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Gut Microbiota-Bone Axis[1]

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[4] **Abbreviations**: (BMC) bone mineral content; (BMD) bone mineral density; (BV/TV%) bone volume as a percentage of total volume; (CONV-D) conventionalized; (CONV-R) conventionally-raised; (CD) Crohn's disease; (DXA) Dual-energy X-ray Absorptiometry; (GF) germ-free; (GIT) gastrointestinal tract; (GM) gut microbiota; (5-HT) 5-hydroxytryptamine; (IBD) inflammatory bowel disease; (IL) interleukin; (IBS) irritable bowel syndrome; (LPS) lipopolysaccharide; (LBP) LPS binding protein; (OCL) osteoclast; (OPG) osteoprotegrin; (Ovx) ovariectomized; (RANK) receptor activator of nuclear factor kappa-B; (RANKL) receptor activator of nuclear factor kappa-B ligand; (SAM) senescence accelerated mice; (SERT) serotonin transporter; (TLRs) toll-like receptors; (Tph1) tryptophan hydroxylase-1; (TNF) tumor necrosis factor; (UC) ulcerative colitis

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ABSTRACT

The gut microbiota (GM) is an important regulator of body homeostasis, including intestinal and extra-intestinal effects. This review focuses on the gut microbiota-bone axis, which we define as the impartial effect of the gut-associated microbial community or the molecules they synthesize, on bone health. While research in this field is limited, findings from preclinical studies support that gut microbes positively impact bone mineral density and strength parameters. Moreover, administration of beneficial bacteria (probiotics) in preclinical models has demonstrated higher

bone mineralization and greater bone strength. The preferential bacterial genus that has shown these beneficial effects in bone is *Lactobacillus* and thus lactobacilli are among the best candidates for future clinical intervention trials. However, their effectiveness is dependent on stage of development, as early life constitutes an important time for impacting bone health, perhaps via modulation of the GM. In addition, sex-specific difference also impacts the efficacy of the probiotics. Although auspicious, many questions regarding the gut microbiota-bone axis require consideration of potential mechanisms; sex-specific efficacy; effective dose of probiotics; and timing and duration of treatment.

Introduction

The gastrointestinal tract (GIT) serves as an important mediator of nutrients and minerals through highly efficient mechanisms. In the context of bone, the traditional role of the GIT in the maintenance of bone health has been through the absorption of minerals including calcium, phosphorous and magnesium. More recent research has suggested a more complex role of the GIT in the maintenance of bone health through a "gut-bone axis" in which several mechanisms have been proposed (Chen and Zhao, 2011, Sjogren, *et al.*, 2012). In addition, the GIT is also home to the largest human-associated microbial community, and in the advent of recognizing the gut microbiota (GM) as an isolated organ system (Vrieze, *et al.*, 2010), which can contribute to host health, has broadened the potential of these interactions. The GM composition is influenced by several factors, including diet, which can be used as a means to manipulate an altered, or dysbiotic (Holzapfel, *et al.*, 1998), state in favour of health maintenance. One strategy used to manipulate the GM to benefit mineral absorption, and ultimately bone health, is through the use of prebiotics. Prebiotics are "non-viable food-components that confer a health benefit on the host associated with modulation of the microbiota" (Pineiro, *et al.*, 2008). Several studies have shown a beneficial effect of prebiotics, such as galacto-oligosaccharides, on the manipulation of the GM composition and improving calcium absorption and bone health in preclinical (Chonan, *et al.*, 1995, Chonan and Watanuki, 1996, Weaver, *et al.*, 2011) and clinical studies (van den Heuvel, *et al.*, 2000, Whisner, *et al.*, 2013). However, it is not certain if the prebiotic indirectly improves calcium absorption through the manipulation of the GM, reduces pH levels as a consequence of its conversion to short-chain fatty acids, or if the prebiotic independently improves absorption by

increasing mucosal surface area, or a combination of these mechanisms. Therefore, the effects of prebiotics alone will not be discussed in this review as the intent is to focus on the gut microbiota-bone axis, defined as the impartial effect of the gut-associated microbial community or the molecules they synthesize on bone health.

Osteoporosis

In 1994, the World Health Organization defined osteoporosis as a disease characterized by low bone mass and microarchitectural deterioration of bone tissue, leading to enhanced bone fragility and a consequent increase in fracture risk (1994). More than a decade later, experts modified the definition from one that encompassed the use of BMD as a sole predictor of fragility fracture, to one that incorporates multiple fracture risk factors including, but not limited to BMD, genetics, family history of fracture, age, and sex (Czerwinski, *et al.*, 2007). Within the context of this definition, an absolute fracture risk at the site of the proximal femur, is calculated for a 10-year perspective for the patient (Czerwinski, *et al.*, 2007). Worldwide estimates suggest that 1 in 2 women and 1 in 5 men will suffer an osteoporotic fracture at some point during their lifetime (Akesson, *et al.*, 2013), with an astounding 8.9 million osteoporosis-related fractures reported annually (Johnell and Kanis, 2006). The overall worldwide economic burden of treating osteoporosis totaled over US$34.8 billion in 1990, and is set to rise to $131.5 billion by 2050 (Johnell, 1997). These estimates account for acute care costs, outpatient care, prescription drugs and indirect cost. In addition, osteoporosis-related fragility fracture often results in poor quality of life due to chronic pain, disfigurement, lowered self-esteem, reduction or loss of mobility, and decreased independence. While a variety of pharmacological agents are used to treat osteoporosis and reduce risk of fragility fractures, with varying effectiveness and potential

adverse side-effects, preventive strategies using a non-pharmacological approach, such as dietary interventions, are also of interest.

Early life, including the intrauterine and/or postnatal period, may provide a critical window of opportunity for diet to ensure the growth and development of a healthy skeleton (Harvey, *et al.*, 2014). Specifically, attainment of peak bone mass, the maximal amount of bone mineral accumulated by the end of skeletal maturation, may provide a prevention strategy against development of osteoporosis during later life. Mathematical modeling has identified peak bone mass to be a strong predictor of osteoporosis risk later in life (Hernandez, *et al.*, 2003). Moreover, a recent systematic review and meta-analysis has confirmed previous observational conclusions regarding the positive association between birth weight and adult bone mass; for every 1 kg increase in birth weight there is a corresponding 1.41g increase in hip BMC in adulthood (Baird, *et al.*, 2011). From a gut microbiota-bone axis perspective, a novel approach for a higher peak bone mass and lower risk of osteoporosis in later life may be through probiotics (i.e. beneficial microbes) in order to support establishment and maintenance of a eubiotic GM (Kao and Levytam, 2009).

The Gut Microbiota

The GIT is an intricate ecosystem where both biotic and abiotic factors interact to maintain homeostatic equilibrium. Within the GIT, is the most diverse and dense microbiota comprised of a consortium of prokaryotic (bacteria, Archaea) and eukaryotic (yeasts, parasites) cells and viruses that together outnumber human germ and somatic cells by a factor of 10 and genomic potential by a factor of 100 (Savage, 1977, Xu and Gordon, 2003). The Gram-positive

Firmicutes and Actinobacteria, and the Gram-negative Bacteroidetes and Proteobacteria, are the four most represented phyla within the intestinal microbiota, followed by Verrucomicrobia and Synergistetes (Backhed, *et al.*, 2012). Over 1000 species have been identified including several probiotics such as lactobacilli (Firmicutes phylum), bifidobacteria (Actinobacteria), and *Escherichia coli* Nissle 1917 (Proteobacteria), that stem from genera that are well represented in the GIT. Several factors influence the GM composition including genetics, sex, diet, age, environment, health or disease state, and pharmacological agents. For example, modifiable variables such as the use of pharmacological agents including omeprazole, a proton pump inhibitor, is thought to increase gastric pH levels leading to bacterial overgrowth and infection including *Clostridium difficile*-associated diarrhea (Dial, *et al.*, 2004, Yearsley, *et al.*, 2006). In contrast, sex-specific microbiome profiles that emerge after sexual maturation, directly alters sex hormone levels including testosterone (Markle, *et al.*, 2013).

In addition, age is a non-modifiable variable that impacts the GM. In fact, the GM is remarkably stable and resilient in the adult (Faith, *et al.*, 2013) and that is important for maintaining the functional diversity within the microbial community. Though, the pattern is not shared for infants and the elderly as it is a dynamic population altered by selective pressures dependent on the life phase. The developmental pattern of the GM manifests itself as life phases including *in utero*, birth, adulthood and elderly. As a fetus, the *in utero* GIT was once deemed void of bacteria with first exposure occurring only upon delivery. However, it has recently been suggested that the fetus may be exposed to maternal microbes or their products (Valles, *et al.*, 2014) that could explain the presence of bacteria in the human meconium, amniotic fluid and umbilical cord in elective Cesareans (Gosalbes, *et al.*, 2013, Moles, *et al.*, 2013). At birth, the

infant becomes massively exposed to microbes; in vaginally delivered infants, microbes are mainly maternally derived and vertical transmission from mother to child is an important determinant of the infant gut microbial composition (Ley, *et al.*, 2005). In contrast, cesarean section-delivered infants are inoculated with microbes originating from the hospital environment (horizontal transfer) and experience delayed microbial colonization (Mackie, *et al.*, 1999). Consequentially, Cesareans succumb to lower bifidobacterial and lactobacilli counts and higher proportions of *Clostridium difficile*; this has been associated with enteropathogenic infections (Voth and Ballard, 2005) and excessive weight gain (Kalliomaki, *et al.*, 2008).

The composition of the infant GM is highly dynamic, unstable, and less diverse compared to the adult microbial composition. The first colonizers of the infant intestinal tract are commonly facultative anaerobic and anaerobic bacteria including *Escherichia coli*, *Streptococcus thermophilus*, *Enterococcus raffinosus*, *Ruminococcus gnavus* and bifidobacterial species (Favier, *et al.*, 2002, Park, *et al.*, 2005). As the environment becomes progressively depleted of oxygen, the growth of obligately anaerobic bacteria is favored and the ratio switches to 100-1000:1 in favour of anaerobic bacteria (Adlerberth and Wold, 2009). In parallel, dietary shifts at weaning from breast milk or formula to solid food provide additional carbon sources and induce a microbiota compositional change towards the diverse and complex adult composition. This shift includes the establishment of dominant bacterial phyla such as Bacteroidetes (*B. thetaiotaomicron*), and Firmicutes including Clostridia and lactobacilli (Avershina, *et al.*, 2014, Favier, *et al.*, 2002, Valles, *et al.*, 2014, Wang, *et al.*, 2004). An adult-like composition is achieved between 1 and 3 years of life (Palmer, *et al.*, 2007, Yatsunenko, *et al.*, 2012). However, the elderly display greater inter-individual variation relative to young adults (age ranging from

28-46 years old) (Claesson, *et al.*, 2012) and a Firmicutes:Bacteroidetes ratio which is closer to infants than adults (Mariat, *et al.*, 2009). In centenarian subjects (mean 100.5 years old), significant compositional changes have been observed compared to both elderly and young adult subjects (Biagi, *et al.*, 2010). For example, a remodeling of *Clostridium* cluster IV in centenarians that favour *C. leptum* with a corresponding decrease in *Faecalibacterium prausznitzii*, were associated with increased systemic inflammation (Biagi, *et al.*, 2010). This may be partly explained by the anti-inflammatory properties of *F. prausznitzii*, which have been shown both locally (for example via production of butyrate) and systemically (negative correlation with pro-inflammatory cytokines) (Furet, *et al.*, 2010, Sokol, *et al.*, 2008, Zhang, *et al.*, 2014).

In line with this, recent studies highlight an association between GM composition and host inflammatory status. In particular, several chronic diseases including inflammatory bowel disease (IBD), irritable bowel syndrome (IBS), celiac disease, and obesity have an immune component and concomitant inflammation that are generally associated with a decreased diversity of the GM. For example, a reduction in the richness and diversity of Firmicutes was found in Crohn's disease (CD) patients (Manichanh, *et al.*, 2006), including decreased counts of *F. prausnitzii* (Sokol, *et al.*, 2008), while Bacteroidetes were reduced in a subset of CD and ulcerative colitis (UC) patients (Frank, *et al.*, 2007). Similarly, UC and IBS patients are characterized by a decrease in bacterial species biodiversity compared to controls with a disappearance of *Bacteroides* species including *B. uniformis*, *B. ovatus*, *B. vulgatus*, and *Parabacteroides* sp. (Noor, *et al.*, 2010). Children with celiac disease have a lower ratio of *Lactobacillus-Bifidobaterium* to *Bacteroides*/*Prevotella-E.coli* in their duodenum compared to

controls independent of whether the disease was active, although these bacterial deviations were corrected following a long-term gluten-free diet (De Palma, *et al.*, 2010, Nadal, *et al.*, 2007). Moreover, obese individuals (BMI >30) harbour an altered GM composition characterized by significantly different abundances of specific taxa including an increased proportion of Firmicutes to Bacteroidetes, and functional genes such as glycoside hydrolases involved in the degradation of dietary starches (Ley, *et al.*, 2006, Turnbaugh, *et al.*, 2006, Turnbaugh, *et al.*, 2008, Turnbaugh, *et al.*, 2009). Interestingly, obesity and CD have been associated with low bone mineral density and increased fracture risk, (Greco, *et al.*, 2010, Gupta and Shen, 2013, Kim, *et al.*, 2012, Mosca, *et al.*, 2014, Vestergaard, *et al.*, 2000, Yoo, *et al.*, 2012), compatible with a diagnosis of osteoporosis.

Because, the adult microbiota is relatively resilient and refractory to manipulation, determining critical windows during development may provide the potential for manipulation as a means to prevent disease by maintaining diversity within the gut. This is especially intriguing in the context of the gut microbiota-bone axis given that exposure or restriction to environmental factors during intrauterine and early postnatal life, has been shown to be predictive of bone mineralization, growth retardation, and both body and gut microbial composition in the offspring (Engelbregt, *et al.*, 2004, Fanca-Berthon, *et al.*, 2010, Javaid, *et al.*, 2006, Tobias, *et al.*, 2005, Xu, *et al.*, 2006, Yin, *et al.*, 2010).

Microbial Impact on Bone Health

A few preclinical studies have investigated the relationship between GM and bone health (summarized in Table 1). One mouse study has investigated differences in bone health between

germ-free (GF) (mice born and housed in sterile conditions and void of GM throughout life) and conventionally-raised (CONV-R) mice (born and living in the natural environment and developing a normal GM) while other studies have administered probiotics to rat and mouse models.

Effect of gut microbiota on bone outcomes: The strongest direct evidence that GM modulates bone health comes from a study that compared BMD and microstructure in GF versus CONV-R mice. Seven-week old GF female mice had femurs with a more favorable bone structure and density than CONV-R mice: higher trabecular bone volume to tissue volume (BV/TV), higher trabecular number, less trabecular separation and higher trabecular BMD. Consistent with these findings, GF mice also had higher rates of bone formation and a lower number of osteoclasts per bone perimeter as shown through histomorphometrical analysis. There was also a lower expression of pro-inflammatory cytokines (IL-6, TNF-alpha) in the bone tissue of GF mice (Sjogren, *et al.*, 2012). To confirm the results observed between GF and CONV-R mice, and ensure differences in bone parameters were the result of the GM, 3-week-old GF mice were colonized with the GM from C57Bl6/J donor mice through fecal transplantation experiments previously described (Backhed, *et al.*, 2004), to establish a new subset of mice known as conventionalized (CONV-D) mice. Interestingly, CONV-D mice display normalized bone mass and frequency of T-lymphocytes and osteoclast precursor cells, indicating that the GM influences bone through immune function. The causative role linking GM and bone inflammatory status opens the way to intriguing studies evaluating if administration of selected beneficial bacteria – probiotics – defined as "live microorganisms that, when administered in

adequate amounts, confer a health benefit on the host" (FAO/WHO, 2001, Hill, *et al.*, 2014), improve or support bone health.

Effect of probiotics intervention on bone outcomes: 14 week old C57Bl/6J male mice receiving the probiotic *Lactobacillus reuteri* ATCC PTA 6475 for 4 weeks displayed an increase in femoral trabecular BV/TV, BMD, BMC, trabecular number, spacing, and thickness, although the same was not seen in cortical bone (McCabe, *et al.*, 2013). In addition, the trabecular region of the lumbar vertebrae also displayed increased BV/TV, BMD, BMC, trabecular number, spacing and thickness in the presence of *L. reuteri*. These benefits were accompanied by suppressed basal TNF-α mRNA expression in the jejunum and ileum (McCabe, *et al.*, 2013). None of the protective effects of *L. reuteri* were observed in female mice.

The same probiotic was also shown to protect against ovariectomy (Ovx)-induced effects on bone. Specifically, trabecular bone from the distal femur and lumbar vertebrae of Ovx Balb/c mice provided with *L. reuteri* for 4 weeks had a similar BV/TV%, thickness, number, and spacing compared to control mice (Britton, *et al.*, 2014). A gut microbiota compositional change was associated with *L. reuteri* administration, including an increase in *Clostridiales* and subsequent decrease in *Bacteroidales* in the jejunum and ileum (Britton, *et al.*, 2014). In addition, C57Bl/6N Ovx mice treated with probiotic *L. paracasei* DSM13434 alone or in a mixture with *L. plantarum* DSM 15312 and *L. plantarum* DSM 15313 for 8 weeks were protected from a reduction in cortical BMC and cortical cross sectional bone area in the mid-diaphyseal region of the femur (Ohlsson, *et al.*, 2014). The attenuation of bone resorption in response to ovariectomy may be explained by a reduction in pro-inflammatory cytokines (TNF-α

and IL-β1) and increase in osteoprotegerin (OPG) serum concentrations (Ohlsson, *et al.*, 2014). One study utilized male senescence accelerated mouse 6 strain that develop normally but show early onset of senescence. At 9 months of age (aged), mice were provided viable and heat-killed *Lactococcus lactis* subsp. *cremoris* H61 for 5 months to determine any protective effects of the bacterium on femur BMD with ageing (Kimoto-Nira, *et al.*, 2007). Heat-killed H61 displayed significantly higher BMD in femurs compared to controls at 14 months of age. Interestingly, the same was not observed when providing viable cells, suggesting a role of a membrane-bound protein or intracellular factor released only upon cellular death and thus a paraprobiotic or postbiotic effect (Taverniti and Guglielmetti, 2011, Tsilingiri and Rescigno, 2013). Moreover, this difference was not detected between 1-month old (young) mice provided with heat-killed H61 for 2 months compared to controls. This shows that time-sensitive periods exist for probiotic interventions in terms of improving bone mineralization, and that it is possible to protect and support bone health even after the developmental period has passed. "Young" mice were followed until 3 months of age, which may not have been sufficient time before observing any benefit of the probiotic compared to 14 months as observed with "aged" mice. There were also no direct comparisons of providing the probiotic intervention to "young" and "aged" mice at 14 months in order to determine greater protective effects of the probiotic intervention on bone mineralization.

Other animal models have also shown positive effects of probiotics on bone health including chickens (Mutus, *et al.*, 2006, Sadeghi, 2014) and zebrafish (Avella, *et al.*, 2012, Maradonna, *et al.*, 2013). In a study using broiler chickens, greater medial and lateral wall thickness of the tibiotarsi was found in chickens supplemented with *Bacillus licheniformis* and *B.*

subtilis (BioPlus 2B) for 6 weeks (Mutus, *et al.*, 2006). Interestingly, broiler chickens that were challenged with *Salmonella enteritidis* and treated with *B. subtilis* (strain identity not provided), had higher ash and calcium contents of the tibia compared to the challenged group alone at 21 days of age, although significance was lost after 42 days of age (Sadeghi, 2014). Zebrafish larvae that received *L. rhamnosus* IMC 501 for 10 weeks exhibited earlier backbone calcification compared to controls (Avella, *et al.*, 2012). This effect was mediated by stimulation of bone formation transcription factors (runt-related transcription factor 2 and Sp7 transcription factor), osteoblast and osteocyte differentiation kinases (mitogen-activated protein kinase 1 and 3), proteins (matrix Gla protein and bone gamma-carboxyglutamate protein), and through the inhibition of sclerostin, an inhibitor of bone formation (Maradonna, *et al.*, 2013). Although zebrafish are genotypically and phenotypically further from humans than a mammalian model and do not share a similar microbial community structure with mice or humans (Rawls, *et al.*, 2006), these studies further confirms that probiotic treatment positively impacts bone health, specifically mineralization.

In summary, the aforementioned studies provide a basis for future study using preclinical models as well as a basis for human studies. The preclinical studies demonstrate a solid link between GM and bone health.

Potential Mechanisms linking the Gut Microbiota to Bone Health

Several mechanisms may underlie the dependency link between bone and the GM and include induced production of gut-derived serotonin; maturation of the immune system during

development, and lipopolysaccharide (LPS)-induced systemic inflammation (Cani, *et al.*, 2007, Cani, *et al.*, 2007).

The majority of circulating serotonin, or 5-hydroxytryptamine (5-HT), is synthesized in the gut by the enterochromaffin cells (Gershon and Tack, 2007). The enzyme tryptophan hydroxylase-1 (Tph1) is responsible for catalyzing the synthesis of 5-HT in the duodenum (Cote, *et al.*, 2003, Walther, *et al.*, 2003). The GM may induce enteroendocrine cells to produce serotonin as shown in a GF model (Sjogren, *et al.*, 2012), although the microbial taxa accountable for stimulating 5-HT production have yet to be elucidated. Reuptake of serotonin into crypt epithelial cells and serotonergic neurons in the gastrointestinal tract, via the serotonin transporter (SERT) (Wade, *et al.*, 1996), results in 5-HT breakdown and ultimately in its dysfunction (Martel, 2006). Functional serotonin receptors are expressed on bone cells and previous studies have shown that 5-HT has negative effects on bone formation in mice (Bliziotes, *et al.*, 2006, Yadav, *et al.*, 2008). In addition, several studies demonstrated that inhibiting production of 5-HT via inhibition of Tph1 prevented bone loss in an ovariectomized rodent model (Cui, *et al.*, 2011, Inose, *et al.*, 2011, Yadav, *et al.*, 2010).

Another mechanism involves the contribution of GM to the maturation of the immune system in early life. One study in GF mice demonstrated that in the absence of the GM, the mucosal immune system was characterized by hypoplastic Peyer's patches containing minimal germinal centers and a reduced number of IgA-producing plasma cells and lamina propria $CD4^+$ T cells (Macpherson and Harris, 2004). In addition, absence of the GM results in immature systemic immunity with fewer and smaller germinal centers and reduced number of $CD4^+$ T cells in the spleen (Dobber, *et al.*, 1992, Mazmanian, *et al.*, 2005). In bone, hematopoietic stem cells

can either differentiate into bone-resorbing osteoclasts or other immune cells, including a macrophage or myeloid dendritic cell, based on the microenvironment (Lorenzo, *et al.*, 2008). In the presence of macrophage colony-stimulating factor (M-CSF), precursor cells increase expression of RANK, which allows RANK ligand (RANKL) to bind and initiate the signaling cascade leading to osteoclast formation (Arai, *et al.*, 1999). Both M-CSF and RANKL are expressed by stromal cells found in the bone marrow, and also in osteoblasts, in response to cytokines and hormones that stimulate bone resorption (Boyle, *et al.*, 2003). Altered immunity as a result of the GM may have profound implications on bone given the dependency of bone stem cell lineages on systemic factors such as cytokines. For example, TNF-α, and its downstream regulator IL-1, have been implicated in promoting osteoclastogenesis (Wei, *et al.*, 2005, Yarilina, *et al.*, 2011).

Finally, the GM and its cell membrane components, including LPS and peptidoglycan, can impact the host via toll-like receptors (TLRs), specifically TLR-4 and TLR-2, respectively. LPS is composed of both a variable carbohydrate-containing domain and a highly conserved immunogenic lipid A domain (Kelly, *et al.*, 2012), which allows the host to recognize LPS as a microbe-associated molecular pattern (Yang, *et al.*, 1998). To elicit an inflammatory response, LPS forms a complex with the lipopolysaccharide binding protein (LBP) and cluster of differentiation 14 (CD14) protein factor (Yang, *et al.*, 1998), in order to signal through TLR-4 (Dixon and Darveau, 2005).

Osteoclasts, like macrophages, express TLR-4 as a consequence of their hematopoietic stem cell lineage (Kajiya, *et al.*, 2010). Pre-osteoclasts express both TLR-4 and receptor activator of nuclear factor kappa-B ligand (RANKL), and their fate depends on RANKL,

osteoprotegerin (OPG; a decoy receptor for RANK) and LPS. During bone resorption, the ratio of RANKL to OPG is high, which leads to the maturation of pre-osteoclasts to a multinucleated osteoclast cell that begins the process of bone resorption. The bifunctional role of LPS in osteoclastogenesis is complex (Liu, *et al.*, 2003). In a situation where pre-osteoclasts are only exposed to LPS and not RANKL, pre-osteoclasts may differentiate into phagocytes and not a mature multinucleated osteoclast cell (Liu, *et al.*, 2003). Therefore, LPS has the potential to suppress osteoclast production, and prevent bone resorption. Likewise, if RANKL binds to RANK in the absence of LPS stimulation, pre-osteoclast cells differentiate into mature osteoclast cells and begin bone resorption. However, if LPS bind to TLR-4 in the presence of RANKL stimulation, osteoclastogenesis is accelerated causing more intensive induction of osteoclast differentiation and cell survival, ultimately resulting in even more severe bone destruction (Liu, *et al.*, 2003).

Additional mechanisms have also been proposed, many of which include increasing mineral availability and absorption either through production of short-chain fatty acids (Campbell, *et al.*, 1997), production of phytase enzyme (Lan, *et al.*, 2002), hydrolysis of glycoside bonds of estrogenic food (Chiang and Pan, 2011) or reducing intestinal inflammation (Sjogren, *et al.*, 2012). The aforementioned mechanisms are still under investigation but provide a mechanistic link between gut and bone.

Knowledge Gaps and Future Directions

Findings to date have provided a basis for future pre-clinical studies to more fully elucidate the relationship between the gut and bone. As summarized in Table 2, there are several

key knowledge gaps related to the gut-bone axis. With respect to probiotic intervention strategies, imminent research will need to clarify dose-response and strain-specific effects that benefit bone health, which may be a challenging endeavor given the specificity of bacterial strains and the vastly differing effects they may have. Thus, dose-response studies will be appropriate once an effective strain is identified. In addition, given that there may be critical windows of opportunity for modulating bone health during the lifespan, timing of exposure as well as duration of exposure are also areas for future investigation. Sex-specific responses also require investigation. In so doing, changes in expression of these regulators can be determined and directly linked to microbial influence.

Conclusion

Studies conducted to date offer substantial evidence that a gut microbiota-bone axis exists. The study in germ free mice illustrates that the composition of GM directly impacts bone mineral density and structure, and because the measurements are done at a relatively young age, these findings demonstrate that microbiota or lack thereof have an effect during development. Thus, targeting early life with interventions that modulate GM may indeed provide a strategy for a stronger, healthier skeleton at adulthood. In addition, probiotics used in the current studies have also shown promise in improving bone health, and with further understanding regarding mechanisms of action, may be a useful strategy alone or in conjunction with drug therapies to promote and support bone health.

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Table 1 Overview of Studies Linking the Gut Microbiota to Bone Health

Animal Model (sex), Sample Size, Groups, Age	Objective	Approach	Main Findings	Main Conclusions	Reference
Germ-Free versus Conventionally Raised Mice					

C57Bl/6J mice (F); n=4-14/ group; CONV-R, GF, CONV-D; 3 weeks old	To determine whether the GM modulates bone health	**GF vs. CONV-R mice**: Compared bone parameters and serum serotonin levels in GF versus CONV-R mice. **GF vs. CONV-D mice**: GF mice were colonized with donor microbiota	**GF vs. CONV-R mice**: ↑ trabecular proximal tibia volumetric BMD ↑ trabecular BV/TV in the femur ↑ trabecular number and ↓ separation ↑ cortical bone area of the femur ↓ serum serotonin levels ↓ Tph1 and ↑ SERT mRNA expression in the proximal colon ↓ frequency of CD4+ T cells	The GM regulates bone mass in mice via an altered immune status, independent of gut-derived serotonin, that effects osteoclast-mediated bone reabsorption.	(Sjogren, *et al.*, 2012)

	at weaning (3 weeks of age).	and OCL precursor cells in bone marrow ↓ TNF-α & IL-6 in femur and ↓ TNF-α in proximal colon
		GF vs. CONV-D mice: 4 weeks after colonization, mice had ↓ volumetric BMD and indistinguishable from CONV-R mice. Normalization of ↓ frequency of CD4+ T cells and OCL

	precursor cells in bone marrow. ↔ in serotonin levels

Effect of Intervention with Probiotic on Bone Outcomes

Exp. 1: a) SAMP6 (M); n= 6-7/ group; control and probiotic [heat-killed *Lactococcus lactis* subsp. *cremoris* H61]; 9 months old b) SAMP6 (M); n= 9/group; control and heat-killed probiotic; 1 month old **Exp. 2**: SAMP6 (M); n= 4-5/group;	To investigate the effects of oral administration of the heat-killed probiotic (**Exp.1**) on a) aged and b) young mice and viable (**Exp. 2**) probiotic strain H61 on physiological variables in mice, including bone density loss.	**Exp. 1**: Heat-killed cells of H61 bacteria were provided to mice in their diet for a) 5 months and b) 2 months. **Exp. 2:** Live H61 bacterial	**Exp. 1a) Heat killed H61 vs. control:** ↑ weight ↓ *Lactobacillus* sp. and *Staphylococcus* species in the feces ↑ IL-12 protein levels in spleen cells ↑ BMD in femurs. **Exp. 1b) Heat killed H61 vs. control:** ↔ in weight and right femur.	Only heat-killed H61probiotic cells attenuated decline in femur BMD and weight loss in aged mice.	(Kimoto-Nira, *et al.*, 2007)

control and probiotic [live *Lactococcus lactis* subsp. *cremoris* H61]; 8 months old		cells (2 x 10^7 cells/mouse) were provided every 2-3 days for 4 months by intragastric gavage.	**Exp. 2 live H61vs. control:** ↔ in BMD between groups		
C57Bl/6 (M and F); n= >8mice/group; control and probiotic (*Lactobacillus reuteri* ATCC PTA 6475); 14 weeks old	Investigate the effects of an anti-inflammatory probiotic strain on bone health in male and female mice moved from pathogen-free	10^9 CFU/mL *L. reuteri* by oral gavage 3 times per week for one month.	**Probiotic vs. control in males:** ↓ in visceral fat weight ↓ TNF-α mRNA in the jejunum and ileum ↑ trabecular BMD, BMC, BV/TV, volume	*L. reuteri* maintains bone health in males but not females by promoting bone formation.	(McCabe, *et al.*, 2013)

facilities to standard animal facilities.	fraction, trabecular number and thickness in the distal femur and lumbar vertebrae ↔ cortical bone parameters in the femur ↑ bone formation rate and osteocalcin in the tibia
	<u>Probiotic vs. control in females</u>: ↔ in all bone parameters

C57Bl/6N (F); n= 10 mice/group; 6 groups: 1. Veh-Ovx, 2. Veh-Sham, 3. L. Para-Ovx, 4. *Lactobacillus paracasei* DSM13434 (L. Para)-Sham, 5. *L. para* DSM13434, *L. plantarum* DSM 15312 and DSM 15313 (L. Mix)-Ovx, 6. L. Mix-Sham; 8 weeks old	Investigate the effects of a single probiotic strain (L. para) and probiotic mixture of three strains (L. mix) on bone health	10^9 CFU/mL of each strain was given to mice in the drinking water 2 weeks prior to Ovx surgery and for 4 weeks after.	**Probiotic or probiotic mix vs. Ovx mice:** ↑ femoral cortical BMC and cortical cross sectional bone area ↓ TNFα, IL-1β, and RANKL/OPG ratio in femoral cortical bone ↑ TGFβ1 expression in femoral bone marrow	Probiotic treatments promote bone health via a reduction of inflammatory cytokines and increased expression of OPG.	(Ohlsson, *et al.*, 2014)
Balb/c (F); n	To determine	1 x 10^9	**Probiotic vs.**	*L. reuteri*	(Britton,

=8/group; 3 groups: 1. control (not Ovx) 2. Ovx + *Lactobacillus reuteri* ATCC PTA 6475 (*L. reuteri*) and 3. Ovx-control (without *L. reuteri*); 12 weeks old.	if the probiotic can suppress, prevent, or attenuate Ovx-induced bone loss.	CFU/ml of *L. reuteri* was gavaged 3 times/week for 4 weeks and 1.5 x 10^8 CFU/ml were added to the drinking water and given ad libitum.	**Ovx mice:** ↑ trabecular BMC of the distal femur and L3 vertebrae ↑ BV/TV, trabecular thickness, number ↓ trabecular spacing ↑ mRNA expression of RANKL and ↓ TRAP5 ↓ osteoclast differentiation ↑ Clostridiales and ↓ Bacteriodales in the jejunum and ileum.	protects against Ovx induced bone loss through the suppression of osteoclast activity and possibly through the manipulation of the GM.	*et al.*, 2014)

Abbreviations: GM: gut microbiota; GF: germ-free; CONV-R: conventionally-raised; CONV-D: conventionalized; BV/TV: bone volume as a percentage of total volume; Tph1: tryptophan hydroxylase-1; SERT: serotonin transporter; OCL: osteoclast; BMD: bone mineral density; BMC: bone mineral content; Exp.: experiment; Ovx: ovariectomized; Veh: vehicle; IL: interleukin; RANKL: receptor activator of nuclear factor kappa-B ligand; OPG: osteoprotegrin; TGFβ1: transforming growth factor beta 1; M: Male; F: Female.

Table 2 Overview of Knowledge Gaps and Future Directions

Research Area	Current State	Knowledge Gaps	Future Directions
Dose and Type of Probiotic Strain	• Probiotic products currently used on the market use a dosage of 10^9 CFU/serving. The preclinical studies use a dosage of 10^7 to 10^9 CFU. • Microbial strains have vastly different effects on several outcomes.	• Does the GM influence bone health in humans? • Which strains specifically influence bone health and to what degree? • What is the optimal probiotic	• Provide candidate probiotic strain to humans at risk for bone disease. • Establish meaningful endpoints to evaluate efficacy of the probiotic. • Dose-response studies to determine effective dose on bone health.

	• Findings from preclinical studies show that commensal GM and some probiotic strains have a favourable effect on bone health.	dosage to elicit health benefits for bone?	
Timing of Exposure/Duration of Treatment	• The commensal microbiota of the infant is highly influenced by a mother's microbiota, type of feeding, diet, genetics, drugs and environment. • Exposure to microbes occurs soon after birth,	• Can early life nutrition 'program' the microbial profile and thereby promote healthy bone development ? • At what life	• Measure biomarkers of bone metabolism to study and compare acute versus chronic exposure to probiotics on bone health • Identify microbial biomarkers of

	which is necessary to ensure proper development of the immune system.	stage and how often should a probiotic be taken?	bone health. • Compare doses at different life stages (*in utero*, post-natal, childhood, adultdhood, and in the elderly).
Sex Effects	There are sex-specific responses to probiotic administration.	• Does the GM affect bone differently depending on sex?	• Studies need to include both sexes.
Commensal Gut Microbiota	• The dominant GM has been established for healthy humans and rodents. • Functional redundancy has been determined	• What is the "normal" GM and correspondin g metabolome for bone health?	• Test the effects of the probiotic strains or groups of strains used the in the pre-clinical studies discussed in this review on bone

	in healthy individuals with varying microbial compositions.	• Are there any harmful effects of the commensal microbiota on bone health?	health in well-established randomized controlled trials. • Determine links between GM and microbiome on bone health. • Test adverse effects of candidate probiotic strains on bone.
Mechanism for Microbial Effects on Bone	• LPS, gut-derived serotonin, immune development, and increased mineral absorption are candidates for linking the GM	• What are the specific mechanisms linking the GM and bone? • Is the	• *In vitro* analysis to screen for candidate microbial components (i.e. LPS) using bone cell lines and determining the

	to bone health.	mechanism through a microbial secreted molecule, membrane component or some other microbial-associated molecule(s)? • What are the mechanisms underlying fetal programing of the GM in ensuring bone health?	corresponding regulation of genes of interest.

Abbreviations: GM: gut-microbiota