Calcified Tissue International (2018) 102:480–488
https://doi.org/10.1007/s00223-018-0403-7

REVIEW

Advances in Probiotic Regulation of Bone and Mineral Metabolism

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Received: 27 December 2017 / Accepted: 8 February 2018 / Published online: 16 February 2018

Abstract
Probiotics have been consumed by humans for thousands of years because they are beneficial for long-term storage of foods and promote the health of their host. Ingested probiotics reside in the gastrointestinal tract where they have many effects including modifying the microbiota composition, intestinal barrier function, and the immune system which result in systemic benefits to the host, including bone health. Probiotics benefit bone growth, density, and structure under conditions of dysbiosis, intestinal permeability, and inflammation (recognized mediators of bone loss and osteoporosis). It is likely that multiple mechanisms are involved in mediating probiotic signals from the gut to the bone. Studies indicate a role for the microbiota (composition and activity), intestinal barrier function, and immune cells in the signaling process. These mechanisms are not mutually exclusive, but rather, may synergize to provide benefits to the skeletal system of the host and serve as a starting point for investigation. Given that probiotics hold great promise for supporting bone health and are generally regarded as safe, future studies identifying mechanisms are warranted.

Keywords Probiotic · Microbiota · Osteoporosis · Lactobacillus · Intestine · Bone · Inflammation · Barrier · Permeability · Colon

History of Probiotics

Probiotics have been consumed by humans dating back to before 7000 BCE [1]. Probiotics are contained in many fermented products made to undergo long-term storage, such as beer, bread, wine, kefir, kumis, and cheese [2–4]. For example, probiotic *lactobacillus* strains are found in the brine of fermented olives, pickled juices, kefir, and yogurt [5]. Probiotics are also found in non-fermented foods such as meat and fruits as well as in the gastrointestinal tract of animals such as pigs, rats, and poultry [5]. Probiotic intake has long been associated with a longer, healthier life [1, 2, 6]. In the late 1800s, it was hypothesized that *lactobacillus* strains produce factors that benefit gastrointestinal metabolism, the intestinal epithelium, and health [1–3, 6, 7]. It was also thought that ingestion of beneficial bacteria could help treat disease through the restoration of the microbiota composition. The concept that beneficial microbes mediate health continues to be supported today. In fact, probiotics are defined by the Food and Agricultural Organization/World Health Organization (FAO/WHO) as "live microorganisms that when administered in adequate amounts will confer a health benefit on the host." This definition is broad without distinction between organic/inorganic, single versus multiple mediators, effects on bacterial growth, or increased resistance to infection. At the same time the definition imposes some core requirements [6, 8].

The nomenclature for bacteria, and thus probiotics, is based on a taxonomic/genetic hierarchy where bacteria are divided into phylum, class, order, family, genus, species, and subspecies and/or strain. With more than 23 bacteria phyla, it is easy to see the abundance of specific probiotics and the complexity of their names. Evidence indicate that probiotic effects can be strain-specific. In addition, not all bacteria within a species act the same and/or can be regarded as a probiotic. Lactic acid-producing bacteria, which include lactobacilli strains, are one of the most well-known groups

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of bacteria/probiotics with health benefits that have been used for centuries. They are gram-positive, obtained from fermented milk products and are usually found in carbohydrate-rich environments and normal intestinal flora of many animals [3]. The genus *Lactobacillus* belongs to the phylum *Firmicutes*, class *Bacilli*, order *Lactobacillales*, family *Lactobacillaceae*. The most commonly isolated species are *Lactobacillus acidophilus, L. salivarius, L. casei, L. plantarum, L. fermentum, L. reuteri, L. rhamnosus, L. gasseri*, and *L. brevis* from human intestine. Several of these, *Lactobacillus acidophilus, Lactobacillus rhamnosus, Lactobacillus casei*, and *Lactobacillus reuteri*, have been extensively studied and well documented. *Lactobacillus acidophilus* can colonize the human colon, has antimicrobial effects, and is used to treat intestinal infections [9]. *Lactobacillus rhamnosus GG* or *Lactobacillus GG* (LGG) tolerates low pH environment, adheres to the gastrointestinal tract, and is effective in treating diarrhea [2, 10].

Role of Probiotics in Bone Health

Bone health depends on the balance of bone resorption by osteoclasts and bone formation by osteoblasts. Hormones, immune cells, and the gastrointestinal system can regulate these processes [11–13]. While the intestine is known for its key role in calcium, phosphorous, and magnesium absorption (contributors to bone mineralization) [14], the intestine also produces endocrine factors that signal to bone cells, such as incretins [15] and serotonin [16]. Recent studies, including some from our lab, indicate that the intestinal microbiota and physiology can also regulate bone health [17–22]. Thus, probiotics which modify the microbiota composition and/or function and promote intestinal health can also benefit bone health. This is supported by numerous studies in a variety of animal models (Table 1). Several recent clinical studies confirm the benefits of probiotics to human skeletal health [23–29]. The mechanisms accounting for probiotic benefits on bone are not fully known, but likely involve (1) changing the intestinal microbiome, (2) modifying intestinal barrier function, and (3) impacting the immune system/immune cells (Figs. 1, 2). We discuss these possible mechanisms below.

Probiotics Modify the Microbiome to Regulate Bone Health

The human body is the host for ~ 100 trillion microbes comprising ~ 1000 species and 28 different phyla [30]. In addition to microbes outnumbering human cells, the gut microbiota also express 100-fold more genes compared to human genome [30]. Changes in microbiota composition can have beneficial or harmful consequences on human health [31]. Therefore, the microbiome–host relationship is an important target for therapeutic purposes, and probiotics may be a key therapy based on their ability to influence the intestine.

Ingested probiotics enter and populate the GI tract. Depending upon the diet, the dose of naturally ingested probiotic bacteria can range from 10^8 to 10^{12} CFU per day [32]. While bacteria are robust and hearty, the acid environment of the stomach and bile and enzyme secretion in the intestine can decrease the number of viable probiotics in the gut by 50% or greater [33]. The abundance of ingested bacteria relative to residential bacteria is much greater in the small intestine where the ratio can be 1:1, whereas in the colon the ingested probiotics are greatly outnumbered by the existing resident bacteria and may comprise only 0.0001-fold of the total bacteria [32, 34]. Ingested probiotics are maintained in the gastrointestinal tract for a few days after ingestion but are rarely maintained after 1 week [32, 35, 36]. Thus, probiotics likely need to be continuously ingested to obtain maximum benefits [37].

Probiotics can modify the microbiota composition simply by the increased presence and proliferation of the probiotic in the gastrointestinal tract, as well as through their ability to compete with other bacterial strains. Probiotics can digest complex carbohydrates and generate oligosaccharides that can be further metabolized by other bacteria that subsequently proliferate and modify the microbiota composition. Probiotic bacteria also secrete antimicrobial agents that can kill certain bacterial strains in the gastrointestinal tract. For example, *Lactobacilli* strains produce several lactic acid, bacteriocins (antimicrobial peptides), and hydrogen peroxide [38].

Changes in the composition of the microbiota affect bone parameters under a variety of conditions [39–41]. A clear link has been demonstrated between microbiota composition and bone growth. For example, the microbiota of undernourished children with stunted growth differs dramatically from healthy children [42] and when transferred to germ-free mice (mice lacking a microbiome) cause poor growth [42]. More importantly, supplementation with two bacterial strains (*Ruminococcus gnavus* and *Clostridium symbiosum*) ameliorated growth abnormalities in the mice receiving the microbiota from undernourished children [42]. Similarly, treatment with the probiotic *Lactobacillus plantarum* restored normal growth rates in undernourished mice [43]. The *L. plantarum* treatment raised serum IGF-1 and IGFBP-3 to normal levels [43] consistent with reports demonstrating the microbiota regulates the IGF-1–IGF-1R axis [44]. This is a general phenomenon since germ-free drosophila repopulated with *lactobacilli* strains regain a normal IGF axis and grow at normal rates [45, 46]. In humans, the probiotic *Bifidobacterium lactis* benefited the growth of infants born to mothers with human immunodeficiency virus [47]. Taken together, these studies demonstrate

Table 1 Studies demonstraing probiotic benefits to skeletal health

Strain	Species (and model)	Sex	Treatment time	Analysis method	Bone effects	Reference
Lactobacillus reuteri (*ATCC 6475*)	Mice	Male	4 weeks	µCT	↑ BV/TV ↑ Tb.N. ↑ Tb.Th. ↑ Osteocalcin ↑ BFR	[22]
Lactobacillus reuteri (*ATCC 6475*)	Mice(OVX)	Female	4 weeks	µCT	↑ BV/TV ↓ RANKL mRNA ↓ TRAP5 mRNA	[19]
Lactobacillus reuteri (*ATCC 6475*)	Mice (Dorsal Surgery)	Female	8 weeks	µCT	↑ BV/TV ↑ MAR ↓ RANKL mRNA ↑ OPG mRNA ↑ IL-10 mRNA	[20]
Lactobacillus reuteri (*ATCC 6475*)	Mice (STZ-induced Type 1 Diabetes)	Male	4 weeks	µCT	↑ BV/TV ↑ MAR ↑ Osteocalcin ↑ Wnt10b mRNA	[21]
Lactobacillus rhamnosus (*HN001*)	Mice (OVX)	Female	4 weeks	µCT	↑ BV/TV ↑ Osteocalcin ↓ RANKL mRNA ↓ TNFα mRNA ↓ IL-17 mRNA	[50]
L. paracasei (*NTU 101*) *or* *L. plantarum* (*NTU 102*)*-fermented soy milk*	Mice (OVX)	Female	8 weeks	µCT SEM	↑ BV/TV ↑ Tb.N.	[100]
L. paracasei or *L. paracasei* and *L. plantarum*	Mice (OVX)	Female	6 weeks	µCT	↑ Cortical BMC ↑ Cortical area ↑ OPG mRNA	[17]
L. Casei	Mice	Unknown	10 weeks	µCT	Prevents wear debris-induced osteolysis	[98]
L. rhamnosus GG	Mice (periodontitis)	Female	6 weeks	µCT	↓ Bone loss ↓ Inflammation ↓ Osteoclasts	[52]
L. gasseri SBT2055	Mice (periodontitis)	Female	5 weeks	Histomorphometry	↓ Bone loss ↓ Inflammation	[54]
L. brevis CD2	Mice (periodontitis)	Male	< 1 week	Histomorphometry	↓ Bone loss ↓ Inflammation	[101]
Bacillus subtilis	Rats (periodontitis)	Male	6 weeks	Histomorphometry	↓ Bone loss ↓ Inflammation	[102]
Bifidobacterium animalis lactis	Rats (periodontitis)	Male	2 weeks	µCT	↓ Bone loss ↓ Inflammation	[103]
Lactobacillus casei, L. reuteri and *L. gasseri*	Rats				↑ BMC ↑ Ca absorption	[104]
Bifidobacterium longum—fermented broccoli	Wistar Rats	Male	12 weeks	Histomorphometry	↓ $TRAP^+$ osteoclasts	[105]
Bifidobacterium longum (*ATCC 15707*)	Wistar Rats	Male	28 days	Texture analyzer Plasma emission spectrophotometry	↑ Tibial Ca, P, and Mg content ↑ Fracture strength	[106]
Lactobacillus rhamnosus (*HN001*)	Sprague-Dawley Rats	Male	3 weeks	DEXA	↑ Ca and Mg retention	[107]
Lactobacillus rhamnosus (*HN001*)	Sprague-Dawley Rats (OVX)	Female	3 months	DEXA	↓ Bone loss	[107]

Table 1 (continued)

Strain	Species (and model)	Sex	Treatment time	Analysis method	Bone effects	Reference
L. casei 393-fermented milk	Sprague-Dawley Rats (OVX)	Female	6 weeks	DEXA Texture Analyzer Plasma emission spectrophotometry	↑ BMD ↑ Bone strength ↑ Ca content ↓ TRAP activity	[108]
L. helveticus-fermented milk	Spontaneously hypertensive rats	Male	14 weeks	DEXA Plasma emission spectrophotometry	↑ BMD ↑ BMC	[109]
L. casei and *L. acidophilus*	Wistar rat (adjuvant-induced arthritis)	Female	12 days	X-ray	↓ Bone loss	[110]
Enterococcus faecium (*with methotrexate*)	Lewis rat (adjuvant-induced arthritis)	Male	50 days	X-ray	↓ Arthritic score	[111]
Bacillus licheniformis and *Bacillus subtilis*	Broiler		6 weeks	Measuring Calipers	↑ Tibia lateral and medial wall thickness	[112]
Brewer's yeast	Broiler		9 weeks	Visual	↓ Tibial dyschondroplasia	[113]
Lactobacillus	Broiler		28 days	Atomic absorption spectrophotometry Phosphomolybdic Acid method	↑ Ca and P retention	[114]

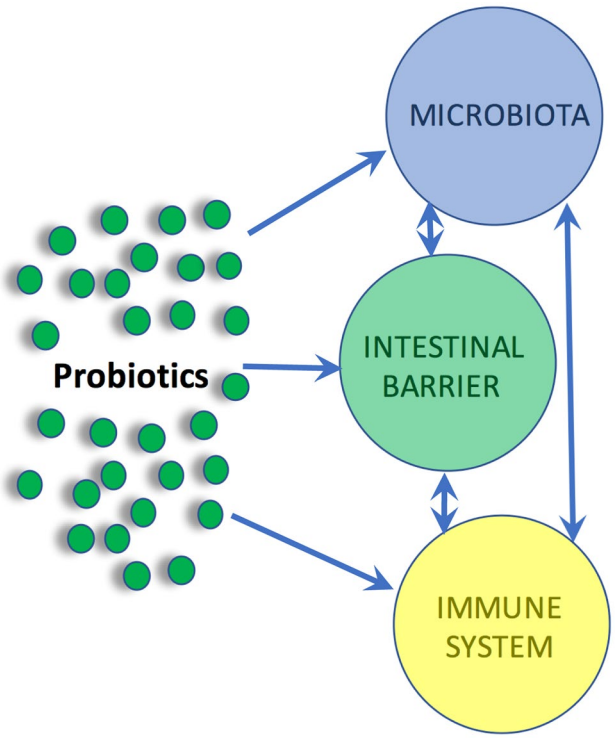

Fig. 1 Probiotic ingestion alters the gastrointestinal microbiota composition, permeability, and local and systemic immune cells. Probiotic-induced changes in microbiota composition can beneficially influence intestinal barrier function and inflammation. Similarly, increasing intestinal barrier function can benefit the immune and possibly microbiota composition. Finally, a strong immune system is likely to benefit intestinal barrier function and microbiota composition

that manipulation of the microbiota with probiotics can benefit bone growth during development.

Microbiota shifts are also associated with changes in bone density. A negative association exists between bone density and intestinal dysbiosis (microbiota imbalance favoring pathogenic over beneficial bacterial strains) observed in inflammatory bowel disease [48]. There are also positive associations, whereby shifts in microbiota composition can enhance bone density. Our lab has shown that treatment of ovariectomized mice with probiotic *Lactobacillus reuteri* causes a significant change in the microbiota composition and prevents estrogen deficiency-induced bone loss [19]. Shifts in microbiota composition induced by prebiotic use also support a role for the microbiota composition in regulating bone health parameters in both mice and humans [49]. In addition, many papers that indicate the probiotic ingestion can modify bone density, though links with specific microbiota components were not determined [17, 21, 22, 50]. In the past year, studies also demonstrate the role of the microbiota in periodontal disease. Diabetes was shown to shift oral microbiota composition that was shown, by transfer to germ-free mice, to be pathogenic and promote periodontal disease and tooth loss [51]. Studies also suggest that probiotics, such as *Lactobacillus rhamnosus GG, Bifidobacterium,* or *Lactobacillus gasseri*, may reduce periodontal disease and bone loss, though more work is needed to determine the specific mechanisms [52–54].

Probiotics Modify Intestinal Barrier Function to Regulate Bone Health

The intestinal epithelium is essential for providing a selective barrier that prevents translocation of harmful

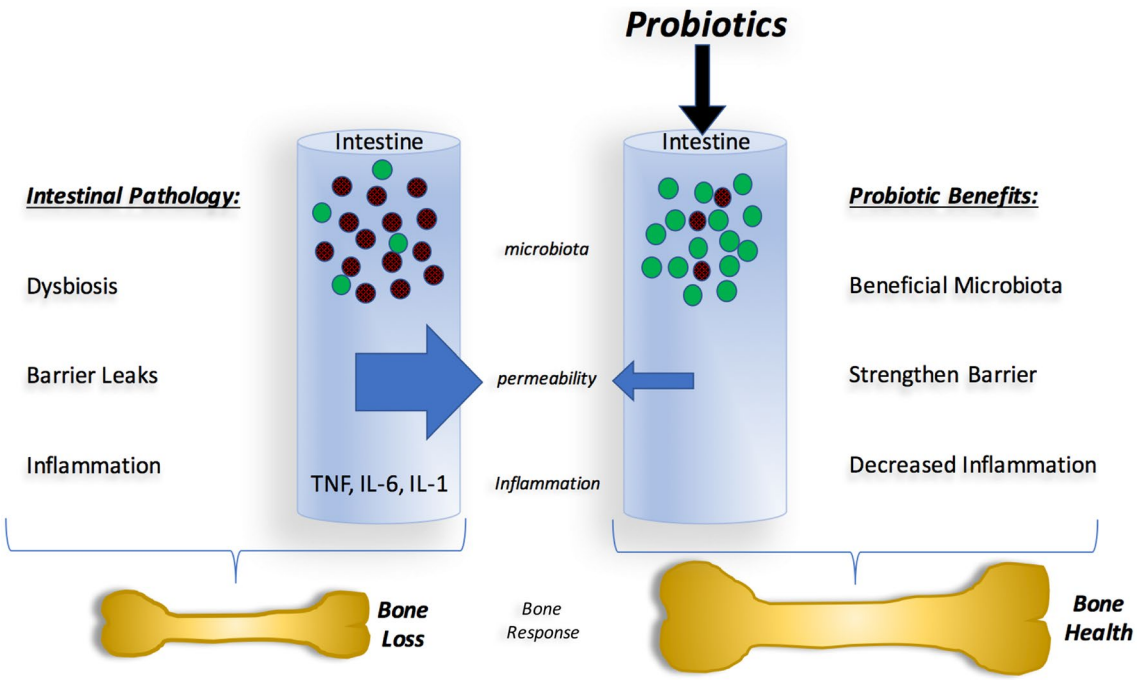

Fig. 2 Probiotic ingestion promotes healthy microbiota composition, barrier integrity, and reduces inflammation, all of which bone health (growth, density, architecture)

substances and pathogens and their products into the blood stream. It is composed of several layers. Importantly, intestinal epithelial cells (IECs) joined together by tight junction proteins provide the key barrier support which allows only selective transport. Additionally, a mucus layer produced by goblet cells covers the surface of the epithelial cells and prevents gut bacteria and pathogen access to host epithelial cells. The intestine also secretes immunoglobulins, defensins, and other antimicrobial products that contribute to maintaining a healthy environment.

Disruption of the epithelial barrier can lead to pathogen translocation into the bloodstream causing systemic inflammation and trigger GI diseases such as inflammatory bowel disease (IBD), celiac disease, and colon cancer [55–57]. Studies from our lab and others demonstrate that GI barrier dysregulation also promotes bone loss [50, 58–61]. Therefore, dysbiosis may promote bone loss by increasing intestinal permeability and serum endotoxin levels [62–64]. The loss of estrogen is known to promote both increased intestinal permeability as well as bone loss [65]. Consistent with this, colonic paracellular permeability decreases during the oestrus phase (high levels of estrogen) of the rat when compared to the dioestrus phase (low levels of estrogen) [65]. Our lab demonstrated increased intestinal permeability as early as 1 week following ovariectomy and estrogen loss in mice [58]. Ex vivo using chambers analyses demonstrated that the ileum had the most dynamic changes and that estrogen deficiency induces region-specific effects on intestinal permeability [58].

Similarly, while probiotic treatment can benefit dysbiosis, probiotics may mediate bone health by enhancing intestinal barrier function [66–70]. In a study causing enteropathogenic *E. coli* (EPEC)-induced dysbiosis, administration of probiotic *E. coli Nissle 1917* increased specific claudin expression and prevented increases in intestinal permeability seen after infection with EPEC [71]. Additional studies also indicate that probiotics can rescue barrier function [72–75]. Consistent with a link between barrier function and bone health, probiotic treatment of estrogen-deficient mice not only prevents intestinal permeability but also prevents bone loss [50]. Probiotic *Lactobacillus rhamnosus* GG (LGG) as well as the probiotic supplement VSL#3 reduced gut permeability, intestinal inflammation, and completely protects against bone loss induced by estrogen deficiency [50]. Together, these data highlight the role that of the gut epithelial barrier and microbiota in bone loss induced by estrogen deficiency.

Role of the Immune System in Mediating Probiotic Effects on Bone

The intestinal immune system, one of the largest in the body, is uniquely positioned to encounter not only dietary components but also gut microbiota and pathogens. The gut immune system must recognize and differentially respond

to pathogens, dietary components, and commensal and beneficial microbes [76, 77]. In addition to immune cells, paneth and goblet cells (specialized epithelial cells) play an important role in this process. Intestinal immune system dysregulation is linked to a number of diseases, including inflammatory bowel disease (IBD) and celiac disease, associated with pathologic expression of cytokines which are also important players in the regulation of bone homeostasis [78, 79]. Thus, it is not surprising that many of these intestinal conditions lead to bone loss [80, 81]. This prompted our lab and others to more closely examine the relationship of the gut and the bone in the context of changes in intestinal inflammation. In early studies, we found that induction of colitis leads to changes in the immune cell composition and cytokine expression within the bone marrow [82]. These changes in the bone marrow can signal to bone cells (stem cells, osteoblasts, and osteoclasts) to significantly alter bone homeostasis. This is indeed the case in animal models of IBD, where dysregulation of cytokines (TNFα, IL1α, IL1β, IL-6) correlates with bone loss [83–87]. Consistent with animal models, patients with IBD have elevated levels of cytokines [83, 84] and exhibit bone loss. Thus, the role of immune system in modulating the gut–bone axis is becoming increasingly clear.

The intestinal microbiota is an important modulator of the gut immune system. Dysbiosis has been shown to increase osteoclastogenic activity including increased IL-17 producing cells and reduced bone mass [51]. Similarly, oral administration of "bad" bacteria to mice causes dysregulated intestinal inflammation and corresponding bone loss [86]. Thus, changes in intestinal microbiota can influence immune responses and bone health. Using germ-free mice, Sjogren et al. showed that germ-free mice have higher bone mass compared to conventional mice [88]. This was associated with reduced numbers of osteoclasts precursors as well as decreased frequency of $CD4^+$ T cells in the bone marrow. However, the bone density findings in germ-free mice have been variable showing more, less, or no change [43, 89–91]; the differing compositions of the microbiota used to populate the conventionalized mice mostly likely contribute to the differing responses.

Previous studies indicate that probiotics can modulate immune function [92, 93]. Because probiotics are orally ingested, they can readily interact with the gut immune components which consequently influence local and distant organ functions. Identification of the dysregulated inflammatory pathways in dysbiosis-induced bone loss prompted us (and others) to examine the role of immune system in mediating the probiotic benefits on bone health. Consistent with its anti-TNFα activity, *L. reuteri* treatment decreases intestinal TNFα expression in male mice and correspondingly increases bone density [94]. Although healthy female mice did not respond to *L. reuteri* treatment in terms of bone density, induction of mild inflammatory state is sufficient to increase bone density following probiotic administration [95]. These results suggest that *L. reuteri* effects on bone are somehow linked to the inflammatory state of the organism. The precise immunological mechanisms by which *L. reuteri* affects bone density is still under active investigation. Interestingly however, *L. reuteri* reversed ovariectomy-induced bone loss and this was associated with corresponding changes in bone marrow $CD4^+$ T cells [19]. This was associated with anti-osteoclastogenic activity of *L. reuteri*. This activity is suggested to be in *L. reuteri*-conditioned media which inhibit the differentiation of monocytic macrophages to osteoclasts [19]. Earlier studies indicate that, histamine secreted by *L. reuteri*, is involved in suppressing TNFα production from human monocytic cells [96]. Thus, the *L. reuteri* effects on bone may involve anti-inflammatory effects on the gut and potentially direct effects on bone cells. Whether these factors influence gut immune or bone immune components in vivo is still under active investigation.

Similar to our findings with *L. reuteri*, other groups have shown that LGG and VSL#3 administration reduces TNFα, IL-17, and RANKL expression in the gut and bone marrow of ovariectomized mice [89]. Ohlsson et al. [97] demonstrated that suppression of bone marrow T-regulatory cells by ovariectomy is partially reversed by probiotic supplementation. Consistent with regulation of anti-inflammatory T cells, bone TNFα and other pro-inflammatory cytokines were reduced by probiotic supplementation in the ovariectomized mice, and enhanced TGFβ1 expression (associated with increased T-regs) was observed. Wang et al. showed that the probiotic *L. casei* alters macrophage phenotype. Specifically, wear debris generated from hip implantation activates macrophages leading to a pro-inflammatory state and osteolysis [98]; however, *L. casei* treatment inhibited osteolysis and the pro-inflammatory state of the macrophages. Such anti-inflammatory effects of probiotics in periodontal models have also been demonstrated [54]. Other studies have indicated that probiotics can beneficially affect hematopoiesis in the bone marrow further suggesting that orally administered probiotics can significantly affect immune cells in the bone marrow that can consequently affect bone health [99]. Taken together, while these studies suggest that probiotics can significantly influence immune system and bone, further definitive studies are necessary to identify specific mechanisms.

Conclusions

The above studies indicate that probiotics hold great promise for supporting bone health. It is likely that multiple mechanisms are involved in linking probiotic signals from the gut to the bone. Here, we discussed studies that indicate a role

for the microbiota (composition and activity), intestinal barrier function, and immune cells in the signaling process. These mechanisms are not mutually exclusive, but rather, may synergize to provide benefits to the skeletal system of the host and serve as a starting point for investigation. Given that probiotics are generally regarded as safe, future studies identifying mechanisms are warranted.

Acknowledgements The authors would like to acknowledge funding from the National Institute of Health, Grants RO1 DK101050 and RO1 AT007695.

Funding This review was supported by NIH grants (RO1 DK101050 and RO1 AT007695) to LRM and NP.

Conflict of interest LRM and NP have no conflict of interest.

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