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REVIEW

Potential of Kefir-Derived Peptides, Probiotics, and Exopolysaccharides for Osteoporosis Management

Jen-Chieh Lai[1,2] · Gary Ro-Lin Chang[1] · Min-Yu Tu[1,2] · Abdulkadir Cidem[1,3] · I-Chien Chen[1] · Chuan-Mu Chen[1,4,5,6]

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Abstract

Purpose of Review Osteoporosis is a prevalent skeletal disorder in postmenopausal women and older adults. Kefir has gained attention for its potent antioxidative, anti-inflammatory, and immunomodulatory properties. This review consolidates findings on kefir-derived peptides' interventions in osteoporosis models and evaluates the therapeutic potential of kefir components in preventing osteoporosis, thereby enhancing its application in clinical nutrition strategies for osteoporosis management.

Recent Findings Kefir-derived peptides exhibit osteoprotective potential in various animal models of osteoporosis, in which several antioxidative and ACE-inhibitory peptides have been shown to promote osteoblast differentiation and mineralization. In addition, emerging evidence supports the role of kefir-derived probiotics and exopolysaccharides (kefiran) in mitigating bone loss.

Summary Kefir holds significant promise in the management of osteoporosis due to its unique composition of bioactive components promoting bone health. While research is still in its early stages, evidence suggests kefir's potential as a natural approach to osteoporosis prevention and management.

Keywords Osteoporosis · Kefir · Bioactive peptides · Probiotics · Exopolysaccharides

Abbreviations

KPs	Kefir peptides
OVX	Ovariectomized
AKR1A1	Aldo–keto reductase family 1 member A1
ApoE	Apolipoprotein E
F8	Coagulation factor VIII
KO	Knockout
BMMs	Bone marrow macrophages
BMMSCs	Bone marrow mesenchymal stem cells
BMD	Bone mineral density
BV/TV	Bone volume
Tb.N	Trabecular number
Tb.Sp	Trabecular separation
Tb.Th	Trabecular thickness
CPPs	Caseinophosphopeptides
CTX	C-terminal telopeptides of type I collagen
EPS	Exopolysaccharide
LGG	*L. rhamnosus* GG
LPS	Lipopolysaccharide
PTH	Parathyroid hormone
SCFAs	Short-chain fatty acids
Treg	Regulatory T cells

✉ Chuan-Mu Chen
chchen1@dragon.nchu.edu.tw

Jen-Chieh Lai
shoulia2001@yahoo.com.tw

Gary Ro-Lin Chang
gary590422@yahoo.com.tw

Min-Yu Tu
du0807@yahoo.com.tw

Abdulkadir Cidem
cidema.kadir@gmail.com

I-Chien Chen
chien@leon-bio.com.tw

1 Department of Life Sciences, Doctoral Program in Translational Medicine, National Chung Hsing University, Taichung 402, Taiwan

2 Department of Orthopedic Surgery, Taichung Armed Forces General Hospital, Taichung 411, Taiwan

3 Department of Molecular Biology and Genetics, Erzurum Technical University, Erzurum 25250, Turkey

4 The iEGG and Animal Biotechnology Research Center, National Chung Hsing University, Taichung 402, Taiwan

5 Rong Hsing Research Center for Translational Medicine, Taichung Veterans General Hospital, Taichung 407, Taiwan

6 Center for General Educational, National Quemoy University, Kinmen 892, Taiwan

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Introduction

Osteoporosis is a systemic skeletal pathology distinguished by diminished bone mineral density (BMD) and compromised bone microarchitecture, consequently resulting in heightened susceptibility to bone fragility and fracture. In 2022, the global prevalence of osteoporosis was estimated to be 19.7% [1], with postmenopausal osteoporosis constituting the majority of cases linked to physiological changes resulting from menopause and the aging process. Owing to its asymptomatic characteristic prior to the occurrence of a fracture, osteoporosis is frequently characterized as a "silent" epidemic of the twenty-first century. Common medications for osteoporosis may be antiresorptive, anabolic, or have a dual mode of action [2, 3]. Examples of antiresorptive medications such as bisphosphonates, which inhibit the activity of osteoclasts that break down bone, while anabolic agents like teriparatide stimulate new bone growth to enhance overall skeletal strength. In addition to these, other medications such as denosumab and selective estrogen receptor modulators (SERMs) are also utilized in the management of osteoporosis, each offering unique mechanisms to help maintain bone health and prevent fractures. While medication for osteoporosis can be beneficial, many of the commonly prescribed medications, such as bisphosphonates and anabolic agents, come with potential side effects that can sometimes outweigh their benefits. For instance, long-term use of bisphosphonates has been associated with rare but serious complications like osteonecrosis of the jaw and atypical femur fractures [4]. Additionally, anabolic agents like teriparatide (PTH1-34) are often expensive and may not be accessible for all patients. While pharmaceutical medications can have a role in osteoporosis management, natural non-pharmaceutical alternatives have gained attention for their potential to support bone health without the associated risks. Kefir is one of them that has been extensively studied in various pathological backgrounds and recently been reported with promising effects on osteoporosis.

Kefir is an alcoholic and acidic beverage made by the milk fermentation with kefir grains, which comprise a diverse assemblage of bacteria and yeast that contribute to the probiotic characteristics of kefir with anti-inflammatory, antioxidant, anti-hypertensive, antimicrobial, anticancer, lipid-lowering, and immune modulation activities, alongside the advantageous effects on brain, liver, gastrointestinal, and skeletal health in both animal and human subjects [5, 6]. These health-promoting characteristics are attributed to the abundance of probiotics in kefir grains and the wide range of bioactive substances produced during fermentation, such as bioactive peptides, exopolysaccharides, organic acids, vitamins, and minerals that enhance the overall nutritional profile of the beverage. The unique combination of these components not only supports gut health but also plays a significant role in regulating metabolism and fosters a balanced immune response, thereby reducing the risk of various chronic diseases and rendering kefir a valuable nutritious diet. The type of kefir can vary significantly depending on the source of kefir grains and the fermentation process used, leading to different flavors, textures, and probiotic profiles that can cater to diverse dietary preferences and health needs [7].

Over the past decade, our laboratory has concentrated on using animal models and clinical trials to demonstrate the value of kefir in preventing and treating osteoporosis. In this article, we aim to review the related studies and discuss the advantages of milk protein-derived peptides, probiotics, and exopolysaccharides present in kefir or kefir grains on the bone health.

Kefir Peptides for Osteoporosis Prevention and Treatment

Chen et al. [8] were the pioneering investigators to clarify the efficacy of kefir supplementation in the prevention of osteoporosis in ovariectomized (OVX) rats. OVX models are extensively employed to elucidate the pathophysiologic mechanism underlying menopausal osteoporosis and to assess the efficacy of various natural substances and pharmacological agents for its prophylaxis and therapeutic intervention [9]. Without the implementation of suitable therapeutic measures, OVX rats were shown to exhibit marked osteopenia in the proximal tibial and distal femoral metaphysis within 14 days [10, 11], and further demonstrated enhanced bone resorption in the femoral neck and lumbar vertebrae after 30 and 60 days, respectively [12, 13]. Chen et al. [8]. provided evidence that a 12-week regimen of kefir administered at varying dosages (164, 328, and 656 mg per kilogram of body weight per day (mg/kg BW/day)) caused a significant reduction in the systemic bone resorption biomarker C-terminal telopeptides of type I collagen (CTX) and a notable enhancement in femoral microarchitecture by increasing the parameters of BMD, bone volume (BV/TV), trabecular number (Tb.N), and trabecular thickness (Tb.Th) while decreasing trabecular separation (Tb.Sp). It also enhanced the mechanical properties of cortical bone, with effects comparable to alendronate, a first-line antiresorptive bisphosphonate agent for osteoporosis therapy, at the highest dose [8]. While the underlying mechanism was not fully explored, findings from the Caco-2 cell model suggested that kefir may promote intestinal calcium absorption.

A follow-up clinical assessment conducted by Tu et al. [14] delineated the short-term effects of kefir consumption in those diagnosed with osteoporosis. Tu's study revealed that, over a 6-month period, the administration of kefir

(1,600 mg/day) in conjunction with $CaCO_3$ (1,500 mg/day) significantly improved BMD compared to the administration of $CaCO_3$ alone. Furthermore, the concomitant treatment of kefir and $CaCO_3$ resulted in a reduction of serum β-CTX in patients with T-scores > −1 after 3 months, as well as an elevation of serum osteocalcin (OC) after a 6-month duration, alongside an increase in serum parathyroid hormone (PTH). This pilot study illustrated the advantages of kefir intake in reducing bone resorption and increasing bone formation, resulting in improved hip BMD in patients with osteoporosis. Based on previous findings in Caco-2 cells [8], it is reasonable to suggest that the positive impact of kefir on bone is partly connected to better intestinal calcium uptake. It is essential to note that the principal active constituents in the aforementioned two studies are primarily small peptides, referred to as "kefir peptides (KPs)" (KEFPEP®), which are generated through the hydrolysis of milk protein during the fermentation process, wherein the probiotic elements derived from kefir grains are eliminated subsequent to fermentation.

Subsequently, the identical research group assessed the effectiveness of KPs across a diverse array of murine models of osteoporosis [15–18]. These studies were executed through the daily administration of KPs via the oral route at a dosage of 100 mg/kg BW [15] or at concentrations varying from 164 to 654 mg/kg BW [16–18] over a minimum duration of 8 weeks. In OVX mice, $CaCO_3$ alone conferred limited protection against bone loss; however, KPs exhibited $CaCO_3$-independent osteoprotective effects, as well as altering gut microbiota by enriching beneficial bacteria and reducing potential pathogens, thus enhancing bone health [15]. In the context of the aldo–keto reductase family 1 member A1 (*AKR1A1*) knockout (KO) murine model, the condition of osteoporosis was instigated by a deficiency in vitamin C [19, 20], and KPs proficiently ameliorated this condition [16]. The osteoprotective effects of KPs in AKR1A1-KO mice have been ascribed to the attenuation of systemic inflammation and oxidative stress, alongside the restoration of bone remodeling markers, particularly through the modulation of the ratio of receptor activator of nuclear factor kappa-B ligand (RANKL) and osteoprotegerin (OPG), which serves as a critical indicator of bone mass and structural integrity [16]. Moreover, KPs inhibited RANKL-induced osteoclastogenesis in bone marrow macrophages (BMMs) by downregulating NFATc1, c-Fos, c-Src, and cathepsin K expression, as well as attenuating signal transduction via p38, NF-κB, Akt, PLCγ2, and CREB-1 phosphorylation [16]. Conversely, KPs facilitated osteoblastic differentiation and mineralization in bone marrow mesenchymal stem cells (BMMSCs) through the upregulation of osteogenic factors, including RUNX2, BMP-2, NFATc1, collagen I, HO-1, and β-catenin, while simultaneously downregulating the negative regulator p53 [16]. These findings illuminated the fundamental mechanisms that contribute to the antiresorptive and anabolic properties of KPs.

Hemophilia is also a notable risk factor for the development of osteoporosis, as a substantial number of individuals afflicted with diverse forms of hemophilia are concurrently diagnosed with osteoporosis [21, 22]. A murine model demonstrating significant deficits in coagulation factor VIII (F8) is routinely used to explore impaired bone remodeling in hemophilic subjects [23]. Utilizing this murine model, Yen et al. [17] identified that F8-KO mice exhibited significant bone loss by the age of 20 weeks; however, following an 8-week regimen, KPs facilitated the restoration of the trabecular architecture within the femurs and lumbar vertebrae, concurrently enhancing the mechanical strength of the femoral cortical bones in F8-KO mice. Furthermore, KPs treatments led to a reduction in the serum RANKL/OPG ratio and the concentration of the proinflammatory cytokine IL-6, in addition to an elevation in the bone formation biomarker alkaline phosphatase (ALP) and a decrement in the bone resorption biomarker CTX. These results illustrate the potential of KPs for the management of hemophilia-induced osteoporosis.

Additionally, cardiovascular disease patients with vascular calcification symptoms often have concomitant osteoporosis, a condition referred to as the "bone-vascular axis" [24, 25]. Chang et al. [18] found that apolipoprotein E (ApoE)-KO mice fed an atherogenic diet for 13 weeks developed aortic calcification and severe bone loss in the femur. Intervention with KPs attenuated systemic and vessel-specific inflammation and oxidative stress, reducing lipid, collagen, and calcium mineral deposition in the vessels and mitigating bone loss in the femur.

Beyond osteoporosis prevention, KPs have demonstrated significant benefits in fracture repair. This was evidenced in our previous rat model of femoral fracture with KP intervention [26]. Unlike untreated rats, which showed incomplete bone healing, rats administered 400 mg/kg of KPs daily for 4 weeks exhibited complete bone healing, with bone gaps reunited and initial fracture lines entirely vanished. The expression of osteogenic factors such as Col1a1, Alp, Spp1, Vegfa, and Cox2 was upregulated in these KP-treated rats. Given that osteoporosis is a major cause of clinical fractures, the findings by Lai et al. [26] suggest the potential of KPs as a nutritional supplement to accelerate bone healing in clinical fracture cases.

Table 1 summarizes the relevant experimental outcomes using KPs in the aforementioned studies, highlighting their potential as a nutritional supplement for osteoporosis prevention, treatment, and fracture healing acceleration.

Table 1 Studies using KPs for osteoporosis prevention and treatment

Disease type	Experimental models & Intervention regimens (oral route)	Intervention outcomes
Postmenopausal osteoporosis [8]	In vivo (OVX SD rats): 164, 328, and 656 mg KPs/kg BW/day for 12 weeks In vitro (Caco-2 cells): 400 μg/ml	1. Serum:↓CTX 2. H&E (femur): ↓pathological score 3. μ-CT (femur): ↑BMD, ↑BV/TV, ↑Tb.N, ↑Tb.Th, ↓Tb.Sp 4. Nanoindentation (femoral cortical bone): ↑hardness, ↑elastic modulus 5. Caco-2 cells: ↑TRPV6-dependent Ca^{2+} influx
Postmenopausal osteoporosis [15]	In vivo (OVX C57BL/6 J mice): 100 mg KPs/kg BW/day, or the combination of KPs and 10 mg $CaCO_3$/kg BW/day for 8 weeks	1. ↓Kidney-surrounding fat 2. μ-CT (femur): ↑BMD, ↑BV/TV, ↑Tb.N, ↓Tb.Sp 3. Nanoindentation (femoral cortical bone): ↑hardness, ↑elastic modulus, ↓residual indentation area 4. 16S rRNA analysis: KPs mildly modulate the structure of gut microbiota in OVX mice
Osteoporosis [14]	Double-blinded human trial (n = 40): Control: $CaCO_3$, 1500 mg/day Treatment: $CaCO_3$ + KPs, 1600 mg/day, for 6 months	1. Serum:↓β-CTX, ↑OC, ↑PTH 2. CT (hip): ↑BMD
Vitamin C deficiency-induced osteoporosis [16]	In vivo (♂AKR1A1-KO mice): oral administration of 164 and 656 mg KPs/kg BW/day for 12 weeks In vitro (BMMs, BMMSCs): 0 ~ 20 μg/ml KPs	1. Serum:↓ROS, ↓MDA, ↓IL-1β, ↓IL-6, ↓TNF-α, ↓CTX-1, ↓RANKL, ↑SOD, ↑catalase, ↑P1NP, ↑OC, ↑OPG 2. μ-CT (femur): ↑BMD, ↑BV/TV, ↑Tb.N, ↓Tb.Sp 3. Nanoindentation (femoral cortical bone): ↑hardness, ↑elastic modulus 4. OC differentiation: ↑ALP, ↑bone mineralization, ↑Collagen I, ↑β-catenin, ↑RUNX2, ↑BMP-2, ↑NFATc1, ↑HO-1, ↓p53 5. Osteoclast differentiation: ↓TRAP, ↓bone resorption, ↓c-Src, ↓cathepsin K, ↓NFATc1, ↓c-Fos 6. Intracellular signaling: ↓p38, ↓NF-κB, ↓Akt, ↓PLCγ2, ↓CREB-1
Hemophilia-induced osteoporosis [17]	In vivo (♂ Factor VIII-KO mice): oral administration of 164, 328, and 656 mg KPs/kg BW/day for 8 weeks In vitro (BMMs): 10, 20, and 40 μg/ml KPs	1. Serum: ↓CTX-1, ↓RANKL, ↓IL-6, ↑OPG 2. H&E (femur): ↓pathological lesion, ↓TRAP 3. μ-CT (femur): ↑BMD, ↑BV/TV, ↑Tb.N, ↓Tb.Sp 4. μ-CT (lumbar): ↑Tb.N, ↓Tb.Sp 5. Nanoindentation (femoral cortical bone): ↑hardness, ↑elastic modulus 6. Osteoclast differentiation: ↓TRAP + osteoclasts
Atherosclerosis-induced vascular calcification and osteoporosis [18]	In vivo (♂ ApoE-KO mice with a high-cholesterol diet): oral administration of 328 and 656 mg KPs/kg BW/day for 13 weeks	1. Serum: ↓total cholesterol, ↓ox-LDL, ↓MDA, ↓TNF-α, ↓CTX-1, P1NP ↑ 2. Aortic arc (staining): ↓lipid, ↓collagen, ↓inflammation 3. Aortic arc (Western blot): ↓TNF-α, ↓IL-1β 4. μ-CT (femur): ↑BMD, ↑BV/TV, ↑Tb.N, ↑Tb.Th, ↓Tb.Sp, ↓SMI

Table 1 (continued)

Disease type	Experimental models & Intervention regimens (oral route)	Intervention outcomes
Femoral fracture [26]	In vivo (♂SD rats with femur-fracture surgery): oral administration of 400 mg KPs/kg BW/day for 4 weeks In vitro (BMMSCs): 0 ~ 80 μg/ml KPs In vitro (MC3T3-E1): 400 μg/ml KPs	1. Serum: ↑OC, ↑P1NP 2. X-ray (fracture site): fracture line completely vanished, full remodeling of cortex, ↑Lane & Sandhu score 3. Staining (fracture site): ↓cartilaginous callus, ↑calcified fibrocartilage 4. mRNA (fracture site): ↑Col1a1, ↑ALP, ↑SPP1, ↑VEGFA, ↑Cox-2 5. MC3T3-E1 cultures (mRNA): ↑Col1al, ↑ALP, ↑M-CSF, ↑Phospho1 6. BMMSC cultures: ↑bone mineralization

Abbreviations: bone marrow macrophage (BMM), bone marrow mesenchymal stem cell (BMMSC), C-terminal telopeptides of type I collagen (CTX), bone mineral density (BMD), bone volume (BV/TV), trabecular number (Tb.N), trabecular separation (Tb.Sp), trabecular thickness (Tb.Th), skeletal muscle index (SMI), parathyroid hormone (PTH), reactive oxygen species (ROS), malondialdehyde (MDA), oxidized low density lipoprotein (ox-LDL), superoxide dismutase (SOD), receptor activator of nuclear factor kappa-B ligand (RANKL), osteoprotegerin (OPG), osteocalcin (OC), procollagen type 1 amino-terminal propeptide (P1NP), alkaline phosphatase (ALP), interleukin (IL), tumor necrosis factor (TNF), nuclear factor-kappa B (NF-κB), Runt-related transcription factor 2 (RUNX2), bone morphogenetic protein-2 (BMP-2), nuclear factor of activated T cells 1 (NFATc1), heme oxygenase 1 (HO-1), Tartrate-resistant acid phosphatase (TRAP), phospholipase C gamma 2 (PLCγ2), cAMP response element-binding protein 1 (CREB-1), alpha-1 type I collagen (Col1a1), secreted phosphoprotein 1 (SPP1), vascular endothelial growth factor A (VEGFA), cyclooxygenase 2 (Cox-2), macrophage colony-stimulating factor (M-CSF), phosphoethanolamine/phosphocholine phosphatase 1 (Phospho1)

Individual Bioactive Peptides With Osteoprotective Effects

Although bioactive peptides with osteoprotective effects have not yet been extensively identified from kefir-fermented products, earlier studies on milk protein-derived peptides offer valuable insights. Caseinophosphopeptides (CPPs) are the most thoroughly studied milk casein (CN)-derived bioactive peptides, known for their calcium-binding activity [27–30]. The benefits of CPPs for bone health are based on the premise that these peptides can promote intestinal calcium absorption, thereby enhancing bone calcification. This premise is supported by findings that dietary calcium-bound CPPs prevented bone loss in OVX rats [31]. Subsequently, Liu et al. [32, 33] isolated a calcium-binding peptide, β-CN (16–40), from a commercially available CPP mixture and found that this peptide enhanced calcium transport in Caco-2 cells via the transcellular route by upregulating the expression of the TRPV6 calcium channel protein. Additionally, this peptide improved bone formation and prevented bone resorption in rats.

Ebner et al. [34] identified a peptide profile of bovine milk protein released from a commercial kefir starter culture, which included many casein phosphopeptides with sequences overlapping β-CN (16–40) and other known CPPs, such as αS1-CN (59–79) [27, 29] and β-CN (1–25) [30, 31]. However, most of these peptides have not yet been functionally characterized. The efficacy of CPPs in promoting calcium absorption remains a controversial issue, possibly due to differences in methods used for assessing calcium bioavailability [35].

To date, ACE-inhibitory tripeptides (IPP and VPP) [36–38], casein-derived antioxidative peptides (EDVPSER, NAVPITPTL, VLPVPQK, and HPHPHLSF) [39–43], and whey-derived ACE-inhibitory (YLLF and WLAHK) and antioxidative (YVEEL) peptides [44] have been characterized for their ability to stimulate in vitro osteoblast proliferation and differentiation. Among these peptides, VLPVPQK, YVELL, and YLLF have been further demonstrated for their osteoprotective potential in OVX rats [45]. Oxidative stress plays a significant role in the development of chronic inflammatory bone disease, as excessive generation of reactive oxygen species (ROS) negatively impacts bone remodeling by promoting osteoclastogenesis and inhibiting osteogenesis [46]. This explains why antioxidative peptides often have osteoprotective effects.

The mechanism of ACE-inhibitory peptides on osteoblasts may involve their activity in inhibiting ACE, thereby preventing the conversion of angiotensin I to angiotensin II, which has been shown to induce osteoblast mitochondrial dysfunction and apoptosis, thus decreasing osteoblast differentiation [47]. Notably, all the mentioned casein-derived peptides can be found in previous peptide profiles of bovine

milk-based kefir with complete sequence coverage, suggesting their osteoprotective effects [34, 48]. Undoubtedly, high-throughput screening of osteoprotective peptides from kefir deserves attention in future research.

Earlier, we isolated a bioactive peptide named KFP-1 from the < 3-kDa fraction of KPs [49]. The identity of KFP-1 covers the sequence of κ-CN (138–154) and has been characterized for its functions in binding calcium, promoting calcium influx in Caco-2 cells through TRPV6 channels, and enhancing intestinal calcium absorption in mice. Furthermore, KFP-1 was found to inhibit osteoclastogenesis and promote osteoblastogenesis in vitro, as well as exert osteoprotective effects in the *AKR1A1*-KO mouse model of osteoporosis. Techniques such as LC/MS/MS and high-throughput analysis of antioxidative and anti-inflammatory peptides using Raw264.7 cell models may aid in the screening and characterization of more milk protein-derived bioactive peptides with osteoprotective effects. These experiments are currently being undertaken in our laboratory.

The known milk protein-derived bioactive peptides with osteoprotective effects are summarized in Table 2.

Probiotics with Osteoprotective Benefits

The utilization of molecular biology methodologies in microbiome analysis, including 16S rRNA sequencing and metagenomic next-generation sequencing, has enhanced the investigation of intricate microbial ecosystems in kefir grains and associated kefir products. A recent metagenomic analysis conducted by Zeng et al. [50]. revealed the microflora structure of Tibetan kefir grains obtained from three distinct sources, in which species under the genera *Lactobacillus* comprise the predominant microbial consortium in kefir grains. Many species identified in the study of Zeng et al. align with prior studies and literature [51, 52]. A lot of species identified in kefir grains or milk kefir beverages have been shown to exert osteoprotective effects in animals and humans, including *Lactoacillus acidophilus* [53–58], *Lactoacillus casei* [56–58], *Lactoacillus fermentum* [59], *Lactoacillus helveticus* [60, 61], *Lactoacillus plantarum* [62–64], *Lactoacillus paracasei* [62–64], *Lactoacillus reuteri* [56–58], *Lactoacillus rhamnosus* [65–67], *Bifidoacterium bifidum* [68], *Bifidoacterium animalis subsp. lactis* [68], *Bacillus clausii* [69], and *Bacillus subtilis* [70]. This section will review earlier studies that applied these probiotics for osteoporosis prevention and treatment, with relevant experimental outcomes summarized in Table 3.

The use of *L. acidophilus* to ameliorate menopausal osteoporosis was first reported by Dar et al. [53], who demonstrated that the protective effects of *L. acidophilus* was through the mechanism to modulate the ratio of regulatory T cells (Treg) and T helper 17 cells (Th17) in OVX mice. The administration of *L. acidophilus* for 6 weeks increased $Foxp3^+$ Treg cells while reducing $Ror\gamma t^+$Th17 cells in the bone marrow and spleen, promoting the expression of anti-osteoclastogenic factors (IL-10, IFN-γ) and inhibiting osteoclastogenic factors (IL-6, IL-17, TNF-α, and RANKL). Similarly, Chen et al. [55] demonstrated that treatments of *L. acidophilus* prevented bone loss in OVX mice by inhibiting systemic levels of inflammatory cytokines (IL-1β, IL-6, and TNF-α) and altering gut microbial diversity, leading to a reduced *Firmicutes*/*Bacteroidetes* ratio in probiotic-fed OVX mice compared to controls. Recently, Dong et al. [54] elucidated that the advancement of postmenopausal osteoporosis correlates with diminished levels of intestinal *L. acidophilus* and short-chain fatty acids (SCFAs), such as butyrate. The intervention with *L. acidophilus* and butyrate effectively mitigated systemic bone loss and curtailed osteoclast formation and bone resorption in OVX mice, as well as directly impeding RANKL-mediated osteoclast differentiation and bone resorption activity in an in vitro setting utilizing Raw264.7 cells. Earlier, Montazeri-Najafabady et al. [56, 57] evaluated the bone-protective properties of *L. acidophilus* in OVX rats, along with two other *Lactobacillus* species, *L. casei* and *L. reuteri*, isolated from traditional fermented yogurts. Their results demonstrated the enhancement in OVX rats treated with *L. acidophilus*, *L. casei*, and *L. reuteri* in terms of BMD, bone mineral content, bone area, and biochemical parameters. Furthermore, Montazeri-Najafabady et al. [57] reported that the postbiotics (supernatant and bacterial lysates) from *L. acidophilus*, *L. casei*, and *L. reuteri* also exerted bone protective effects similar to the probiotic treatments in OVX rats. Later, Gholami et al. [58]. indicated that the administration of a mixture of *L. acidophilus*, *L. casei*, and *L. reuteri* resulted in a notable improvement in BMD and bone mineral content in OVX rats, showing apparent synergistic effects among these *Lactobacillus* strains.

L. fermentum has been served as a probiotic agent for a 6-month trial in postmenopausal women who were not on osteoporosis medication [59], where the probiotic supplementation was found to maintain the serum level of bone turnover marker OC and increase femur neck BMD compared to the placebo-controlled group. *L. helveticus* is the third dominant species in the microbial communities of Tibetan kefir grains, accounting for about 4% of total communities [50]. Interventions utilizing *L. helveticus* was observed to mitigate the process of bone remodeling by enhancing bone formation while concurrently diminishing bone resorption, thereby leading to a reduction of bone loss in OVX rats [60, 61]. Meanwhile, Parvaneh et al. [61]. also demonstrated the effectiveness of the *L. helveticus* and *Bifidobacterium longum* combination in their studies, but they noted that single-strain supplementation exhibited superior efficacy on bone. Yang et al. [62]. elucidated the potential of the *L. plantarum* and *L. paracasei* against postmenopausal osteoporosis in OVX mice, suggesting that probiotic

Table 2 Milk protein-derived bioactive peptides with osteoprotective effects

Bioactive peptide (name or sequence)	Peptide source or the parental milk protein	Major findings
Casein phosphopeptides [31, 33]	Tryptic digest of milk casein or Commercial CPP mixtures	1. Supplementation of CPPs in diets prevented the decline of BMD in OVX rats [31] 2. CPP treatments increased serum Ca and OC levels, femur index, and femoral Ca content and decreased serum ALP and PTH levels in normal rats [33]
RELEELNVPGEIVESLSSSEESITR [32]	β-CN (16–40) Phosphorylation at Ser-30 and Ser-32–34	1. β-CN (16–40) treatment increased Ca^{2+} transport and TRPV6 expression in Caco-2 cells 2. β-CN (16–40) supplementation increased serum Ca level, femur length, and femoral Ca content and decreased serum ALP level and urinary pyridinoline content in normal rats
IPP, VPP [36–38]	Synthetic tripeptides or the peptide solutions (< 1 kD) prepared from *L. helveticus*-fermented whey	1. IPP and VPP increased the bone mineralization during BMMSC-based osteoblast differentiation but caused no effects on BMM-based osteoclast differentiation [36] 2. IPP increased bone mineralization in BMMSCs due to enhanced cell survival and matrix formation, and also reduced the RANKL/OPG ratio [37] 3. Microarray analysis indicated that IPP holds the promise to enhance in vitro osteoblast growth, differentiation, and signaling [38]
EDVPSER (PEP1) NAVPITPTL (PEP2) VLPVPQK (PEP3) HPHPHLSF (PEP4) [39–43]	α_{S1}-CN (99−105) α_{S2}-CN (130−138) β-CN (185−191) κ-CN (119−126)	1. These peptides were shown to stimulate the proliferation of preosteoblasts and increase the expression of ALP, OC, and collagen I genes, ALP activity, OC secretion, and bone mineralization during in vitro osteoblast differentiation 2. PEP2 was shown to induce in vitro osteoblast differentiation by the PI3K/Akt signal cascade [39]
YVEEL (P2) YLLF (P3) WLAHK (P6) [44, 45]	β-LG (58−62) β-LG (118−121) α-LA (123−127)	1. These peptides were shown to increase the proliferation of preosteoblasts, ALP activity, and bone mineralization with a trend of P2 > P6 > P3 2. These peptides upregulated the expression of ALP, type I collagen, OC, and RUNX2 during in vitro osteoblastic differentiation [44] 3. P2 and P3 administrations reduced serum ALP, RANKL, and OC levels and prevented bone loss in OVX rats; they also decreased serum TNF-α while elevating TGF-β and IFN-γ levels [45] 4. The antioxidative peptide (P2) shows a greater ability to protect bones compared to the ACE-inhibitory peptide (P3) by reducing inflammation and enhancing bone formation markers [45]
TEVPAINTIASAEPTVH (KFP-1) [49]	κ-CN (138−154)	1. KFP-1 promoted intestinal Ca^{2+} absorption in mice 2. KFP-1 inhibited in vitro RANKL-induced osteoclast differentiation and bone resorption 3. KFP-1 enhanced in vitro osteoblast differentiation and bone mineralization 4. KFP-1 administration prevented bone loss in AKR1A1-KO mice with vitamin C deficiency

Abbreviations: Casein phosphopeptide (CPP), casein (CN), transient receptor potential vanilloid subfamily member 6 (TRPV6), transformation growth factor beta (TGF-β), interferon gamma (IFN-γ), angiotensin-converting enzyme (ACE), aldo–keto reductase family 1 member A1-knockout (AKR1A1-KO)

Table 3 Identifiable probiotic species in kefir with osteoprotective potential and major findings in related intervention studies

Probiotics (strains)	Intervention regimens (oral route)	Major findings
Lactobacillus acidophilus (ATCC4356 [53], LA-02 [54], GDMCC1.412 [55])	1. 2×10^8 CFU/day for 6 weeks in OVX mice [53] 2. 2×10^7 CFU/10 g BW/day for 2 weeks in OVX mice [54] 3. 10^9 CFU/ml in drinking water for 2 weeks in mice prior to OVX operation [55]	1. These studies showed that administration of *L. acidophilus* reduced OVX-induced bone loss and enhanced trabecular and cortical bone microarchitecture [53–55] 2. The treatments skewed Treg/Th17 cell balance by inhibiting osteoclastogenic Th17 cells (↓IL-1β, IL-6, and TNF-α) and promoting anti-osteoclastogenic Treg cells (↑IL-10 and IFN-γ) [53, 54] 3. The treatment enhanced intestinal barrier permeability and reduced RANKL production in B cells of OVX mice [51] 4. The treatment modulated the gut microbiota and altered certain microorganism abundances, which led to an attenuation of bone loss [55]
Lactobacillus acidophilus, *Lactobacillus casei, Lactobacillus reuteri* [56, 57], and the combination of 3 strains [58]	1. 10^9 CFU/day for 4 weeks in OVX rats [56, 57] 2. 10^9 CFU/strain/day for 4 weeks in OVX rats [58]	1. All the treatments with single strain exerted osteoprotective effects in OVX rats, in terms of BMD, bone marrow concentration, bone area, and biochemical parameters [56] 2. Treatments with the postbiotics from these bacteria also ameliorated OVX-induced bone loss [57] 3. The treatment with a combination of 3 strains showed significant enhancement in OVX rats in terms of BMD and bone mineral content [58]
Lactobacillus fermentum SRK414 [59]	4×10^9 CFU/capsule, twice a day for 6 months in postmenopausal osteoporosis women who are not on osteoporosis medications	The treatment was found to maintain OC levels and increase femur neck BMD during a 6-month trial in postmenopausal women but not in the placebo-treated group
Lactobacillus helveticus ATCC27558 [60, 61]	10^8–10^9 CFU/day for 16 weeks in OVX rats	The treatments attenuated bone remodeling and consequently improved bone health in OVX rats by increasing bone formation (↑BMP-2, RUNX2, OC, BMD, BV/TV, Tb.Th) along with bone resorption reduction (↓CTX, Tb.Sp, porosity)
Lactobacillus plantarum GKM3, *Lactobacillus paracasei* GKS6 [62]	10^9 CFU/day for 4 weeks in OVX mice	1. Both strains showed anti-osteoporosis effects in OVX mice, with GKS6 outperforming GKM3 2. In vitro, the treatments upregulated osteoblastic marker genes (↑BMP-2, ALP, and OC) while downregulating osteoclastic marker genes (↓RANK, c-Fos, and TRAP), suggesting its anti-osteoporosis benefit
Lactobacillus paracasei (DSM13434) and *Lactobacillus plantarum* (DSM15312, DSM 15313) mix [63, 64]	1. 10^9 CFU/ml in drinking water, average 4.5 ml/day for 2 weeks in ♀ mice before OVX surgery [63] 2. 10^{10} CFU/day for 1 year in early postmenopausal women [64]	1. The treatment prevented OVX-induced cortical bone loss and altered the immune status in bone by reducing TNF-α and IL-1β and increasing OPG expression, resulting in attenuated bone resorption in OVX mice [63] 2. The probiotic treatment significantly reduced lumbar spine bone loss compared with placebo in postmenopausal women [64]

Table 3 (continued)

Probiotics (strains)	Intervention regimens (oral route)	Major findings
Lactobacillus rhamnosus (LGG [65, 67] and UBLR-58 [66])	1. 5×10^8 CFU/mice, twice a week for 8 weeks in tenofovir disoproxil fumarate (TDF)-treated mice [65] 2. 4×10^8 CFU/day for 6 weeks in OVX mice [66] 3. 10^9 CFU/mice, 5 times a week for 4 weeks in ♀ C57BL/6, TCR$\beta^{-/-}$, OT-1, and DEREG mice [67]	1. The osteoprotective effects of *L. rhamnosus* were largely determined by activities to balance the Treg/Th17 cell ratio (↑Treg, ↓Th17) in the bone marrow, spleen, and gut of mice [65–67], which may reduce serum osteoclastogenic cytokines (IL-6, IL-17, TNF-α) while increasing anti-osteoclastogenic cytokines (IL-4, IL-10, IFN-γ) [65, 66], along with the upregulation of Wnt10b expression by $CD8^+$ T cells, which in turn activate Wnt signaling in osteoblasts to stimulate bone formation [67] 2. The concentration of butyrate produced in the gut following LGG administration is linked with the regulation of bone anabolism via Treg cell-mediated $CD8^+$ T cell Wnt10b production [67]
Bifidobacterium bifidum FL228.1, *Bifidobacterium animalis subsp. Lactis* F1-7 [68]	10^9 CFU/day for 10 weeks in OVX mice	1. Both strains prevented OVX-induced bone loss in mice by the mechanisms to protect gut mucosal barrier and reduce pro-inflammatory M1 macrophage growth, thus suppressing excessive osteoclast generation 2. FL228.1 increased the abundance of beneficial bacteria in the colon, including *Lactobacillus* and *Colidextribacter*, while F1-7 increased the abundance of *Bifidobacterium* and decreased the abundance of *Desulfovibrio* and *Ruminococcus* in the colon
Bacillus clausii (commercial spores) [69]	2×10^8 CFU/day for 6 weeks in OVX mice	The treatment prevented bone loss by skewing Treg/Th17 cell balance (↑Treg, ↓Th17) in the bone marrow and spleen of OVX mice, which resulted in the reduction of proinflammatory cytokines (IL-6, IL-17, IFN-γ, and TNF-α) and increased levels of anti-inflammatory cytokine (IL-10)
Bacillus subtilis C-3102 [70]	3.4×10^9 CFU/day for 24 weeks in postmenopausal women without diagnosis of osteoporosis	1. Compared with placebo, the probiotic treatment lowered urinary NTX and serum TRAP levels at 12 weeks of treatment and increased total hip BMD at the end of 24 weeks of treatment in postmenopausal women 2. The probiotic treatment was shown to modulate gut microbiota by increasing *Bifidobacterium* and decreasing *Fusobacterium* abundances

Abbreviations: colony-forming units (CFU), N-terminal telopeptide of collagen I (NTX), regulatory T cell (Treg), T helper 17 cell (Th17)

interventions induce the upregulation of osteoblastic marker genes (BMP-2, ALP, and OC) alongside the downregulation of osteoclastic marker genes (RANK, c-Fos, and TRAP); meanwhile, the efficacy of *L. paracasei* was demonstrated to surpass that of *L. plantarum*. The protection of *L. paracasei* was similarly demonstrated in the study of Ohlsson et al. [63], where both the treatments with a single *L. paracasei* strain and with a mixture of two *L. plantarum* strains and one *L. paracasei* strain protected mice from OVX-induced bone loss, showing that both the probiotic treatments reduced the serum CTX, TNF-α, and IL-1β and urinary fractional calcium excretion, along with an increase in OPG and Treg cells in the bone marrow. However, no notable distinction was observed between single-strain and 3-strain mixture treatments. Subsequently, Jansson et al. [64] conducted a placebo-controlled human trial in early postmenopausal women through the supplementation of the aforementioned 3-strain mixture. Following a 12-month period, the probiotic-treated group experienced approximately a 0.01% reduction in lumbar spine BMD compared to nearly a 0.72% reduction in the placebo-treated group, thereby illustrating the prophylactic effects of the 3-strain mixture of *L. paracasei* and *L. plantarum* on lumbar spine bone loss in early postmenopausal women.

L. rhamnosus has been reported to prevent bone loss induced by tenofovir disoproxil fumarate (TDF) and OVX surgery in mice [65, 66]. TDF serves as primary antiretroviral medication for patients with human immunodeficiency virus (HIV) and hepatitis B virus (HBV) infections, yet concerns regarding its potential to induce osteoporosis are rising [71]. Liu et al. [65] demonstrated that *L. rhamnosus* can be a safe and effective probiotic supplement to prevent and treat TDF-induced osteoporosis by modulating Treg/Th17 cell balance in the bone marrow, spleen, and gut, which will in turn increase intestinal barrier integrity, decrease osteoclastogenesis-related cytokine expression, reconstruct the gut microbiota, and change the metabolite composition, thus reducing TDF-induced inflammatory responses in mice. Similarly, Sapra et al. [66]. reported that administration of *L. rhamnosus* exerted comparable protective effects in OVX mice via the regulation of Treg/Th17 cell balance. Furthermore, Tyagi et al. [67] demonstrated that *L. rhamnosus* stimulated bone formation in eugonadic young mice by increasing the production of butyrate, which will expand the pool of Treg cells in the gut and bone marrow and upregulate the expression of osteogenic Wnt10b by $CD8^+$ T cells and thus activating Wnt signaling in osteoblasts.

B. bifidum and *B. animalis subsp. lactis* has been recently reported to protect mice from OVX-induced one loss in the study of Zhang et al. [68], in which both the probiotic treatments reduced inflammatory cytokine expression, mitigated gut inflammation, and inhibited osteoclast proliferation in OVX mice. The mechanism involves mucosal barrier protective factors such as occludin, Zonula Occludens-1 (ZO-1), claudin-2, and mucus protein 2 (MUC2), along with decreased pro-inflammatory M1 macrophages. The treatments also influenced the gut microbiota of OVX mice, with *B. bifidum* enhancing beneficial bacteria such as *Lactobacillus* and *Colidextribacter* in the colon, while *B. animalis subsp. lactis* augmented *Bifidobacterium* and reduced *Desulfovibrio* and *Ruminococcus* levels in the colon of OVX mice.

The efficacy of *B. clausii* in promoting bone health was evidenced in the study of Dar et al. [69], wherein *B. clausii* also employed the mechanism of Treg/Th17 modulation to mitigate OVX-induced bone loss in mice. *Bacillus subtilis* has undergone evaluation regarding its osteoprotective effects in a cohort of healthy postmenopausal females, as reported by Takimoto et al. [70]. This study demonstrated that a 24-week regimen of probiotic intervention resulted in a significant decrease in urinary and serum markers indicative of bone resorption, an enhancement in total hip BMD, and a modulation of gut microbiota characterized by an increase in the relative abundance of the genus *Bifidobacterium* and a reduction in the relative abundance of the genus *Fusobacterium*.

The typical probiotic intervention dosage for OVX rats is 10^9 CFU daily for over 4 weeks, whereas human subjects generally receive approximately 10^9–10^{10} CFU/day for a minimum duration of 6 months (Table 3). Each gram of kefir grains contains 10^8 CFU of *Lactobacillus sp.* [51], suggesting that consumption of a cup yields approximately one billion to thirty billion CFU of probiotics. Although direct evidence for the anti-osteoporosis effects of kefir-derived probiotics is absent, the benefits of kefir on bone health through its abundant probiotic flora are anticipated. Recent studies have demonstrated the impact of kefir consumption on gut microbiota, highlighting the critical function of gut probiotics in bone health via the "gut-bone axis" [72]. This connection suggests that improving gut health with probiotics may enhance the intestinal mineral absorption and reduce inflammation, ultimately contributing to better bone density and strength [73]. Emerging research also highlights the potential of specific strains of probiotics found in kefir to stimulate the production of beneficial SCFAs, which can further support bone health by promoting calcium absorption and modulating inflammatory responses within the body. As scientists continue to explore this relationship, it becomes increasingly clear that integrating probiotic-rich foods like kefir into the diet could serve as a proactive approach to maintaining and improving bone health throughout life.

Exopolysaccharide (EPS)

EPS is produced during the fermentation of kefir by lactic acid bacteria that utilize diverse carbohydrates as substrates, contributing to enhance the texture and flavor of

kefir beverages while also acting as prebiotics that nurture beneficial microbiota and foster gut health [74]. *Lactobacillus kefiranofaciens* is the predominant probiotic within kefir grains, comprising 83% of the microbial consortia [50], which serves as the most important producer of kefiran, showing a peak productivity of 2.58 g/L when co-culturing with *Saccharomyces cerevisiae* [75]. Kefiran, recognized as the principal EPS in kefir drinks, is a water-soluble branched EPS consisting of roughly equal amounts of D-glucose (Glc) and D-galactose (Gal), with a complex structure composed of (1 → 6)-linked Glc, (1 → 3)-linked Gal, (1 → 4)-linked Gal, (1 → 4)-linked Glc, and (1 → 2, 6)-linked Gal and a molecular weight ranging from 55 to 10^4 kDa [75]. Kefiran has gained significant attention due to its remarkable rheological properties, which have extensive applications in the food industry and medicine. These properties not only enhance the texture and stability of food products but also contribute to potential health benefits, such as anti-inflammatory, antioxidant [76–79], antimicrobial, wound-healing [80], and antitumor activities [81].

The impact of kefiran on osteoporosis has not been thoroughly studied. We assume that the benefits of kefiran to bone health may not rely on the direct action on the bone cells, whereas its remarkable anti-inflammatory properties could offer an indirect mechanism to avert the bone remodeling toward a pathological state. This is due to the fact that inflammation significantly influences bone remodeling and is a crucial factor in the progression of osteoporosis. Clinical findings reveal that postmenopausal women exhibit increased levels of proinflammatory cytokines like IL-1β, IL-6, and TNF-α [82–84], which play a crucial role in promoting osteoclastogenesis. Furuno et al. [76] demonstrated that kefiran inhibited bone marrow-derived mast cell degranulation and TNF-α production through the attenuation of the Akt and ERK pathways, indicating a potential anti-inflammatory effect for kefiran. Mast cells play an important role in osteoporosis development, as evidenced by the increased number of mast cells in the bone marrow of postmenopausal osteoporosis patients and the fact that mast cell-deficient mice are protected from OVX-induced bone loss [85]. Many of the mediators released by mast cells can promote osteoclast formation (histamine, TNF-α, IL-6) and inhibit osteoblast activity (IL-1β, TNF-α), leading to bone resorption [86]. Liao et al. [78] demonstrated that kefiran mitigated lipopolysaccharide (LPS)-induced systemic inflammation via NF-κB signaling inhibition in mice. Bahari et al. [87] revealed that kefiran decreased the expression of proinflammatory cytokines in monocytes, a linage of osteoclast precursor cells. These findings suggest that kefiran hinders the secretion of proinflammatory cytokines, potentially diminishing systemic inflammatory responses and consequently inhibiting osteoclast differentiation and bone resorption.

Several polysaccharides derived from conventional Chinese medicinal plants, including *Astragalus* [88], *Achyranthes bidentata* [89], and *Eucommia ulmoides* [90], have been evidenced to augment the diversity of the gut microbiota to regulate bone metabolism. These studies enable us to further assume that kefiran exerts osteoprotective benefits by altering the gut microbiota. Serafini et al. [91] found that kefiran served as a substrate for bifidobacterial growth and increased the growth capacity of *B. bifidum* PRL2010 on a carbohydrate-free MRS agar plate containing 0.3% kefiran. They further demonstrated that kefiran affected the transcriptome of *B. bifidum*, leading to higher transcription of genes related to dietary glycans metabolism and host–microbe effector molecules like pili [91]. Later, Hamet et al. [92]. demonstrated the bifidogenic effects of kefiran on mouse gut microbiota. Their findings demonstrated that the administration of kefiran modified the gut microbiota by enhancing bifidobacterial levels while leaving *Lactobacillus* unaffected. It should be noted that the aged people and individuals dealing with chronic intestinal inflammation tend to have diminished levels of bifidobacteria in their intestines [93], which may be enhanced through the intake of kefiran. Other lactic acid bacteria present in kefir grains likewise generated EPS [94]. Bengoa et al. [95]. showed that EPS extracted from the fermented milk with *L. paracasei* strains altered the microbiota, leading to increased production of SCFAs, such as propionate and butyrate, which also confer some benefits for gut health. Min et al. [96] demonstrated that the EPS generated by *L. plantarum* influenced the gut microbiota of mice and enhanced the host's immunity, thereby lowering the risk of symptoms associated with inflammatory bowel disease.

The potential mechanisms through which kefiran or non-kefiran EPS exert their effects can be elucidated by referencing the prior review article of Zhou et al. [97], wherein it is highlighted that the ways through which natural resourced polysaccharides regulate intestinal microbiota to facilitate bone metabolism are closely tied to the regulation of SCFAs, immunity, and hormones, encompassing a range of signaling pathways, such as TGF-β, Wnt/β-catenin, BMP/Smads, and RANKL. Although the unique structure and biological activities of kefiran and non-kefiran EPS have been extensively researched, the understanding of operational mechanisms and their use in the management of osteoporosis still requires validation through studies involving animal models and human participants.

Future Directions

The global burden of disability-adjusted life years, osteoporosis-associated deaths, and fractures has been steadily increasing since 1990 [98]. Concurrently, there is a growing trend towards consuming natural food products with health benefits among diverse population segments worldwide.

This trend has motivated increased consumption of kefir and other fermented milk products as part of regular diets.

The health-promoting benefits of kefir are attributed to its abundant array of bioactive peptides, probiotics, exopolysaccharides, vitamins, minerals, and additional compounds that offer a variety of biological functions. In this review, we have highlighted the beneficial impacts of kefir peptides on bone health and extensively discussed the osteoprotective potential of kefir-derived probiotics and kefiran. Undoubtedly, future work is warranted to further validate the beneficial effects of kefir-derived probiotics and kefiran in the prevention of osteoporosis. To date, investigations into the osteoprotective effects of kefir remain in the early stage, and more kefir-derived bioactive peptides and probiotics, as well as the efficacy and application of kefiran, are waiting to be discovered in order to unravel the mystery behind this traditional fermented beverage.

Conclusion

Kefir, a traditional fermented beverage, holds significant promise in the management of osteoporosis due to its unique composition of bioactive components promoting bone health through various mechanisms (Fig. 1). While research is still in early stages, evidence suggests kefir's potential as a natural approach to osteoporosis prevention and management. Its multi-faceted benefits and safety make it an attractive candidate for further investigation. Future research should focus on mechanisms, dosages, and synergistic effects, with clinical studies crucial for practical applications. As our understanding grows, kefir may offer new possibilities for improving bone health and quality of life for millions affected by osteoporosis worldwide.

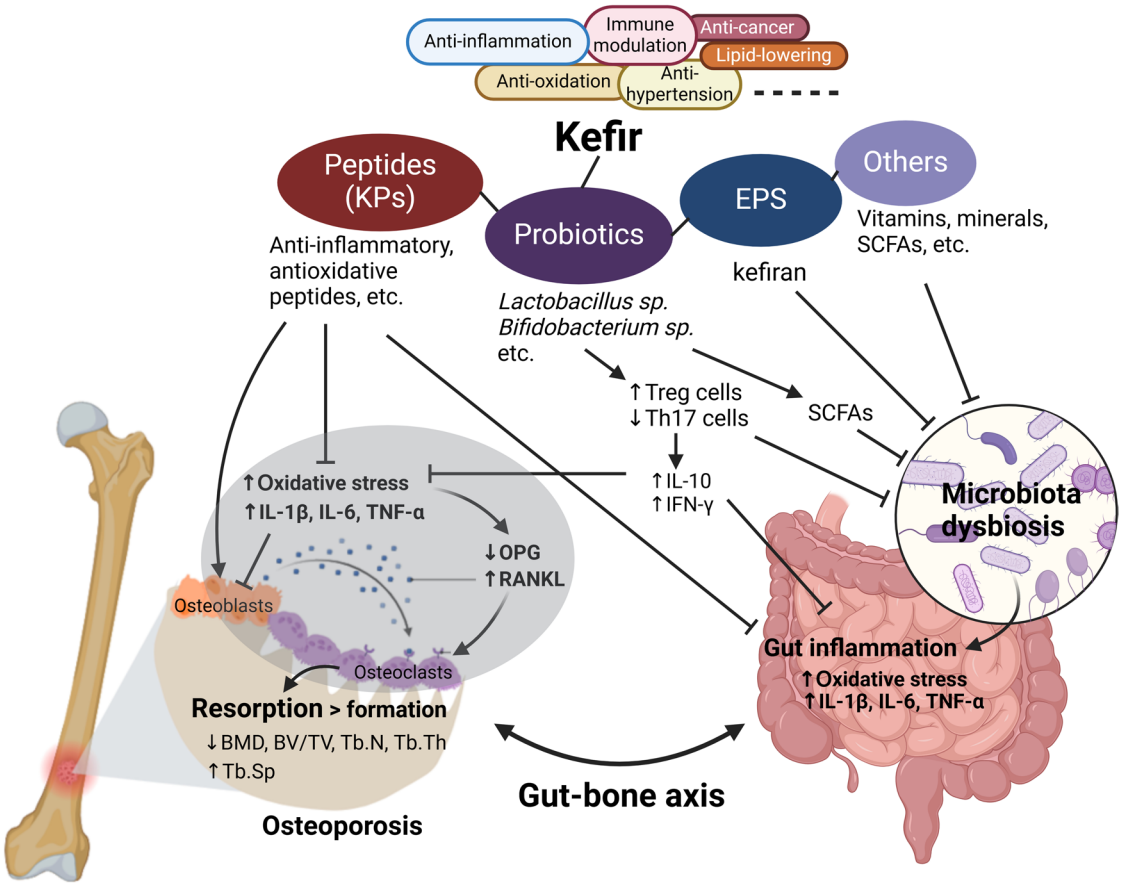

Fig. 1 The benefits of consuming kefir for bone health and gut microbiome. The intake of kefir provides its key functional elements (bioactive peptides, probiotics, EPS, and other essential nutrients) to deliver osteoprotective effects by modifying pathological gut-bone axis. These osteoprotective advantages might arise from the capabilities of certain bioactive peptides to influence the formation and activity of osteoclasts and osteoblasts, as well as the roles of probiotics (and their metabolites, such as SCFAs) and EPS (kefiran) in adjusting the gut microbiota, leading to the reduction of gut inflammation and dysbiosis. This illustration was created in https://BioRender.com

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Authors' Contributions J.C.L., M.Y.T., and C.M.C. conceptualized the study. J.C.L., I.C.C., A.C., and G.R.L.C. prepared Fig. 1 and tables 1–3. J.C.L. and G.R.L.C. wrote the main manuscript. G.R.L.C. and C.M.C. reviewed and edited the manuscript. C.M.C. supervised the project and visualized the results. J.C.L. managed the project administration, while C.M.C. acquired funding. All authors reviewed the manuscript.

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Data Availability Data is provided within the manuscript or supplementary information files.

Declarations

Ethics Approval and Consent to Participate Not applicable.

Consent for Publication The authors consent to publishing this work.

Conflicts of Interest The authors declare no competing interests.

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