Review Article

Bone and the gut microbiome: a new dimension

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Abstract: Our understanding of the link between the human gut microbiome and health outcomes is rapidly expanding. The different microbial communities within the human body affect cellular responses and shape many aspects of our physiology; including that of the skeletal system. Mouse models have been imperative to reveal the underlying mechanisms by which the human gut microbiome affects bone metabolism. Since the gut microbiome is influenced by modifiable factors (e.g., diet or drugs), it embodies great potential as a therapeutic target holding promise for the management of bone diseases. The purpose of the current review is to highlight the complex interplay between the gut microbiota, their metabolites and bone metabolism by presenting a compendium of a remarkable range of pre-clinical and clinical studies in the field. This review also discusses the type of studies that will be essential for translating microbiome research into successful microbiome-based medical interventions.

Keywords: Bone; gut microbiome (GM); pre-clinical studies; clinical trials

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Introduction

Recently, the advent of cost-effective sequencing technologies has exposed the vast number of microbial communities hosted in the human body—comprising as many cells as those present in our body—and unveil them as an integral component of sustainable health (1). Gene products from these microorganisms are responsible for the breakdown of complex molecules in food, protection from pathogens and a healthy development of the immune system (1,2). The term "microbiome" refers to the collection of microorganisms, their genomes, and their interactions, in a given environment. Human microbiome research and datasets have expanded rapidly in recent years with a vast array of applications extending from forensic science to translational health opportunities and beyond.

The human gut microbiota is by far the most studied ecosystem in relation to health and disease processes. Even if microbial communities are present at different body sites, the microbiome within the gastrointestinal tract has attracted the major focus of research efforts, as it contains high microbial density and its signature can be easily assessed from faecal material. Alterations in the human gut microbiome (GM) have been implicated in a wide range of diseases including Crohn's disease, ulcerative colitis, type 2 diabetes mellitus, asthma, obesity, autism, and rheumatoid arthritis (3,4).

The adult GM has an average unique microbial signature that is largely stable over time; in other words, inter-individual differences are much larger than individual temporal microbiome variability. The extensive interpersonal GM variation has been linked to environmental factors (5-7) [especially diet (8)] and to host genetics (9,10). Yet, the complex interaction of host genetics, environment and gut microbiota determining human physiological diversity has not been fully elucidated and will be a fertile area for methods development and discovery in the upcoming years.

Specifically, in the field of bone health and disease, evidence is accumulating about the involvement of the GM in preserving bone homeostasis. In response to this steadily increase in knowledge, earlier this year, the term "Osteomicrobiology" has been coined to bridge the gaps between bone physiology, gastroenterology, immunology

and microbiology (11). The human skeleton plays an important role in the overall function of the body. Besides being a highly specialized supporting framework of the body, bones protect vital organs, provide an environment for marrow (both for blood-forming and fat storage), act as a mineral reservoir for calcium homeostasis and are a storehouse for growth factors and cytokines (12). Therefore, the interest in the interplay between the microbiome and the human skeleton goes beyond the function of bones as a structural organ to also characterize its role on the global health of individuals.

Here, I review the remarkable range of pre-clinical and clinical studies that support the role of gut microbiota in bone metabolism, placing special emphasis on the most recently published studies and on future directions the musculoskeletal field is taking, revitalized by this novel research dimension.

Effects of the GM on bone metabolism

The microbiome-bone relationship is complex and involves several mechanisms including:

(I) The regulation of nutrient absorption from the diet (e.g., calcium, phosphorus);
(II) The translocation of microbial products across the gut endothelium [e.g., lipopolysaccharide (LPS), short-chain fatty acids (SCFAs), peptidoglycan];
(III) Regulation of immunomodulation (e.g., $CD4^+$ T cell activation, control of osteoclastogenic cytokine production).

The impact of GM profiles in bone metabolism is being delineated by perturbation experiments primarily performed in mouse models (13-17). Germ-free (GF) mice, raised in sterile isolators and completely devoid of microbiota, are operational models to study the consequences of microbiota on physiology. GF mice can be used as a "test tube" to examine the effects inflicted by specific microbes or communities of microbes in their host. Depletion of the gut microbiota by antibiotic use has similarly been attempted as an alternative to GF animals but this approach is not free of caveats that have limited its use (18).

In 2012, investigations by Sjogren *et al.* showed, for the first time, that bone density was regulated by the presence of gut microbiota (13). In this study, female GF mice had higher trabecular and cortical bone density when compared to control C57BL/6 mice raised in conventional conditions at 9 weeks of age (13). GF mice also presented with fewer $CD4^+$ T cells and osteoclast precursors in the bone marrow. The authors attributed these observations to impaired osteoclastogenesis leading to reduced bone resorption (13). Favorable bone characteristics in GF C57BL/6 mice were also described later by the same research group (19) and by others (14,20). In addition, these studies showed that the gut microbiota modulates inflammatory responses caused by sex steroid deficiency and resulting in trabecular bone loss (14). Since these first reports, conflicting studies have emerged in the field. A recent study, also using C57BL/6 mice, found no significant differences in bone parameters between GF females and females raised in conventional conditions at 4 weeks of age (15). Likewise, the same authors showed that the introduction of defined microbiota to GF Swiss Webster (SW) male or female mice did not significantly alter bone mass, osteoclast precursor and T cell populations, nor the expression of several inflammatory markers (15). Conversely, gut microbiota has been shown to have an anabolic (bone forming) rather than catabolic (bone resorption) effect on bone in 8-week-old conventionally raised male BALB/c mice. These mice had higher cortical and trabecular volume as compared to their GF counterparts (16).

The discrepancy among these studies can be attributed to differences in the genetic background, age or sex of the mice. Importantly, Yan *et al.* demonstrated that both bone resorption and formation are promoted by microbiota colonization of GF mice and that the net effect of colonization is highly time-dependent (17). Despite an initial increment of bone resorption within the GF females colonized with microbiota from conventional mice as compared to GF siblings, bone formation rate increased, and at 8 months after colonization, the bone mass was similar among both groups of mice (17). Other factors, usually disregarded in these mechanistic studies, as the mode of transplantation of the microbiota or the vendor-location-specific mice characteristics, can result in selective colonization of the GF mice and affect the generalizability of the reported findings (15). Lastly, although not investigated yet in the context of the bone-gut axis, it is known that the timing of the microbiota colonization has important effects on the future immune responses of the host (21), and therefore, should be taken into consideration in GF mice studies. Notwithstanding the limitations of GF models (22), these investigations have provided the backbone to disentangle the role of the gut microbiota in bone metabolism, as described below.

GM effect on bone growth

During childhood and adolescence, longitudinal bone growth occurs at the growth plates, where chondrocytes proliferate and undergo hypertrophy before endochondral ossification takes place. Insulin-like growth factor (IGF1) is a key regulator of growth plate maturation and also plays a role in the maintenance of bone in adults (23). The GM has also been shown to exert an effect on bone growth and development. Yang *et al.* demonstrated that IGF1 serum levels were increased in colonized mice compared to GF mice beyond any potential developmental impairment in GF mice (17). The authors also established that SCFAs (metabolites produced by the microbiota during fermentation of dietary fiber) were responsible for IGF1 induction by the microbiota (17). In line with these findings, SCFAs, more specifically, propionate and butyrate, were able to induce metabolic reprogramming of osteoclasts *in vitro*, leading to enhanced glycolysis at the expense of oxidative phosphorylation. This metabolic change was also shown to downregulate essential osteoclast genes such as *TRAF6* and *NFATc1* (24), and ultimately, to inhibit osteoclast differentiation and bone resorption. From these findings, it has been proposed that SCFAs therapeutic supplementation holds great potential to prevent bone resorption, particularly in elderly individuals (24).

GM effect on calcium absorption

Another possible mechanism by which the GM could influence bone is through the enhancement of calcium absorption and other bone-related minerals critical for development (25). Calcium is absorbed by the intestinal mucosa and deposited as calcium hydroxyapatite ($Ca_{10}[PO_4]_6[OH]_2$) in bones and teeth, where it provides hard tissue with its strength (26). Calcium is particularly important during the process of bone accretion (27,28). It has been shown that fermentation to SCFAs by the gut microbiota results in higher calcium absorption (25) and therefore, constitute an alternative to correct calcium deficiency without the need of an increase in calcium-rich foods or supplements. Studies in adolescents found increased levels of calcium absorption among those using different prebiotics (nutrients capable to modify the gut microbiota): galacto-oligosaccharides (GOS) and soluble corn fiber (SCF), both of which can be fermented to SCFA. Moreover, this increment correlated with relative abundances of *Parabacteroides*, *Bifidobacterium*, *Bacteroides*, *Butyricicoccus*, *Oscillibacter*, and *Dialister* measured in faeces (29-31). Also, a small clinical trial in post-menopausal women showed a dose-response effect in bone calcium retention with SCF dose. A significant increase in bone-specific alkaline phosphatase was observed despite that bone turnover markers were not changed by the intervention (32). Yet, the direct link of these prebiotics and bone mass in humans is still lacking and the evidence from animal models is not conclusive. In growing male Sprague-Dawley rats, GOS feeding resulted in increased calcium and magnesium absorption and retention; as well as higher total and trabecular volumetric bone mineral density (vBMD) and bone area at the distal femur, and increased vBMD at the proximal tibia with consequently greater resistance strength to breaking at the femur and tibia (33). In contrast, it has been reported that GF mice have normal levels of serum calcium and hormones regulating calcium homeostasis and do not present any alteration in bone formation rate (13).

GM effect on bone resorption

As described above, the gut microbiota is an important immunoregulator of osteoclast-osteoblast mediated bone remodeling processes in the healthy adult skeleton of mice. In humans, particular attention has been placed on the skeletal changes occurring during the menopausal transition, typically characterized by bone loss. Soon after the onset of menopause, the normal bone turnover cycle is impaired leading to a decline in bone mass. It is estimated that worldwide 1 in 3 women over age 50 will experience osteoporotic fractures as a consequence of menopause (34). Menopause triggers a rapid decline in circulating estrogen correlated with expansion of Th17 cells (osteoclastogenic population of $CD4^+$ T cells) and increased serum levels of pro-inflammatory cytokines, including tumor necrosis factor α (TNFα), interleukin-1β (IL-1β), IL-8, and IL-6, as well as the osteoclastogenic cytokine receptor activator of nuclear factor-κB ligand (RANKL) (35). Major insight on age-related bone loss has been obtained from studies in ovariectomized mice (ovx), mimicking the post-menopausal status (36). Ovx mice present gut permeabilization, in addition to the above-mentioned immunogenic changes, resulting in severe translocation of microbial and/or microbial products (14,35). Strikingly, ovx rodents raised in GF conditions appear to be protected against bone loss, but microbial recolonization restores the capacity of sex steroid deficiency to induce bone loss (14). Nevertheless, other studies have demonstrated that specific microbes possess

Table 1 Specific probiotics and prebiotics shown to modulate skeletal parameters

Bone modulation agent	Effect	Type of study	Possible mechanism	Reference
Probiotic				
L. rahmnosus GG	Inhibition of bone loss	C57BL/6 mouse model	Tightened gut epithelial barrier integrity and dampened the levels of osteoclastogenic cytokines	Li *et al.* (14)
L. plantarum WJL	Increased femur length	BALB/c mouse model	Increase in serum IGF1 levels	Schwarzer *et al.* (16)
B. *clausii*	Inhibition of bone loss	BALB/c mouse model	Increased number Treg and decreased number of Th17 cells	Dar *et al.* (37)
L. reuteri ATCC PTA 6475	Inhibition of bone loss	BALB/c mouse model	Decreased osteoclastogenesis	Britton *et al.* (38)
L. acidophilus ATCC 4356	Inhibition of bone loss and increase in bone heterogeneity	BALB/c mouse model	Increased number Treg and decreased number of Th17 cells	Dar *et al.* (39)
L. paracasei DSM13434	Inhibition of bone loss	C57BL/6 mouse model	Decreased osteoclastogenesis	Ohlsson *et al.* (40)
L. paracasei DSM13434, *L. plantarum* DSM 15312 and DSM 15313	Inhibition of bone loss	C57BL/6 mouse model	Decreased osteoclastogenesis	Ohlsson *et al.* (40)
L. helveticus ATCC 27558	Increased BMD levels and strength	Sprague-Dawley rat model	Increased osteoblast proliferation and bone formation activity	Parvaneh *et al.* (41)
L. reuteri 6475	Reduced bone loss	Human clinical trial	Unknown	Nillson *et al.* (42)
Prebiotic				
SCFA	Acute increase in bone resorption	BALB/c mouse model	Increase in serum IGF-1 levels	Yan *et al.* (17)
SCFA	Increased BMD levels	C57BL/6 mouse model	Change in the metabolic state of pre-osteoclasts	Lucas *et al.* (24)
GOS	Increased BMD levels and bone strength	Sprague-Dawley rat model	Increased calcium and magnesium retention	Weaver *et al.* (33)

BMD, bone mineral density; SCFA, short-chain fatty acid; GOS, galacto-oligosaccharides.

immunomodulatory properties and can revert the effect of ovx-induced bone loss. Beneficial effects of probiotics (microorganisms which when administered in adequate amounts confer a health benefit on the host) have been demonstrated for *Bacillus clausii* (37), *Lactobacillus reuteri* (38), *Lactobacillus acidophilus* (39), *Lactobacillus rhamnosus GG* (14), *Lactobacillus paracasei* either alone or in combination with *Lactobacillus plantarum* (40), and *Lactobacillus helveticus* (41). These studies are summarized in *Table 1*.

GM effect on bone formation

Despite of its pivotal role in bone metabolism, evidence of an effect of GM on osteoblast formation and activity is not as substantial as that from osteoclasts. Yet, recent studies have established that GF mice present enhanced osteoblast activity (20,41,43). Cultured calvarial osteoblasts from conventionally raised mice showed significantly higher expression of osteocalcin, alkaline phosphatase, IGF1/2, and a decreased ratio of osteoprotegerin/receptor activator of nuclear factor-kappa B ligand (OPG/RANKL) as compared with GF mice. Simultaneously, osteoblasts of conventionally raised mice showed less mineralization, probably as a result of the inhibitory effect of osteocalcin (43). Reduced mineralization and decreased expression of *Col2a1*, *Runx2*, and *Sp7* were also observed in bone marrow stromal cells (osteoblast-progenitors) from conventionally raised mice as compared to GF mice (20). The latter study also showed

that commensal gut microbiota pro-osteoclastic actions are related to sustained alterations in RANKL signaling (20). Nevertheless, in ovx rats, *Lactobacillus helveticus* supplementation resulted in an increment of the osteoblast surface, upregulated expression of *Runx2* and *Bmp2*, increased serum osteocalcin, bone volume/total volume and trabecular thickness (41).

GM effect on bone strength

Bone strength, defined as the capacity of bone to respond to mechanical demands, is ultimately determined by its material composition, quantity and dimension, together with bone distribution and microarchitecture. Up to now, bone mineral density (BMD) has been the preferred phenotype to study the bone-gut axis. Nevertheless, BMD typically explains only 60–80% of bone strength when bone samples are compared in a laboratory setting under controlled loading conditions (44). Bone composition characteristics or bone geometrical parameters might explain why many individuals who fracture have BMD levels similar to those of individuals who do not. For instance, a low BMD can be offset by specific bone morphology or structural geometry or with no, or minimal compromise to mechanical strength. Here, the GM can also play a role shaping differences in bone strength not picked up by the BMD measurement. Interestingly, a recent investigation suggested that alterations to the GM for extended periods during growth may lead not only to changes in bone mass but to impaired bone biomechanical performance (45). These findings are consistent with changes in bone tissue properties beyond mass or geometry. Properties as collagen quality or non-collagenous proteins have been proposed as possible factors mediating the observed effect. There are several publications reporting altered collagen properties associated with fragile bones (46-48), whereby collagen orientation generated changes in fracture energy by two orders of magnitude (49). Consistent with a role of microbiota in bone morphology, the administration of *Lactobacillus acidophilus* to osteoporotic mice resulted in higher BMD levels and enhanced bone heterogeneity as evidenced by infrared spectroscopy (39). The significance of this work lies in the fact that most anti-resorptive drug candidates (bisphosphonates, calcitonin, cathepsin K inhibitors, estrogen) decrease bone heterogeneity, thus increasing its brittleness and risk of fracture (39). Another probiotic, *Lactobacillus helveticus* was also reported to improve bone strength in a three-point bending test of ovx 10-week-old female Sprague-Dawley rats, although the authors attributed this improvement directly to an increase in bone density and a reduced bone porosity (41).

Other GM effects on bone

Although out of the scope of this review, there is now cumulative evidence linking the human microbiome with alterations in other musculoskeletal tissues as joints (50,51) and muscles (52,53). It is well established that the musculoskeletal system is complex, displaying mechanical, biochemical and molecular interaction among cells within different tissues. Given these interactions, it is plausible that the human microbiome exerts both direct and indirect effects in bone homeostasis via the different constituents of the musculoskeletal system. Mechanically, muscle-derived forces drive an adaptive response in bone, affecting its morphology, structure and strength as portrayed by the mechanostat theory (54). Therefore, an increase in muscle mass and strength, as reported in response to *Lactobacillus* species in mice, will definitely result in changes in bone mass and distribution.

Furthermore, the role of the human microbiome on local bone tissue damage has also been investigated. While the gut microbiota has been implicated in the development of osteomyelitis (55), the oral microbiome is a key factor in the localized T-cell-induced bone damage in chronic periodontitis (56,57). Besides, even if it seems not to play a causative role on bisphosphonate-related osteonecrosis of the jaw, the oral microbiome could be a contributing factor in the host response (58,59).

Translational potential of GM studies

Despite the remarkable number of pre-clinical studies in animal models included in this review, only now, these findings are being translated into clinical practice. Earlier this year, Nilsson *et al.* reported that supplementation with *Lactobacillus reuteri 6475* resulted in a reduced bone loss in older women with incipient osteoporosis after following a randomized controlled trial for one year (42). The positive effect of *Lactobacillus reuteri 6475* on the BMD of estrogen-deficient (38) and active-inflammation (60) mouse models was already documented, but it was in this clinical trial, that it was demonstrated for the first time that probiotics can be used to affect the human skeleton. From the 90 postmenopausal women enrolled in the study, those in the treatment group showed reduced volumetric BMD loss at

the tibia (mean difference between groups =1.02%; 95% CI: 0.02–2.03%), a site rich on trabecular bone, after one year (42). While animal models have contributed much to our understanding of pathophysiological mechanisms, in general, their value in predicting the effectiveness of treatment strategies in clinical trials is less striking (61). Therefore, Nilsson *et al.* results are a milestone for the emerging discipline of osteomicrobiology, particularly in the search of targets for therapies that can improve bone structure and quality, and ultimately effectively prevent osteoporosis.

Future directions and concluding remarks

There is ample literature supporting the effect of the GM in bone metabolism based on mechanistic studies in animal models as herein reviewed. Nevertheless, robust data from human studies are still lacking. Encouragingly, besides the successful *Lactobacillus reuteri* clinical trial (42), preliminary results from both, clinical trials and large population-based studies, are starting to be presented at international meetings of bone research with increasing frequency. The steadily growing interest of the scientific community in microbiome studies warrants that resources will be allocated to the generation of this data fomenting the generation of answers and new research questions in the field of osteomicrobiology.

Beyond its mechanical and scaffolding properties, the skeleton has important endocrine functions independent of mineral metabolism. Osteocalcin, produced by the osteoblasts and modulated by the GM (20,43), plays a crucial role in regulating insulin metabolism in a hormonal manner, and also induces testosterone production (62). Moreover, recent studies have discovered that this hormone is necessary for both brain development and brain function in the mouse, implicating osteocalcin in the biology of aging (63). Another protein secreted by osteoblasts, lipocalin 2, which can cross the blood-brain barrier, has recently been shown to possess important metabolic regulatory effects and to control appetite (64). As there is evidence of a link between the GM and osteoblast activity (20,41,43), the described discoveries open a wholly new approach to the treatment of metabolic disorders. Conversely, it is well established that the brain regulates bone homeostasis by controlling the secretion of hypothalamic (e.g., oxytocin and vasopressin) or pituitary hormones (e.g., growth hormone, thyroid stimulating hormone, and follicle-stimulating hormone) (65). In addition, the skeleton is a highly innervated tissue with sensory and autonomic neurons (66). SCFAs from bacteria have been shown to influence the blood-brain-barrier development and maintenance, and *Lactobacillus reuteri* to increase oxytocin production (67). These factors could thus alter the secretion of endocrine factors and efferent neural outflow to the bone tissue. Although our understanding of the interactions between our gut microbes and the nervous system is in the early stages, advances in this field will enlighten the neuroendocrine regulation of our skeletal system

As already mentioned, the human microbiome is shaped by the action of both environmental factors and host genetics, but also by the ecological networks of these microbial communities and their dynamic interaction. In other words, the effect of a particular bacteria can, and most likely will, be mediated by the presence of other external perturbations (i.e., the presence of other bacteria or host individual characteristics). Nevertheless, most of the current approaches consider bacterial communities as a mere collection of independent organisms. Neglecting that these bacteria communicate, cross-feed, recombine and coevolve (68) would compromise the success and stability of microbiome-based therapies to prevent or combat disease. Bayesian statistics and machine learning algorithms are among the new methodologies proposed to map microbial networks and its use will certainly lead to new discoveries.

This review provides a synopsis of studies supporting the influence of gut bacterial communities on bone physiology. Nevertheless, it is important to emphasize that the GM comprises not only bacteria, but also archaea, protozoa, fungi, and viruses that coexist in a dynamic equilibrium. These communities offer an additional dimension to the investigation of host-microorganism interactions. For instance, bacteriophages (i.e., viruses that infect bacteria) are the most abundant biological entities in the human gut ecosystem (ten times more abundant than bacterial cells) (69). Phages play a key role in shaping microbial communities and exert direct immunomodulatory effects on the host by translocating across the mucosal barrier of the gut into lymph, blood and tissues (70). Yet, there has been paucity in the study of phages given the challenges imposed by their vast diversity and lack of a genetic universal marker that allows profiling, such as the bacterial 16S rRNA genes, in order to facilitate their investigation (71). Therefore, even if in its infancy, the incorporation of non-bacterial communities residing in the human body will potentially be the treasure trove for advancing our understanding of human health and disease processes, particularly of the musculoskeletal system.

Besides our developments in the analysis of microbiome data, the last decade has also provided us with substantial evidence of genetic (72) and epigenetic (73) factors influencing the bone tissue. The mechanisms by which our genetic information shape our gut microbiota (74), whose metabolites, in turn, can influence levels of gene expression (75) and modulate host epigenetic processes (76) are still to be elucidated. Even if it is rather evident that the performance of large exploratory integrative studies is too ambitious based on our current knowledge; it is important to keep in mind that these layers of information describing multiple levels of cell regulation ultimately generate the observable phenotypic variability and reflect the integral complexity of living systems.

In conclusion, pre-clinical studies have already provided remarkable mechanistic evidence of the influence of the human GM on bone physiology. These studies have shown that bacteria and bacterial products can modulate processes of bone gain and bone loss via effects on both osteoblasts and osteoclasts. On top of these discoveries, the GM seems to also modulate bone morphology, ultimately affecting bone strength and consequently fracture risk. Recently, a probiotic has been shown, for the first time, to inhibit bone loss in post-menopausal women, strengthening the hopes about the potential of the osteomicrobiology field. However, more studies in humans are needed. Microbiome, genetic and metabolic studies in large populations are underway and will provide the basis of understanding how the microbiome determine skeletal health. The field still needs to go beyond the simplistic assessment of independent bacterial associations and implement analyses able to model the underlying complexity of the microbiome interactions (i.e., using ecological networks, multi-omics and integrative algorithms) in order to bring the field closer to medical interventions and preventive strategies directed to modulate the human microbiome.

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