnt10b by GC is a critical component in mediating GIO. To test this, mice with targeted osteoblast Wnt10b overexpression (OC-Wnt10b TG mice, created by fusing the Wnt10b gene with the OC promoter to target Wnt10b expression to osteoblasts (53)) underwent the standard GC treatment (or vehicle) protocol. After 8 weeks, analyses of femoral trabecular bone volume fraction revealed that Wnt10b overexpression prevents bone loss following GC treatment (Fig 8B-C). Trabecular architectural measures corresponded with the changes in bone volume (Fig 8D). Taken together these data support the key role of Wnt10b suppression in mediating GIO and demonstrate that both LR and MDY are able to prevent GC-mediated Wnt10b suppression and bone loss.

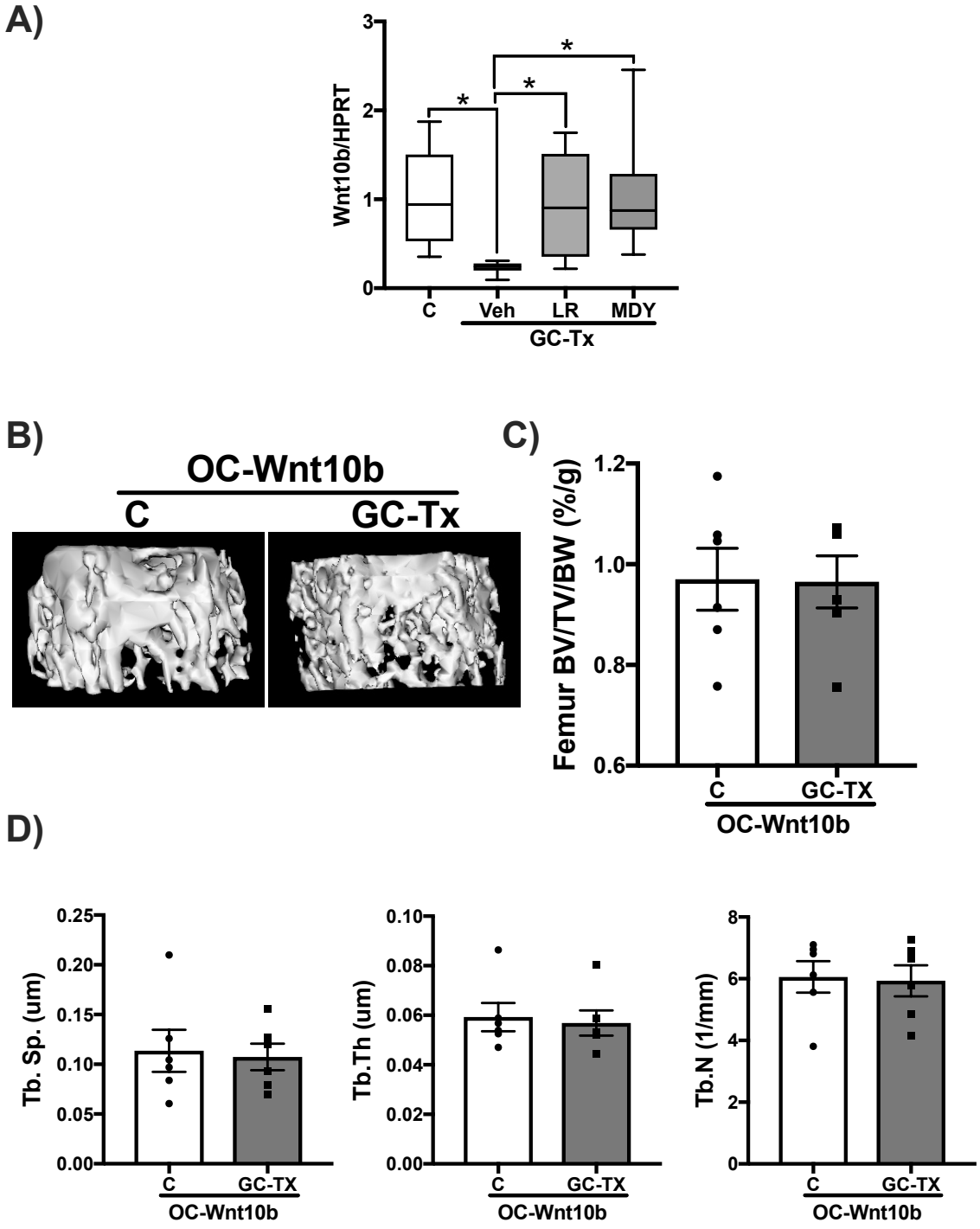

Figure 3.8: Role for Wnt10b in *L.reuteri* and MDY prevention of GIO
A) Gene expression of Wnt10b in whole bone tibia. 16 week old male Wnt10b TG mice were treated with either placebo or prednisolone pellet for 8 weeks B) Representative µCT iso-surface images of distal femur. C) Analysis of femoral trabecular bone volume fraction (n=6 students t-test). D) Bone femur microarchitecture µCT analyses. Values are average ±

Figure 3.8. (cont'd)
SE. n = 5-9 per group Statistical analysis was performed with 1 way ANOVA with Tukey post-test. *$p<0.05$.

3.5 Discussion

GIO is the most common cause of secondary osteoporosis with 30-50% of patients undergoing chronic prednisolone treatment sustaining a fracture (1,6). Increased risk of fracture accompanying GC's is thought to be do to alterations to osteoblastogenesis towards adipogenesis as well as osteoblast and osteocyte apoptosis (12,14,26,84,86,88–90). In addition to its direct affects on bone, GC treatment also alters the gut microbiome (33,34). Previous studies demonstrate a role for intestinal microbiome composition in the regulation of bone (37,43,45,49–51,91–94). In the present study we identify that GC treatment in addition to its direct effects on bone cells, resulted in an altered gut microbiome and increased intestinal permeability accompanying with GC induced bone loss. We further reveal that oral supplementation with *L. reuteri* 6475, continuous ABX or direct inhibition of gut barrier leakage significantly blunts trabecular bone loss, suggesting the role of the gut microbiome as a mediator of GIO.

Prolonged exposure to GC's can result in various side effects outside of osteoporosis including gastrointestinal complications, thus because of its pleiotropic nature, many GC-mediated effects have not been well characterized (33,95,96). Recent studies have even suggested that GC signaling derives a portion of its effects through alterations to the gut microbes (82,97). In the current study, GC treatment was demonstrated to cause a significant overall shift in microbial composition as evidenced through nonmetric multidimensional scaling (NMDS). Interestingly, our probiotic treatments each significantly altered the composition compared to GC alone and to each other. We hypothesize that each of these compositions have a different balance of healthy verses disease promoting bacteria which could be playing a role in its differential effects on the

intestinal barrier and furthermore bone health. Further studies are needed to understand which specific bacterial populations are beneficial to bone.

In our study, alteration of the microbiota via GC's was accompanied by an increase in serum endotoxin levels, suggesting a decreased barrier function. Previous studies have shown that increases in cortisol can alter the gut microbiome and alter the gut barrier function (98). Barrier function has been proposed as a mechanism in which the gut microbiome influences bone health in other models of microbial alteration (37,43,44,94). Consistent with this, our analyses indicate that serum endotoxin significantly correlates with trabecular bone density. Interestingly, by directly inhibiting barrier permeability with MDY we prevented GC induced bone loss. This data demonstrates that GC-induced changes to gut barrier function is mechanistically important for GC induced bone loss. This is consistent with our previous study, in which chickens infected with intestinal salmonella do not lose trabecular bone when treated with MDY (99). Recently, in post-ABX induced microbial dysbiosis, prevention of barrier leak with MDY treatment, prevents bone loss (44). Because MDY is not absorbed, its benefits on bone health in this model are a consequence of its effects specifically on the intestine, thereby underscoring the importance of the gut-bone signaling axis as a therapeutic target for GIO.

While previous studies have shown that probiotics can alter the gut microbiome, promote intestinal barrier function and benefit bone health (43,44,100–104). In the present study, only LR had a beneficial effect on barrier function and accompanying GC induced bone loss. LR's ability to strengthen the intestinal barrier and benefit bone could be due to its ability to produce biologically active metabolites or proteins (45,105,106). Probiotic LGG has been shown to require epithelial cell interaction for its effects (107).

Furthermore, recent studies suggest LGG works through increasing levels of the butyrate producing bacteria *Clostridia* (46). However our LGG treatment did not affect *Clostridia* levels compared to GC or control cohorts (data not shown). In addition, LGG and LR altered the gut microbiome significantly from all other treatment groups and additional work needs to be done looking into specific changes to the gut microbiota and how that can benefit GC induced bone loss. It could be that specific bacteria present in the LR group that are beneficial to barrier function and bone are absent in the LGG cohort.

In further support of GC's alterations to the gut microbiota/barrier function contributing to GC induced bone loss, we found that continuous ABX administration during GC treatment depleted the gut microbiota, prevented barrier leak and bone loss. This is consistent with other previous reports demonstrating that antibiotic treatment can prevent serum endotoxin levels and severity of inflammatory bowel disease (108–110).

Our studies demonstrate that GC treatment has a marked effect on trabecular bone parameters at both long bone and vertebral sites, which was observed in repeated experiments and prevented by LR, ABX and MDY treatments. Interestingly, cortical bone microarchitecture was affected by GC treatment and not altered by any of our gut treatments. This could be do to cortical bone being less metabolically active than trabecular bone (111) and that our treatments could not affect slow remodeling cortical bone. Additionally, GC treatment decreased cortical thickness and bone mineral density. However, treatment increased marrow area, total area, inner perimeter and outer perimeter suggesting an altered bone structure. Furthermore, GC treatment had no effect on moment of inertia or bone mechanical strength. This is consistent with studies showing

GC treatment affects on cortical or strength parameters are dose and time dependent (77,112).

As previously mentioned, past studies have shown that endogenous GC's induce bone loss via rapid bone resorption, followed by prolonged and profound suppression of bone formation (4,7,8). This phenomenon could be the reason we only saw preventative changes to anabolic bone remodeling processes with LR and MDY in our 8-week mouse model. This is consistent with pervious reports showing LR and MDY treatment can increase mineral apposition, bone formation, osteoblast number and serum osteocalcin (44,45,49,51).

Our observation that GC's act directly on osteoblast and osteocytes *in vivo* is consistent with pervious studies (84,113). However, for the first time we show that supplementation with ABX, probiotic LR and MDY can prevent GC induced osteoblast and osteocyte apoptosis. Interestingly, these gut treatments prevented increases to serum endotoxin, which has been shown to induce osteoblast apoptosis (114). Additionally, serum endotoxin correlated with bone density as well as osteoblast and osteocyte apoptosis, further suggesting that changes to barrier function could be playing a role in GC induced bone loss.

Similar to previous studies, we observed GC treatment decreasing the WNT10b signaling pathway and promoting adipogenesis (26,86,88). Thus, we hypothesized that in addition to the gut effects; LR and MDY were preventing GCs direct effects on WNT signaling and adipogenesis. This hypothesis was supported by our findings *in vivo;* that treatment with LR and MDY prevented GC induced increases to bone marrow adipocytes as well as decreased WNT10b gene expression. This is consistent with pervious reports

showing treatment with LR can prevent suppression of WNT10b and increased adipogenesis in mouse models of type 1 diabetes (51). In further support that WNT10b signaling is playing a key role in GC induced bone loss, we found that OC-WNT10b TG mice treated with GC ‘s did not display trabecular bone loss.

In summary, it is generally accepted that GCs induce bone loss via direct effects on bone remodeling cells. Our studies highlight the importance of the gut microbiome and intestinal barrier function in GIO. Our GC model serves as a way to study the role of gut microbiome in GIO and highlights the gut microbiome as potential therapeutic target for GIO. Discovering the connection between the gut microbiota and bone health can speed identification for new treatments not only for GIO but also for osteoporosis all together.

3.6 Acknowledgements

The authors thank the Investigative Histology Laboratory in the Department of Physiology, Division of Human Pathology and the Biomedical Imaging Center at Michigan State University for their assistance with histology and imaging, respectively. The authors are also grateful to the staff of Campus Animal Resources for the excellent care of our animals. These studies were supported by funding from the National Institute of Health, grants RO1 DK101050 and RO1 AT007695.

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CHAPTER 4: SUMMARY AND CONCLUSIONS

4.1 Specific Aims and Study Outcomes

As described in Chapter 1, bone remodeling is an ongoing, dynamic process that is regulated by many non-skeletal factors such as the gut microbiota. Several studies have linked alterations in the gut microbiome to changes in bone density, yet there is still a lack of understanding as to which specific alterations benefit bone vs. harm bone. Therefore, the overall aim of this study was to investigate the role of the gut microbiota in two *in vivo* models of bone loss: 1) Acute antibiotic depletion followed by a natural repopulation of the gut microbiota (Chapter 2) and 2) pharmacologic glucocorticoid treatment (Chapter 3). We hypothesized that alterations to the gut microbiome induced by these models would play a role in the detrimental effect on bone density. Therefore, this study would elucidate a mechanism behind gut microbiome alterations and bone loss in two distinctly different models. Furthermore, it would investigate the use of novel therapeutics to prevent bone loss by targeting the gut, by using probiotics and a mucus-like supplement. This chapter will summarize the research objectives and obtained results.

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4.2 Study Outcome

This dissertation set out to investigate the link between the gut microbiome and bone health in two separate studies: 1) investigate how ABX induced dysbiosis affects bone health and 2) the role of the gut microbiome in the pathogenesis of GIO. The data presented in this dissertation demonstrates a link between the gut microbiome and bone density in male mice; though further studies are required to fully understand the specific alterations in the microbiota that drive these changes. In addition, we reveal that probiotics, microbiome composition, and intestinal barrier function play a vital role in bone density.

The intestinal gut microbiota contains approximately 100 trillion bacteria as well as fungi and viruses, which can provide many beneficial effects to the host. These include producing essential nutrients, digesting otherwise indigestible food components and educating the host immune system (1–3). However, the gut microbiota has been linked to a variety of metabolic, inflammatory and immunologic diseases (4–6). Thus, a delicate homeostasis between the host and gut microbes determines commensalism or pathogenicity. The intestinal barrier is an essential factor in establishing this homeostasis, as it prevents the translocation of bacterial products and pathogenic bacteria into the systemic circulation. This barrier can be affected by alterations to the gut microbiome, and decreases in barrier function have been observed in early life stress and diseases such as IBD (7, 8). In the past ten years, there have been numerous studies linking alterations to the gut microbiome in the regulation of bone health (6, 9–13). Antibiotics are known to significantly alter the gut microbiota with changes in the composition being detected up to four years after treatment has stopped (13, 14). However, to date, no one has investigated the role of ABX induced dysbiosis on bone health. We demonstrate that 2 weeks of ABX

depletion of the gut microbiota did not affect bone density. However, 4 weeks of microbial repopulation led to an increase in the *Firmicutes:Bacteroidetes* ratio and a 30% decrease in trabecular bone volume, caused by a decrease in bone formation and an increase in bone resorption. Interestingly, repopulation was accompanied by an increase in intestinal permeability. This increase in intestinal permeability was shown to be mechanistically important in repopulation induced bone loss as it was prevented following treatment with the mucus supplement MDY. Treatment with the probiotic LR was the only bacterial supplement that significantly prevented the increase in the *Firmicutes:Bacteroidetes* ratio, intestinal permeability, and bone loss. Furthermore, LR and MDY increased anabolic and decreased catabolic parameters, leading to their prevention of bone loss. **Our data highlight the importance of the gut microbiota and intestinal barrier function in bone loss during gut bacterial dysbiosis.**

Since their introduction into medicine, GCs have been used for their anti-inflammatory and immunosuppressive effects. However, it is now known that they have significant side effects with long-term treatment, such as osteoporosis. Interestingly, pharmacologic doses of GCs have been shown to alter the gut microbiome and decrease mucus production (15–17). This shift in the gut microbiome is noteworthy as previous studies support a role for the gut microbiota in the regulation of bone health (9, 10, 18–22). However, there have been no studies investigating the role of the gut microbiome in the pathophysiology of GIO. In chapter 3, we demonstrated that the gut microbiome is important in GIO as depletion or alteration of the gut microbes with chronic ABX and probiotic LR respectively, prevented bone loss. Interestingly, GC treated mice displayed increased serum endotoxin levels, suggesting a decrease in barrier integrity, which was

prevented following treatment with ABX and LR. We found that MDY treatment prevented elevated serum endotoxin levels and bone loss, suggesting barrier function is a key component of GIO. Treatment with ABX, LR and MDY also prevented GCs direct effects on bone. As gut treatments prevented GC induced apoptosis of osteoblasts and osteocytes. Furthermore, LR and MDY treatment prevented GC induced suppression of Wnt10b. Furthermore, over expression of Wnt10b was also able to prevent bone loss. This data suggests that suppression of Wnt10b signaling by GCs is a critical component in mediating bone loss. **The work performed in this aim is the first to demonstrate that the gut microbiome is important in the pathophysiology of GIO. Furthermore, we are the first to show that GC treated mice present with higher serum endotoxin levels suggesting a decreased barrier function. We also offer the probiotic LR and a mucus supplement as a potential therapeutic for GIO.**

4.3 Limitations of the Studies

Although many important conclusions can be drawn from this study, there are some limitations to be considered.

4.3.1 Limitations in ABX Studies

1. Antibiotic treatment was provided to healthy male mice, a treatment that rarely occurs in a clinical setting. However, this study was investigating the effect of dysbiosis on bone health and 2-week treatment with antibiotics was used as a tool to induce dysbiosis. Additionally, 2-weeks of antibiotic treatment did not influence bone health.

2. As experiments investigating the gut microbiome or probiotics effects on bone health have been inconsistent, reproducibility has become an essential aspect in this area of study. Inconsistency could be due to experiments being performed in separate facilities leading to mice having different microbiomes, making comparisons difficult. This study can be used as a means to examine specific alterations to the microbiome composition and their subsequent impact on bone health, providing further insight into the gut-bone axis.

4.3.2 Limitations in GIO Studies

1. In clinical studies, GC treatment is administered orally, while in our model GCs were delivered subcutaneously via a slow releasing pellet. This could cause alterations to conclusions as oral treatment could have drastically different effects on the gut microbiota compared to subcutaneous treatment. However, in pilot studies we treated mice orally with GCs and observed results similar to subcutaneous treatment.

2. GC treatment is not given to healthy people but our studies were done in apparently healthy mice. Our future studies will include mice with arthritis or IBD to determine if GIO in these mice is mediated by the gut microbiota.

4.4 Future Directions

The data presented in this thesis offers insight into how alterations to the gut microbiota can regulate bone density. These studies will hopefully guide future research, which aims to further validate and understand the gut-bone axis, allowing for the development of novel therapeutics for the prevention of osteoporosis. To allow for this, more studies are required to further understand how the gut microbiota regulates bone health.

Our results from chapter 2 suggest that an increase in the *Firmicutes:Bacteroidetes* ratio following antibiotic treatment can contribute to bone loss via dysfunction of the epithelial barrier. Future experiments are needed to better understand which specific species within the gut microbiome can affect bone health. To begin to address this, following broad-spectrum ABXs, we can treat with various bacterial specific ABX treatments to regulate the 4-week microbiota repopulation. This will yield different intestinal microbial compositions, which, in theory, will result in varying bone density measurements. This data would potentially provide links between which microbial communities have beneficial or harmful effects on bone health. Transferring these altered microbiomes into germ-free mice would further prove microbiome functionality.

To further understand dysbiosis induced bone loss, it will be interesting to study the time course over which bone is lost. In the present study, we look at 4 weeks post ABX. Examining additional time points of 2, 8 and 12 weeks post ABX would allow us to

determine whether there are acute and chronic phases of bone responses and if dysbiosis has long-lasting consequences on bone health.

We are the first group to identify that the gut microbiota is playing a role in the pathogenesis of GIO, as the absence of the gut microbiome prevented bone loss (Chapter 3). However, to further understand the functionality of the microbiome, fecal transfers should be performed. Germ-free or ABX depleted microbiome mice would be given fecal samples from GC treated mice throughout the experiment. If the microbiome alterations are playing a significant role in GIO, we would expect to see bone loss in mice administered the GC microbiome compared to control mouse microbiota. To further make our GC model clinically relevant, we could also administer GC treatment orally (23) and perform similar experiments to the ones outlined in this thesis. This would provide us with more data to further support the role of the microbiota in this disease.

In chapter 3 we report for the first time that GC treated mice present with increased serum endotoxin levels. This has not been researched clinically and thus provides a fascinating avenue to explore. Clinical collaborations are needed to obtain serum from patients who receive GCs, across many different conditions, and compare them to normal healthy age and sex-matched controls. If our hypothesis is correct, the GC treated patients should present with higher endotoxin levels, adding support to the idea that GCs adversely affect intestinal barrier integrity, which can have a subsequent detrimental effect on bone.

In vivo data presented in the current study demonstrates that LR treatment prevents GC induced osteoblast and osteocyte apoptosis. To further address this, glucocorticoid-induced osteoblast/osteocyte apoptotic pathway can be assessed in culture. This will tell us the mechanism by which LR inhibits GC induced osteoblast apoptosis. In addition, recent

studies have shown that probiotics can affect the metabolomic profiles of the gut and serum to have systemic effects on bone (24, 25). Metabolomic analysis of gut and serum samples from LR treated mice could provide an idea as to how LR alters metabolomics of the gut and which products have beneficial effects on bone remodeling.

4.5 Conclusion

As the human populations' life expectancy has increased, there has been an increase in age-related diseases such as osteoporosis. This has led to more than 300 million people being affected by both type 1 and 2 osteoporosis, accounting for over 2 million bone fractures (26–28). As outlined in chapter 1, current treatments for osteoporosis have several shortcomings due to many side effects. This makes the development of novel therapeutics for the prevention of osteoporosis of utmost importance. Recently, studies have shown that the gut microbiota can regulate bone remodeling and has had promising results as a potential therapeutic target. However, a better understanding of the gut-bone axis is required before therapeutics can be developed. The studies presented in this thesis demonstrate the effects of the gut microbiome, on bone remodeling in two distinctly different models. We reveal that dysbiosis following antibiotics or glucocorticoid treatment plays a key role in bone loss. Furthermore, we demonstrate that treatment with probiotic LR can prevent bone loss; building on preexisting studies in which probiotic consumption has beneficial bone effects (11, 18, 20, 29, 30). Disruption to the intestinal barrier is mechanistically significant for bone loss, as treatment with a mucus supplement restored barrier function and prevented bone loss in both models. These studies provide new approaches to further understand the gut microbiome-bone link.

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