Published in final edited form as:

Calcif Tissue Int. 2018 May ; 102(5): 512–521. doi:10.1007/s00223-017-0321-0.

FROM OSTEOIMMUNOLOGY TO OSTEOMICROBIOLOGY: HOW THE MICROBIOTA AND THE IMMUNE SYSTEM REGULATE BONE

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Abstract

Osteomicrobiology refers to the role of microbiota in bone health and the mechanisms by which the microbiota regulates post-natal skeletal development, bone aging and pathologic bone loss. Here, we review recent reports linking gut microbiota to changes in bone phenotype. A pro-inflammatory cytokine milieu drives bone resorption in conditions such as sex-steroid hormone deficiency. The response of the immune system to activation by the microbiome results in increased circulating osteoclastogenic cytokines in a T-cell dependent mechanism. Additionally, gut microbiota affect bone homeostasis through nutrient absorption, mediation of the IGF-1 pathway, and short chain fatty acid and metabolic products. Manipulation of microbiota through prebiotics or probiotics reduces inflammatory cytokine production, leading to changes in bone density. One mechanism of probiotic action is through upregulating tight junction proteins, increasing the strength of the gut epithelial layer and leading to less antigen presentation and less activation of intestinal immune cells. Thus, prebiotics or probiotics may represent a future therapeutic avenue for ameliorating the risk of post-menopausal bone loss in humans.

Keywords

Estrogen; Sex steroids; microbiota; Intestine; bone loss; probiotics; LGG; VSL#3™

Introduction

The gut microbiota consists of trillions of microbial organisms including bacteria, viruses, and fungi that live in close contact with human surfaces. The largest microbiota is the intestinal microbiota. These microbes live in proximity with the intestinal wall, a boundary between the self and the foreign of the host. Symbiotic gut microbes increase nutrient absorption from food, and make it difficult for pathogenic bacteria to colonize the gut. In recent years, especial attention has been made to the role that the gut microbiota, especially bacteria, play in interfacing with human pathophysiology. The sheer quantity of bacteria and

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The authors have declared that no conflict of interest exists.

their genetic material – it is estimated there may be 100 times as many bacterial genes as there are human ones – not to mention the difficulty in isolating and culturing many of these species, has made the study of microbiota formidable [1–3]. Inter-individual variation and even intra-individual variation add to the complexity of study. With the advent of more rapid and cheaper sequencing technologies, the diversity of bacterial composition within and between human hosts has come to light. The importance of microbiota has become definite; many would even call microbiota our largest organ. Specific strains have been found to have effects on immune cells [4], and new insights have been made on overall microbiota effects on systemic immune responses. Gut microbes have now been shown to modulate local intestinal as well as systemic immune responses, with oftentimes surprising associations with distant and diverse organ systems and disease processes such as hematopoiesis [5], brain microglia [6], renal ischemia [7], periodontal disease [8], and graft versus host disease [9]. In this review, we will focus on recent discoveries of how these bacteria interact with the immune system to regulate bone in health and in disease.

The recognition that both the immune system and the microbiota are critical for bone homeostasis signifies the evolution from the field of osteoimmunology to that of “Osteomicrobiology”, a term introduced by Ohlsson et al [10], to refer to investigations on the role of microbiota in bone health and the mechanisms by which the microbiota regulates postnatal skeletal development, bone aging and pathologic bone loss.

Bone as an Immunological Organ

Bone undergoes continual turnover, with a balance in formation and resorption crucial to bone health [11]. The importance of the immune system in regulating bone remodeling was recognized three decades ago [12]. A strong association between inflammatory conditions and bone loss has long been established clinically [13, 14]. The most common cause of osteoporosis is due to estrogen deficiency [15]. Postmenopausal bone loss results from a decrease in estrogen levels, leading to increases in bone resorption which is, in part, countered by increases in bone formation. In mice, menopause is modeled by surgical removal of the ovaries. The mechanism by which sex steroid deficiency leads to bone loss is primarily increased osteoclast formation, activity and lifespan driven by increased production of the inflammatory cytokines IL-1, IL-17, TNF α (TNF) and RANKL in the bone marrow (BM) [16–19]. A role for IL-1 and TNF in humans is supported by reports that menopause increases the levels of these factors [20–24], while treatment with inhibitors of IL-1 and TNF prevents the increase in bone resorption induced by estrogen deficiency [25]. The causal role of TNF in ovx-induced bone loss in mice has been demonstrated in a variety of experimental models [26–28]. Key mechanisms by which TNF stimulates bone resorption include potentiation of the activity of RANKL [29, 30] and induction of Th17 cells [31–33]. Osteoblastic cells are one major source of RANKL. Osteocytes are probably the most relevant source of RANKL in the mouse [34, 35].

Significant experimental evidence supports the notion that T cells are a pivotal source of TNF in ovx mice and postmenopausal women [36, 37]. Evidence backing a mechanistic role for T cells include the fact that ovx is unable to induce bone loss in T cell deficient nude mice [26, 29, 38, 39], in wild-type (WT) mice depleted of T cells [40], in mice treated with

the costimulation inhibitor CTLA4-Ig [41], and in mice lacking the costimulatory molecule CD40L [40]. The relevance of T cells for ovariectomy induced bone loss has been confirmed by several laboratories [42, 43]. Evidence for a role of T cell produced TNF in ovx induced bone loss has been provided by studies with mice lacking the capacity to produce TNF or lacking the expression of TNF receptors [26].

One T cell lineage pivotal for the bone loss of estrogen deficiency is Th17 cells. Th17 cells are a subset of CD4⁺ cells which produce IL-17 and exert pro-osteoclastogenic effects. In women, there is an association with increased levels of serum IL-17 and osteoporosis [44–46]. Ovx drives differentiation of naïve CD4⁺ helper cells into mature Th17 cells [47], a process which is inhibited by estrogen binding to ER α and stimulated by cytokines such as TGF β , IL-1 β and TNF [32, 33, 48, 49]. In addition, ovx leads to increases in conventional CD4⁺ and CD8⁺ T cells, which produce TNF and RANKL. Silencing of IL-17R [50] or use of anti-IL17 antibodies [51] result in protection against bone loss.

Another T cell lineage relevant for bone is regulatory T cells (Tregs), a suppressive population of CD4⁺ T cells defined by the expression of the transcription factor FoxP3 and the ability to block conventional T cell proliferation and production of effector cytokines [52]. Defects in Treg numbers and/or activity have been implicated in several chronic inflammatory diseases. Tregs regulate OC formation [53–56] and blunt bone resorption [54, 57] through the secretion of IL-4, IL-10 and TGF β 1 [56]. Importantly, estrogen is known to directly increase the relative number of Tregs [58] while Tregs prevent ovx-induced bone loss [59].

The Microbiota Affects Bone Health

The microbiota regulates metabolism [60, 61] and affects the production of hormones critical for bone health including sex-steroids [62], vitamin D [63] and serotonin [64]. More importantly, the gut microbiota is critical for the development and the function of the immune system. A link between microbiota and bone was first recognized in 2012 [65]. In their seminal paper K. Sjogren et al demonstrated an increase in trabecular bone mass in mice raised in germ-free conditions, as compared to controls raised in conventional conditions. As this phenotype was reversed by colonization with gut flora from conventionally raised mice, the evidence was convincing that the results were not due to innate abnormalities of germ-free mice. Additional findings of this initial investigation were a lower number of CD4⁺ T cells in the bone marrow and lower levels of bone marrow TNF in germ-free mice. This corresponded with fewer osteoclast precursors and a higher bone mass. In keeping with the study of K. Sjogren et al, we reported that 20-week-old female germ-free C57Bl/6 mice have higher trabecular bone volume than congenic age matched mice raised in conventional conditions [66]. These initial observations have been recently confirmed in a study demonstrating that commensal gut microbiota stimulates bone resorption and inhibits bone formation, thus lowering bone mass [67].

Mechanistic studies have disclosed that the microbiota regulates bone formation by altering the production of insulin-like growth factor 1 (IGF-1), an important regulator of bone remodeling. The exact role of IGF-1 remains controversial because in one study mice raised

in conventional conditions were found to have lower bone formation and lower serum and bone marrow levels of IGF-1 compared to GF mice [67]. By contrast, in a second investigation colonization of GF mice with physiologic microbiota increased liver-derived IGF-1 [61, 64]. In the short-term IGF-1 stimulated bone resorption causing bone loss [64]. However, over a longer period of time (8 months) IGF-1 stimulated bone formation leading to a net gain in bone mass [64]. Investigations have also shown that commensal microbiota induces sustained changes to in RANKL mediated osteoclastogenesis [67].

Recent work has also disclosed that regulation of bone mass by the microbiota is dependent on NOD1 and NOD2 signaling [68]. That study has indeed disclosed that cortical bone thickness in mice lacking Nod1 or Nod2 was not increased under germ-free conditions as compared to conventionally raised controls. Moreover, the expression of TNF and RANKL, which is below normal in germ-free mice, is within normal limits in Nod1^{-/-} or Nod2^{-/-} GF mice, indicating that the effect of the microbiota is dependent on both NOD1 and NOD2 signaling [68].

It is now clear that various factors may affect the bone density of germ-free mice. One important factor is genetic strain. Another is gender. While female germ-free C57Bl/6 mice have higher bone mass than controls, in male BALB/c mice the effect of the microbiota on bone mass is opposite [61]. Adding further complexity to this issue, bacterial colonization of adult CB6F1 mice was found to stimulate bone resorption and decrease bone mass in the first 4 weeks after colonization [69]. However, long-term colonization leads to an equalization of bone mass, indicating that duration of colonization is another critical variable that influences bone turnover and bone mass [69]. To determine whether endogenous microbiota contribute to the regulation of bone remodeling at baseline, the same investigators administered antibiotics to conventionally raised mice. The consequent elimination of the microbiota caused an increase in bone mass and a lowering of the rate of bone turnover [69]. These findings indicate that in a short-term setting the ablation of the microbiota increases bone mass, whereas reconstitution of the microbiota in former germ-free mice causes bone waste.

Adding further complexity, the results of these investigations are not in line with a series of studies conducted to analyze the effect of malnourishment on body growth and skeletal development. Stool samples from children from Malawi with severe malnutrition contained an immature microbiome. Colonization of germ-free mice with stool samples from malnourished children resulted in stunted body growth and shorter bones, whereas germ-free mice on the same diet given “mature” microbiomes of healthy children underwent normal body growth [70]. Interestingly, colonization of germ-free mice with stools from malnourished children caused an increase in cortical bone density [70]. Germ-free mice colonized with stools from healthy 6 months old children acquired a higher bone density than those colonized with stools from healthy 18 months old children [70], further suggesting that immature microbiota induces bone anabolism.

In another investigation, it was found that the reduced skeletal development of germ-free mice was partially reversed by colonization with selected lactobacilli [61]. Moreover, sialylated human milk oligosaccharides (HMOs) are significantly less abundant in the milk

of mothers with severely malnourished infants [71]. HMOs are not significant nutrients for humans, but gut bacteria utilize HMOs as an energy source. Colonization of germ-free mice with stools from 6-month-old stunted Malawian infants resulted in blunted body growth. Feeding the colonized mice with HMOs produced a microbiota-related increase in body mass, and altered muscle and liver, metabolism according to a pattern suggestive of a greater capacity to exploit nutrients [71]. Among the changes induced by HMOs supplementation was an increase in bone volume [71], indicating that positive modification of the microbiota induced by a prebiotic substance stimulates bone growth. Together, these studies demonstrate that changes in the microbiota regulate independently bone growth and bone mass acquisition.

The notion that the gut microbiota plays a critical role in bone growth is strengthened by antibiotic studies in mice. Short-term administration of low doses of antibiotics at weaning resulted in elevated levels of bone mass [60, 72]. Treatment with antibiotics that specifically decrease intestinal bacteria also increases bone mass [69]. In addition, administration of a cocktail of tetracyclines were found to prevent ovariectomy induced bone loss [73]. Some antibiotic studies revealed unexpected differences on the bone response to microbiota reduction in male and female mice [72]. Moreover, an increase in bone density in response to antibiotics was more consistently observed in young animals [60, 74]. These studies clearly demonstrate that antibiotic treatment influences bone mass. Because antibiotics lower the number of bacteria, and/or alter the diversity of microbial taxa within the intestinal lumen, it can be inferred that the bacterial load and diversity within the gut are significant contributing variables to the mechanisms by which the gut microbiota regulates bone mass.

A great deal of differences exists in the microbiome of mice housed in different facilities and/or fed different types of chow [75–77]. Therefore, the diversity of the microbiome in the particular facility is a potential confounding factor to data interpretation when assessing the influences of the microbiome on bone density. For example, at sacrifice the trabecular bone volume of the conventionally raised mice used in our studies was less than 50% of that of the BALB/c male mice used by Schwarzer et al [61]. Further attesting to the relevance of local housing conditions, we found C57Bl/6 mice from Jackson Laboratory have a higher bone volume than syngeneic mice bought from Taconic Biosciences. However, following 4 weeks of co-housing in our animal facility, the bone volume of mice from Jackson Laboratory decreased to the level of that of Taconic Biosciences mice. Since mice are coprophagic, and transfer their microbiomes from mouse to mouse by this behavior, the bone density decrease observed in mice from Jackson Laboratory is perhaps due to colonization of Jackson Laboratory mice with fecal material from Taconic Biosciences mice. These observations clearly point to the critical need to account for reciprocal host-microbiome interactions in experimental approaches that investigate the microbiome and bone density.

Microbiota and the bone loss induced by sex steroid deficiency

Postmenopausal osteoporosis is the most common form of bone wasting disorder [15]. Fractures due to osteoporosis are a frequent cause of disability, particularly in the elderly. Declining estrogen levels at menopause result in a potent stimulation of bone resorption and,

to a lesser extent, bone formation leading to a period of rapid bone loss [11]. This initial phase is followed by a slower but more prolonged period of bone loss that affects mostly the cortical compartment of the skeleton. Osteoporosis due to androgen deficiency is also relatively common in men [78]. However, androgens regulate bone homeostasis in men mostly via their peripheral conversion to estrogen [79] and estrogen deficiency has a dramatic effect on bone homeostasis in men [80, 81].

The role of the microbiome in the bone loss induced by sex steroid deficiency was investigated in a study employing mice raised in germ-free conditions. Mice raised in conventional conditions and germ-free mice colonized with commensal gut microbiota, were used as control groups. Sex steroid deficiency was induced pharmacologically using the GnRH agonist Leuprolide. This investigation revealed that germ-free mice do not develop the loss of trabecular bone typically induced by sex steroid withdrawal [66]. Colonization of germ-free mice with the microbiota of conventionally raised animals made the skeleton of these animals as sensitive to sex steroid deprivation as the skeleton of conventionally raised mice, demonstrating that the protection of germ-free mice against bone loss is not due to intrinsic, irreversible immune abnormalities conferred by the germ-free status. Interestingly, Leuprolide treatment caused equal cortical bone loss in germ-free mice and control mice, indicating that cortical bone loss occurs via a microbiota-independent mechanism [66]. Leuprolide increased bone resorption in conventional and colonized mice, but not in germ-free mice. In addition, germ-free sex steroid depleted mice had lower rates of compensatory bone formation than conventional sex steroid depleted mice [66].

Assessment of cytokine production revealed that sex steroid deficiency expands TNF and IL-17 producing T cells, and increases the levels of TNF, RANKL and IL-17 protein in the bone marrow and in the small intestine of conventionally raised mice. By contrast, in germ-free mice, no increase in these cytokine levels occurred in the bone marrow, nor in tissues of the small intestine following sex steroid deficiency [66]. Overall, the data showed that the microbiota is required for sex steroid withdrawal to induce bone loss. Moreover, the data indicated that this may occur due to the gut microbiome driving the expansion of intestinal T cells and Th17 cells, which produce TNF, RANKL and IL-17.

Still unclear is whether bone loss is induced by cytokines that travel to the bone marrow but are produced by gut immune cells, or bone loss is induced by immune cells activated in the gut that home to the bone marrow. A third possibility is that foreign antigens of intestinal origin reach the bone marrow causing an immune response. Bacterial translocation, the passage of viable bacteria across the intestinal wall into the systemic circulation, is a recognized phenomenon, although very rare in healthy individuals [82]. Translocation of gut microbiota derived ligands into the circulation occurs physiologically [83], and this may be sufficient to activate bone marrow T cells. Based on preliminary cell migration studies, it appears likely that immune cells, first activated in the intestine, home to the bone marrow where they produce osteoclastogenic cytokines.

The intestinal wall is the border between the mammalian host and the luminal microbiota. The intestinal wall is in contact with the gut microbiota and constantly samples and respond to the antigenic load within the intestinal lumen [84]. Gut permeability, the passage of small

molecules between the gut lumen and submucosa, is essential for health. Increased permeability allows larger molecules and potential antigenic load to enter the gut wall and initiate intestinal inflammatory responses [85–88]. Maintaining tight gut permeability is critical in the context bone formation because osteoclastogenic cytokines are produced by immune cells that reside in intestinal sub-epithelial compartments of the intestine, and any change in gut permeability is thus likely to elevate levels of osteoclastogenic cytokines and influence bone density.

This is relevant because estrogen withdrawal increases gut permeability, leading to increased bacterial translocation and increased levels of inflammatory markers [66, 89]. Specifically, sex-steroid deficiency resulted in reduced transcript levels of a number of the gap junction proteins of the Claudin family that regulate gut permeability [90, 91]. Thus, increased gut permeability after sex-steroid depletion results in a higher antigenic load entering the epithelial submucosa, thereby initiating the production of inflammatory cytokines. Therefore, any therapeutic approach that can tighten gut permeability would offer a promising therapy for preventing sex steroid depletion-induced bone loss.

Manipulation of Microbiota Changes Bone Remodeling

Since bone health is affected by the lack or pathologic alteration of the intestinal microbiota, it is logical to assume that strategies may be developed to alter the microbiota in a way to induce beneficial skeletal effects. One such strategy is nutritional supplementation with probiotics. Probiotics are defined as live microorganisms that provide certain health benefits to the host, when administered in appropriate amounts [92]. Earlier work had already revealed positive effects of probiotics on bone mass. For example, a 2004 study revealed that rats receiving *Lactobacillus helveticus* – fermented milk developed a higher bone density as compared to controls [93]. However, the idea that microbiota manipulation directly impacted the inflammatory response and bone phenotype was not widely accepted until several groups in Europe and in the United States independently demonstrated that probiotics could prevent the loss of bone mass induced by ovariectomy by reducing inflammatory cytokine production [94, 95]. However, the effects of probiotics in bone were found to be influenced by many factors. For example, in one study probiotics appeared to decrease intestinal inflammation and increase bone density in eugonadic male but not female mice [96]. In female mice, probiotics improved bone density under inflammatory stress but not in normal conditions [95]. In fact, treatment with *Lactobacillus reuteri* increased bone density in mice subjected to a dorsal surgical incision mimicking the incision carried out to perform ovx, whereas it had no effect in non-operated mice [97]. This suggests that the presence of an inflammatory state is required for probiotic to increase bone density. However, caution must be taken when generalizing these studies, as the specific strain of probiotic, the species of the animal model, and even factors such as the location of the laboratory appear to complicate phenotypic findings.

In further exploring the mechanism of probiotic effect, Li et al demonstrated that probiotics interact with the epithelial layer of the small intestine, and change regulation of tight junction proteins that form the gut epithelial layer [66]. Through treatment with either *Lactobacillus rhamnosus* GG (LGG) or a commercially available mix, VSL#3, which

contains eight different strains, probiotics prevented ovx induced bone loss, and increased bone mass in mice without ovx (figure 1). These effects correlated with a decrease in gut permeability and intestinal and systemic inflammation. The intestinal epithelium uses tight and gap junction proteins of the claudins and Jam families to maintain a physiological barrier between the gut lumen and submucosa. Estrogen deficiency leads to decreased expression of junction proteins causing increased gut permeability and increased levels of inflammation. Probiotics act, in part, by strengthening the gut barrier (figure 2). A stronger intestinal barrier leads to less antigen presentation and less activation of intestinal immune cells. Further studies are needed to elucidate this mechanism of action of probiotics in estrogen deficient and estrogen replete animals and humans, but it appears that the link between gut inflammation and production of osteoclastogenic cytokines could be ripe for therapeutic targeting with probiotics.

While numerous animal studies have documented the positive skeletal effects of probiotics, little information is available in humans. Recently, a short-term prospective double-blind study has been conducted in postmenopausal women [98]. In this study 50 women were treated with placebo or the multispecies probiotic supplement GeriLact for 6 months. No changes in bone density were detected probably because of the short duration of the study. However, patients treated with probiotics had a reduction in indices of bone turnover, and serum levels of TNF and PTH [98].

The microbiota can also be manipulated via nutritional supplementation with prebiotics. Prebiotics are non-digestible fermentable food ingredients that promote the growth of beneficial microbes and/or promote beneficial changes in the activity of the microbiome [99]. Prebiotics are contained in food items such as dandelion greens, garlic, leek, banana, onion, artichoke and chicory [100]. In many cases, a significant amount of the food is needed to get enough prebiotic for activity, therefore prebiotics, such as inulin, have been developed into capsule, or shake forms [100]. Prebiotics include non-digestible oligosaccharides such as polydextrose, fructo-oligosaccharides (FOS), inulin, xylo-oligosaccharides, galacto-oligosaccharides (GOS) and soybean oligosaccharides. The mechanism of action of prebiotics is not clear but these substances are known to increase calcium absorption in healthy animals [101, 102], healthy humans [103, 104], ovx animals [105] and postmenopausal women [106]. In experimental animals FOS and inulin increase trabecular and cortical bone volume in intact rodents [107–109], and prevent ovx-induced bone loss in mice [110]. Studies in humans have revealed that prebiotics increase whole body bone mineral density in adolescent girls [104] and decrease bone loss in postmenopausal women [111]. Prebiotics act by regulating both bone formation and bone resorption. The mechanism of action of prebiotics in bone remains unknown. However, it is likely that metabolic and immunological pathway may be involved [112]. First, dietary fiber is fermented to short chain fatty acids (SCFA) in the lower gut by resident microbiota. SCFAs lower intestinal pH, which causes an increase in calcium absorption [112]. Moreover, SCFAs serve as energy sources for gut epithelial cells, and this may lead to improved gut health and gut barrier function [113].

Conclusions

In the last five years, it has become clear that the intestinal microbiota is relevant for bone health. Most critical data have been obtained in experimental animals and confirmation in humans is needed. As of yet, there is no FDA-approved treatment for osteoporosis based on manipulation of microbiota. However, advances in other areas suggest that manipulations of the microbiota may be used in the future to treat common skeletal disease. Such interventions might include simple dietary modifications that affect the gut microbiota. For example, a low-glycemic diet was found to alter microbiota composition in a way that offered protection against age-related macular degeneration in the mouse, leading to the creation of the term “gut-retina axis” [114]. Perhaps dietary modifications that alter the composition of the microbiota will be found to be useful to prevent bone loss. Prebiotics, and probiotics, are already widely in use for a variety of conditions. If clinical trials currently in progress establish their efficacy, prebiotics and probiotics might become an inexpensive means to prevent bone loss in humans.

Fecal microbial transplant (FMT), a procedure in which stool, and its associated microbiota, are taken from a healthy donor and placed into a recipient, has already become an accepted treatment for *Clostridium difficile* infections in humans. Research highlights putative roles of similar human microbiota manipulations in atherosclerosis, obesity, and oncologic disorders [115]. Bone diseases might become another application for simplified stool transplant procedures. Speculating on the growth of personalized medicine, perhaps in the future, patients with severe osteoporosis would have their microbiota analyzed, and means to shift the microbiota to a more bone-protective composition would be attempted through diet, prebiotics, probiotics, or fecal transplant.

Nonstandard abbreviations used

BM	Bone marrow
GF	Germ-Free
Conv.R	conventionally raised
TNF	Tumor necrosis factor α

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Figure 1. Probiotic supplementation prevents sex-steroid induced bone loss

Conventional ovariectomized (ovx) and sham operated mice were supplemented twice a week with either VSL#3™, *Lactobacillus rhamnosus* GG (LGG), or vehicle. The figure shows in vivo prospective measurements of spinal bone volume (BV/TV), as measured by micro-computed tomography (μCT) scanning at baseline and 2 and 4 weeks after surgery. n = 10 to 14 mice per group. Data are expressed as Mean + SEM. * = p < 0.05, and *** = p < 0.001 compared to sham vehicle, a = p < 0.0001 compared to baseline. Figure and legend reproduced with permission from JCI.

Figure 2. Diagrammatic representation of the effects of sex steroids deficiency and probiotics on gut permeability

In physiologic conditions, proteins of the Jam and Claudin families seal the space between intestinal epithelial cells preventing bacteria and bacterial products from penetrating the intestinal wall and activating immune cells. Sex steroid deprivation downregulates the expression of gap junction proteins leading to increased gut permeability. The resulting increased bacterial translocation induces a local and systemic immune response that causes an increased production of osteoclastogenic cytokines. Probiotic supplementation upregulates expression of Claudins and Jams, thus restoring normal gut barrier function. Figure and legend reproduced with permission of Cold Spring Harbor.