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Living probiotic biomaterials for osteoporosis therapy

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ABSTRACT

Osteoporosis (OP) is a chronic metabolic bone disease characterized by diminished bone mass, decreased bone strength, deterioration of bone microarchitecture, and increased bone fragility with fracture risk. The aging population has made OP a public health problem that has serious effects on individuals, families, and society as a whole. It is urgent that new strategies that are safe, effective, and inexpensive for the treatment of OP should be developed. Increasing evidence has suggested that the gut microbiota (GM) is inextricably linked to bone homeostasis through cross-talk between host and microbiota. During the development of OP, GM perturbations can initiate and reinforce the disruption of bone remodeling balance. In this review, we first review the current knowledge of how the GM affects bone metabolism, and conclude that GM changes bone metabolism and participates in the formation of OP by affecting intestinal barrier, host metabolites, immune system and endocrine system. Then, we discuss that probiotics are expected to be a potential oral therapeutic strategy for OP, but there are limitations. Furthermore, we discuss how bioactive functional materials for providing probiotics to the gut can be constructed based on the chemical barrier, biological barrier, immune barrier, and mechanical barrier in the gut. This review is anticipated to stimulate further innovative thinking focusing on the intestinal barrier as a key target for delivery of probiotics and treatment of OP.

1. Introduction

Osteoporosis (OP), a systemic metabolic bone disease, is characterized by decreased bone mass and degenerative bone microstructure that results in impaired bone strength, increased fracture risk, and a decreased ability to bear weight [1]. There are approximately 71.8 million OP people over 50 in China, with a prevalence rate of 21.3%, according to the most recent survey [2]. Bedsores, pneumonia, urinary tract infections, and even death can result from osteoporotic fractures. A growing number of patients in China suffer from OP as a result of the aging population, putting great strain on both patients and society. It is urgent that an OP treatment that is safe, effective, and low-cost be developed.

There are a large number of species of microorganisms inhabiting the intestinal tracts of animals, collectively referred to as gut microbiota (GM). A human gut colonizes more than 100 trillion microorganisms, including bacteria, fungi and viruses, primarily Firmicutes, Bacteroidetes, Proteobacteria, and Actinomycetes [3]. GM affects host metabolism by regulating the function of the intestinal epithelium, the immune system, the metabolism of nutrients, drugs and exogenous substance, and by maintaining the integrity of the intestinal mucosal barrier [4]. Through its interaction with the host, the GM ecosystem also plays a role in the mechanism of bone and joint disease [5,6]. Its effects can be extensive, affecting host metabolism, bone immunity, microbial metabolism in the intestinal tract, and the brain-gut axis related to bone health [7]. As the plasticity of GM is known, manipulating external influencing factors may be a distinct means of reshaping the GM architecture and its associated biological influences.

Nowadays, researchers are focusing on the "gut-bone axis," a target to inhibit the development of OP, in order to improve intestinal microecology and bone metabolism microenvironment. Studies have shown that OP patients have a significantly different GM composition from

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healthy individuals [8]. When intestinal probiotics (e.g., Lactobacillus reuteri and Lactobacillus rhamnosus) are supplemented orally, intestinal microecology is modulated, intestinal permeability is reduced, osteoclasts are reduced, and bone loss from sex steroid (estrogen) depletion is prevented. However, the complex environment of the digestive tract limits the effectiveness of oral probiotics: for example, intestinal mucus is constantly changing in the gastrointestinal tract, which makes colonization difficult for probiotics. Rapid peristalsis (from the mouth to the anus for 30 h) reduces the effect of probiotics, and the production of acidic gastric juice keeps them inactive. In order to treat OP effectively, it is expected that the development of a smart biomaterial system capable of efficiently delivering probiotics via convenient, safe and direct oral administration will become a new strategy, which is worth investigating in depth. This review first discusses how GM affects bone metabolism and contributes to the development of OP, limitations of current clinical treatments for OP, and most importantly, we discuss how bioactive functional materials for providing living probiotics to the gut could be constructed based on the chemical barrier, biological barrier, immune barrier, and mechanical barrier in the gut (Fig. 1).

2. Effects of gut microbiota on Osteoporosis

2.1. Bone metabolism and Osteoporosis

Bone modeling and bone remodeling are two basic physiological processes involved in bone development, bone metabolism and bone homeostasis. It is believed that bone remodeling is the main physiological process during bone growth and development (Fig. 2 A), as it involves the process of morphological adaptation and programmed bone construction as a result of external stress and physiological needs through membrane internalization and cartilage internalization [9]. Bone remodeling dominates after maturation, and new bone replaces old bone. Bone formation and bone resorption are in a dynamic balance, which maintains bone structure, keeps bone morphology unaffected, and regulates the body's bone metabolism [10]. Bone remodeling relies on the tight coupling of bone formation and bone resorption, which are carried out by osteoblasts and osteoclasts, to maintain homeostasis. This dynamic balance between the two cell functions is what allows OP to take place [11]. Several metabolic bone diseases are caused when osteoblastic bone formation and osteoclastic bone resorption are imbalanced, including OP [12].

As the process of bone remodeling progresses, cell-to-cell contacts, extracellular matrix, and cytokines play a key role in the spatial and temporal interaction between osteoblast-mediated bone formation and osteoclast-mediated bone resorption [13]. Osteoblasts originate from bone marrow mesenchymal stem cells (BMSCs), and they synthesize bone matrix and produce bone tissue [14]. Wnt and bone morphogenetic protein (BMP) signaling pathways are required for differentiation of BMSCs into osteoprogenitor cells, which are mediated by osteoblast-specific transcription factors such as runt-associated transcription factor 2 (RUNX2) and osterix (Osx), etc [15–17]. There are several pathways by which osteoblasts affect osteoclast development, differentiation, and apoptosis, including intercellular contact pathways such as Fas/FasL, Ephrin2/ephB4 and semaphorin3A/neuropilin-1, as well as cytokine secretion pathways such as OPG/RANKL/RANK and RANKL/LGR4/RANK [18]. Osteoclasts are derived from the monocyte/macrophage hematopoietic lineage [19]. Osteoclast precursors and mature osteoclasts are stimulated by macrophage colony-stimulating factor (M-CSF) in the presence of its receptor c-Fms [20].NF-κB receptor activator ligand (RANKL) is a key factor in osteoclastogenesis and differentiation [21]. RANKL can be anchored on the surface of the cell membrane in a membrane-bound form, or secreted in the form of a soluble protein, and binds to RANK distributed on the surface of osteoclast precursors to activate downstream signaling pathways related to proliferation and differentiation [22]. Osteoprotegerin (OPG), as a decoy receptor, can bind to RANKL and competitively block its binding to RANK, thereby inhibiting the excessive activation of RANKL [23]. Osteoclasts and their precursor cells also affect osteoblastic bone formation by secreting transforming growth factor-β (TGF-β), insulin-like growth factor 1 (IGF-1), complement C3a, semaphorin 4D, and vesicular ATPase D2 isoforms [18].

In summary, osteoblasts, osteoclasts, and cytokines form a complex and precise coordination network to maintain bone metabolism. When bone resorption exceeds bone formation in an imbalance, OP will develop.

2.2. Gut microbiota

As the “second largest genome in human history,” GM is a rich and diverse microbial community composed of bacteria, fungi, viruses, and protozoa [24]. It is estimated that the human gut contains more than 1000 different types of bacteria, including Bacteroides, Firmicutes, Actinomycetes, Proteobacteria, and Verrucomicrobia [25]. Multiple biological processes are regulated by GM, such as gut physiology, nutrient absorption, host growth, energy balance, metabolic function, immune system function, brain-behavioral function, and inflammation [26–31]. The composition differences of the GM have also been shown to be associated with a variety of complex human diseases, including obesity, irritable bowel syndrome (IBS), diabetes, colorectal cancer, Parkinson's disease, and rheumatoid arthritis [32–37].

The GM plays a crucial role in coordinating the dynamic processes of osteoblastic bone synthesis and osteoclastic bone resorption in the skeletal system (Fig. 2 B). As is shown in Fig. 2 B, GM influences bone mass via mechanisms including 1) affecting intestinal barrier; 2) producing host metabolites; 3) altering immune system; 4) impacting endocrine system. Bone metabolism and bone mineral absorption are closely related to GM under physiological conditions, but it is also pathologically involved in OP progression. From the perspective of physiopathology, we discussed GM's impact on skeletal homeostasis and OP, which involves intestinal barrier, host metabolism, immune system, and endocrine system.

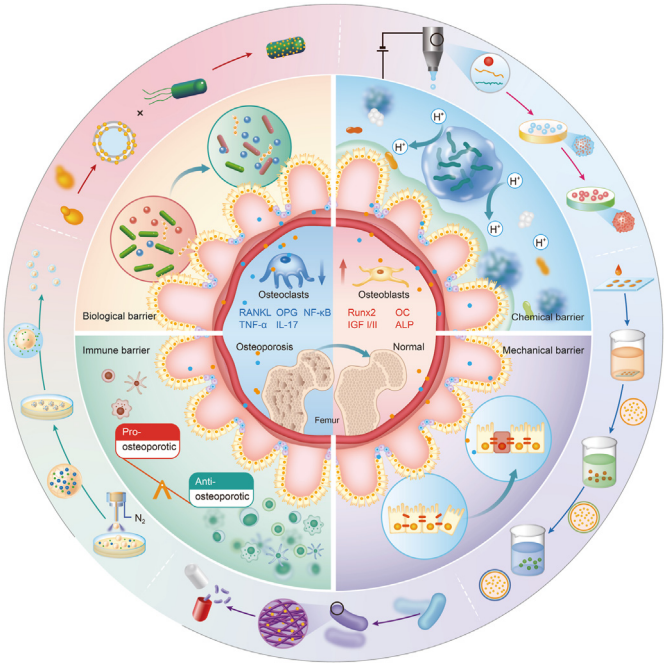

Fig. 1. Living probiotic biomaterials for OP and their strategies aimed at intestinal barriers.

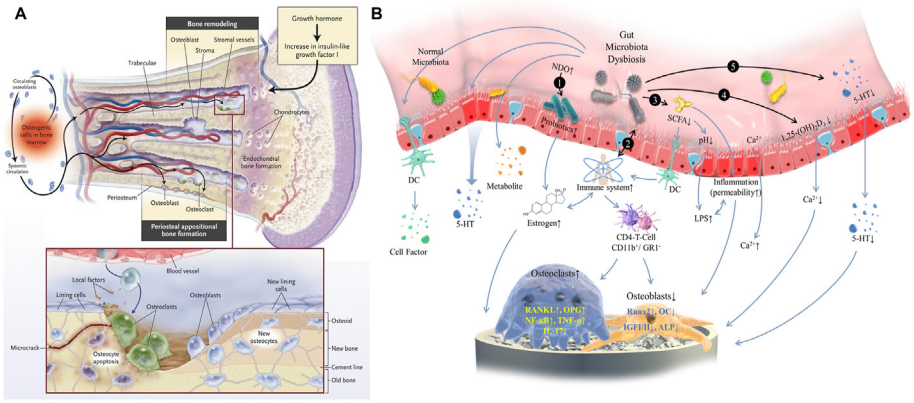

Fig. 2. Bone metabolism, modeling and remodeling and the mechanism of GM in the development of OP. A) The role of osteoblasts and osteoclasts in bone modeling and remodeling. In basic multicellular units, osteoclasts collaborate with osteoblasts to model bone. Remodeling of the bone is required to maintain calcium homeostasis and restore microdamage and microcracks. By uncoupling osteoblasts and osteoclasts, uncoupled bone shape is determined B) The mechanism of GM in OP development. GM influences bone mass via mechanisms including 1) affecting intestinal barrier such as changing intestinal permeability; 2) producing host metabolites such as SCFAs; 3) altering immune system such as increasing the expression of inflammatory cytokine responses; 4) impacting endocrine system such as regulating the level of endocrine factors.

2.3. Gut microbiota-intestinal barrier-bone metabolism

The dysregulation of GM compromises the integrity of the intestinal barrier, impairs the permeability of intestinal epithelial cells, facilitates pathogen translocation into the bloodstream, and causes inflammation throughout the body [38]. This plays a key role in bone metabolism, and any change in gut permeability may cause osteoclast factors produced by gut epithelial immune cells to enter the bloodstream, increasing the level of osteoclastic factors, affecting bone homeostasis, and reducing bone density [39]. For example, lipopolysaccharide (LPS), which is a part of Gram-negative bacteria's cell walls, can upregulate the permeability of the intestinal barrier and facilitate the translocation of GM metabolites [40]. In an OVX-induced OP rat model, the serum LPS increased by 20% after the intestinal barrier function was impaired, accompanied by significant periodontal bone loss [41]. Conversely, GM-derived short-chain fatty acids (SCFAs) can stimulate the expression of intercellular tight junction proteins, resulting in tighter intercellular binding that reduces intestinal permeability [42]. Therefore, GM-regulated intestinal barrier homeostasis plays an important role in bone metabolism.

2.4. Gut microbiota-host metabolites-bone metabolism

The glycolysis of indigestible dietary fiber by GM produces SCFAs, mainly acetate and propionate, which have important regulatory effects on bone metabolism and bone mass [43]. SCFAs can induce metabolic remodeling of osteoclasts, reduce the expression of osteoclast-related genes like TRAF6 and NFATc1, inhibit osteoclast differentiation, and reduce bone resorption [44]. Also, SCFAs play a key role in regulating bone formation by promoting osteogenic differentiation of BMSCs and improving bone mineral density [45,46]. Tyagi et al. [47] showed that butyrate enhances bone anabolism by increasing the level of TGF-β1 in $CD4^+$ T cells and Treg cells and promoting the generation of Wnt10b in adjacent $CD8^+$ T cells. Further, SCFAs can increase intestinal permeability and decrease intestinal pH, which allows calcium to be absorbed more readily. It has been shown that feeding mice SCFAs and a high-fiber diet can increase bone mass, prevent bone loss, and significantly improve OP in mice [48].

LPS is the main component of the cell wall of Gram-negative bacteria, which can be recognized by intestinal epithelial cells and intestinal immune cells [49] by activating transforming growth factor (TGF) and toll-like receptor 4 to stimulate inflammation [50], thereby affecting bone metabolism. LPS is more likely to enter the bloodstream when intestinal permeability is increased due to GM disturbance [51]. The LPS particles implanted by Smith et al. [52] were timed released and induced bone loss in rats' tibias and femurs, accompanied by an increase in inflammatory mediators in bone metaphysis, such as interleukin-1, COX-2, and tumor necrosis factor (TNF), etc. It was also found that LPS significantly reduced the volume of trabecular bone and the mineral density of the lumbar vertebra [53].

2.5. Gut microbiota-immune system-bone metabolism

It is widely accepted that both the immune system and GM play crucial roles in maintaining bone homeostasis, and thus the field of "bone immunology" has evolved into "bone microbiology", which links the pathophysiology, microbiology, and immunology of bone with its effects on bone development, bone aging, and pathological bone loss [54–56]。

A balance between regulatory T (Treg) cells and T helper 17 (Th17) cells is crucial in the regulation of bone immunity. By targeting Th17 cells or Treg cells, GM can modulate bone immunity [57]. The segmental filamentous bacteria (SFB) [58] in the gut encode antigens that can be recognized by T cell antigen receptors (TCRs) specifically expressed by Th17 cells, which negatively regulates skeletal maturation [59]. Clostridium and Bacteroides are enriched in the large intestine and can induce Treg cell responses [43]. *Helicobacter pylori* can promote the production of antigen-specific Treg cells in the colon [60]. c-MAF-dependent Treg cells respond specifically to Helicobacter hepatica and mediate immune tolerance [61]. Bifidobacterium and Streptococcus thermophilus can increase the concentration of TGF-β, thereby regulating the differentiation of Tregs/Th17 cells [62]. GM and its derived metabolites act as bridges between Th17 cells and Treg cells, promoting or inhibiting each other in bone metabolism, thus stabilizing immunity and inflammation in a dynamic manner [47,63].

Upon encountering foreign pathogenic microorganisms in the gut, the body's innate immune system recognizes them via pattern recognition receptors (PRRs), such as the NOD-like receptors (NLRs) [64]. When NOD1 and NOD2 bind to GM polypeptides, they activate the NF-κB signaling pathway, resulting in the gene expression of chemokines and cytokines that affect bone metabolism [65,66]. NOD1 induces pro-inflammatory signaling by recognizing peptidoglycan mainly present in Gram-negative bacteria [67]. As a result of binding to both Gram-positive and Gram-negative peptidoglycans, NOD2 activates NF–B signaling, causing osteoclasts to release inflammatory substances which trigger osteoclast formation [68]. In a porphyromonas-induced periodontitis model, Prates et al. [69] validated NOD2's effect on bone resorption. In a NOD2-knockout mouse model of actinobacteria-induced periodontitis, Souza et al. [68] demonstrated that actinobacteria-induced bone resorption is dependent on NOD2 signaling, which is important for osteoclast differentiation and inflammatory bone resorption.

2.6. Gut microbiota-endocrine system-bone metabolism

It has been proposed that GM is a virtual "endocrine organ" that influences the synthesis of neuroendocrine hormones such as cortisol, gut hormones, and neurotransmitters [70]. By virtue of this endocrine influence, GM has sophisticated and crucial effects on bone metabolism and bone health.

Bone metabolism can be affected by GM through sex hormones like estrogen and androgen. In postmenopausal OP, estrogen levels decrease,

resulting in rapid bone loss. The loss of bone associated with estrogen deficiency is GM-dependent and can be prevented by supplementation with intestinal probiotics, such as Lactobacillus rhamnosus [71]. Sex hormones can also be affected by GM's own metabolism. For example, the intestinal commensal Clostridium can convert glucocorticoids into androgens through enzymes such as hydroxysteroid hydrolase [72], and Slagella enterica can regulate estrogen levels [73].

GM plays an important role in regulating the synthesis of serotonin (5-HT), which functions in a complex and contradictory manner in the development and formation of bones [74]. Although 5-HT produced in the brain acts as a neurotransmitter to promote bone formation and inhibit bone resorption, it inhibits bone formation in the blood circulation [75]. In germ-free (GF) mice, 5-HT levels decreased, while trabecular bone volume increased [76]. However, transplanting spore-forming bacteria (Sp) into GF mice can completely restore 5-HT levels in serum, colon, and feces [77]. Furthermore, Corynebacterium, Streptococcus, and *Escherichia coli* can also produce 5-HT [78].

Glucagon-like peptide 1 (GLP-1) and its analogs can affect bone metabolism by promoting the proliferation and differentiation of osteoblasts [79]. By producing SCFAs [80] or binding bile acids [81], GM induces GLP-1 secretion from enteroendocrine cells, thereby increasing bone mineral density, bone strength, and trabecular bone volume in OVX rats [82,83]. In addition, GM can affect the growth and differentiation of osteoclasts, osteoblasts and chondrocytes by regulating the secretion of insulin-like growth factor 1 (IGF-1) [84]. Compared with GF mice, conventionally reared neonatal mice have higher levels of IGF-1 in the blood circulation [85], and the transplantation of conventional GM into GF mice can significantly increase IGF-1 levels and promote bone formation and bone remodeling [86].

Overall, GM affects endocrine functions, host self-metabolites, immune systems, and intestinal barrier functions, thereby influencing bone homeostasis and contributing to OP by altering the relative activity of osteoclasts and osteoblasts [87].

3. Living probiotics as a treatment for osteoporosis

The clinical treatment for OP is currently divided into three main categories: 1. bone resorption inhibitors (estrogen, calcitonin, bisphosphonates, etc.); 2. osteogenesis drugs (parathyroid hormone-related drugs and peptide analogs such as Teripat, etc.); 3. bone mineralization drugs (calcium, vitamin D, etc.) [88]. Despite good results in treating OP, there are potential side effects and off-target effects in non-bone sites, leading to adverse reactions such as osteonecrosis of the jaw, atypical femoral fractures, and cardiovascular events [89,90]. Since many drugs for the treatment of OP have severe side effects and are not suitable for long-term use, it is of great significance to find more effective and safer treatment strategies. GM disorders are one of the important pathogenic factors for OP, which makes developing GM-targeted treatment strategies highly appealing. It is expected that probiotics will eventually become a highly effective alternative to the current clinical drugs for OP treatment.

Probiotics, which are primarily beneficial bacteria in GM, exert beneficial effects on the host through four main mechanisms: (1) competitive colonization and reproduction, eliminating pathogenic bacteria; (2) improving intestinal barrier function such as strengthening the epithelial cell walls and increasing mucosal viscosity; (3) immune regulation by reducing pro-inflammatory factors IL-6 and TNF-α, etc; (4) secretion of antimicrobial peptides, SCFAs, and other antibacterial substances into the serum [91].

It has been widely reported that probiotics are key components of GM in regulating bone metabolism [92]. Several probiotics (Table 1) have been shown to promote bone growth, bone mineralization and bone structure in animals, such as rodents [93,94]. Furthermore, probiotics have been extensively studied for their therapeutic effect in OP caused by estrogen deficiency, and the mechanisms are as follows: (1) Probiotics such as Lactobacillus and Bifidobacterium can enhance the absorption of

Table 1
Effects of probiotics on bone (animal studies).

Probiotic strain	Animal model	Bone effects	Reference
Bacillus licheniformis and Bacillus subtilis	Broiler chicks	↑Thickness of the medial and lateral wall of the tibia, tibiotarsal index, percentage ash, phosphorus content	[102]
Bifidobacterium longum (ATCC 15707)	Wistar rats	↑Tibial calcium, phosphorus, magnesium content ↑Biomechanical properties	[103]
Bifidobacterium longum-fermented broccoli	Wistar rats	↓Osteoclast differentiation	[104]
Lactobacillus reuteri (ATCC 6475)	Male mice	↑Trabecular bone parameters in the distal femur metaphyseal and lumbar vertebra ↑Osteoblast serum markers and dynamic measures of bone formation	[105]
	Male type 1 diabetic mice	↑Wnt10b and osteoblast maturation markers	[106]
	Female mice (inflammatory state)	↑OPG and IL-10 ↓RANKL	[107]
	Female mice (estrogen deficiency)	↓Osteoclast bone resorption markers and activators (Trap5 and RANKL)	[96]
Lactobacillus rhamnosus (HN001)	Sprague-Dawley rats (OVX)	↑Mineral bioavailability, bone mineral density and mineral content.	[108]
Lactobacillus rhamnosus GG (ATCC 53103)	C57BL6/J Mice	↑Trabecular bone microarchitecture, cortical bone volume and biomechanical properties ↓Osteoclastogenesis-related cytokines	[109]
Lactobacillus rhamnosus UBLR-58 (MTCC 5402)	BALB/c mice	↑IL-4, IL-10 and IFN-γ ↓IL-6, IL-17, TNF-α and RANKL	[97]
Lactobacillus paracasei and Lactobacillus plantarum	Female mice (OVX)	↑Bone mineral density and the microstructure of femoral bone	[110]
Lactobacillus helveticus	Spontaneously hypertensive male rats	↑Bone mineral density and bone mineral content	[111]
L. casei 393	Sprague-Dawley rats (OVX)	↑Bone weight, bone mineral density and bone breaking force ↓Tartrate resistant acid phosphatase	[112]
Lactobacillus casei and Lactobacillus acidophilus	Wistar rat (induction of arthritis)	↓bone damage	[113]
Lactobacillus paracasei DSM13434 (single)/ Lactobacillus paracasei DSM13434, Lactobacillus plantarum DSM 15312 and DSM 15313 (mix)	Female mice (OVX)	↑Cortical bone mineral content ↓Cortical bone loss and bone resorption	[114]
Lacticaseibacillus paracasei DSM13434, Lactiplantibacillus plantarum DSM	Female mice (OVX)	↑Trabecular thickness in the proximal metaphyseal region of tibia	[115]

(*continued on next page*)

Table 1 (*continued*)

Probiotic strain	Animal model	Bone effects	Reference
15312, and DSM 15313		↑Cortical thickness and cortical area of the middiaphyseal part of the tibia	

vitamin D, increase the number of osteoblasts, and at the same time suppress the differentiation of osteoclasts, thereby delaying the reduction of bone mass in OVX-induced estrogen-deficient OP rats [95,96]. (2) Probiotics can improve intestinal permeability damaged by estrogen deficiency, thereby relieving OP. When administered twice-weekly, Lactobacillus rhamnosus GG or the commercial probiotic supplement VSL#3 can reduce intestinal permeability, inhibit intestinal and bone marrow inflammation, thereby reducing bone loss [71]. (3) Probiotics can reduce osteoclastic factors by immunomodulation, thus reducing bone loss caused by estrogen deficiency. In an OVX-induced mouse model of postmenopausal OP, Sapra et al. [97] investigated the effects of oral probiotic Lactobacillus rhamnosus (LR) on bone health. They found that oral administration of LR resulted in a significant increase in pro-osteoclastic $CD4^{+}Ror\gamma t^{+}$Th17 cells in immune tissues such as bone marrow, spleen, and lymph nodes, while anti-osteoclastic $CD4^{+}Foxp3^{+}$-Tregs cells and $CD8^{+}Foxp3^{+}$Tregs cells significantly increased, thereby reducing bone loss and increasing cortical bone and trabecular bone in mice. In addition, LR decreased serum levels of osteoclastic factors (IL-6, IL-17, and TNF-α) and increased serum levels of anti-osteoclastic factors (IL-4, IL-10, IFN-γ).

Probiotics have also been shown to regulate bone metabolism in clinical trials, not only animal experiments. The representative clinical trials are as follows: (1) In high cholesterol adults, oral administration of the probiotic L. reuteri NCIMB 30242 increases serum 25-hydroxyvitamin D levels. It can be converted into 1,25-hydroxyvitamin D3, which promotes calcium absorption in the small intestine, accelerates bone mineralization, and relieves OP [98]. (2) In a randomized, placebo-controlled, double-blind clinical trial, Lactobacillus reuteri ATCCPTA 6475 can reduce bone loss in elderly women [99]. (3) In another clinical trial, isoflavone aglycone-rich and probiotics-rich red clover extract can effectively improve bone turnover and promote estrogen metabolites, thus relieving bone loss in 78 postmenopausal OP patients [100]. (4) Furthermore, Jafarnejad et al. [101] found in a randomized, double-blind, controlled clinical trial that oral supplementation with multiple probiotics reduced the rate of bone turnover and improved bone health in postmenopausal women. Probiotics' clinical feasibility in preventing bone loss and OP has been preliminary confirmed in these clinical trials. However, it is still necessary to investigate the intrinsic mechanisms by which probiotics treat OP in humans. There are four types of defense barriers with different structures and biological functions between the GM and the lamina propria of gut: chemical barriers, biological barriers, immune barriers, and mechanical barriers. (1) The chemical barrier is the first line of defense against the invasion of microorganisms and pathogenic factors, which covers the epithelium by forming protective gel-like substances. It mainly includes mucus, immunoglobulins, bacteriostatic substances and various digestive juices and enzymes. (2) The biological barrier consists of trillions of interdependent and interconnected microorganisms that colonize the gastrointestinal tract. These microbes, including bacteria, fungi, viruses, and archaea, maintain a symbiotic relationship with the host, which compete for nutrients and colonization space in the gut and produce antimicrobial compounds, thereby resisting invading pathogens. (3) The immune barrier is formed by intestinal lymphoid cells and their secretions, such as cytokines and immunoglobulins. There are three distinct tissue compartments that contain intestinal lymphoid cells, including the gut-associated lymphoid tissue (GALT), the mucosal lamina propria, and the epitheliums. GALT is the largest lymphoid tissue in the body, which includes Peyer's patches, mesenteric lymph nodes, and isolated lymphoid follicles. (4) The mechanical barrier mainly consists of intestinal epithelial cells and tight junction-related proteins (TJs), which seals the spaces between cells. It not only prevents the gut from potentially harmful substances, but also maintain permeability of the intestinal epithelium. TJs are composed of transmembrane proteins, including occludins, claudins, junctional adhesion molecules, tricellulins and zonula occludens.

Limited by these four layers of defense, simple oral probiotics have limitations: 1. constantly updated intestinal mucus in the gastrointestinal tract makes it difficult for probiotics to colonize; 2. rapid gastrointestinal peristalsis (30h from the mouth to the anus) shortens the effect of probiotics; 3. continuous production of acidic gastric juices can easily inactivate probiotics. Therefore, the bioavailability and therapeutic efficacy of probiotics are confronted with enormous challenges. However, biomaterials can be used to intelligently regulate four major barriers of the gut, thereby better exerting the regulatory effects of probiotics. Combining suitable biomaterials and probiotics can create oral probiotic bioactive smart materials that are expected to break through the limitations of current oral drugs and safely affect GM in the long term.

4. Therapeutic strategies of living probiotic biomaterials

4.1. Living probiotic biomaterials for chemical barriers

The rapid peristalsis of the gastrointestinal tract and the constant renewal of mucus within the gastrointestinal tract make it difficult for probiotics to colonize and play a beneficial role. Mucins are glycosylated proteins with negatively charged oligosaccharide chains that are found in the mucus on surface of the gut [116]. Biomaterials designed for intestinal mucus can increase the retention time of probiotics in the intestine and avoid rapid clearance. (1) The viscosity and elasticity of intestinal mucus determine its transport capacity and are fundamental rheological properties [117,118]. Depending on the surfactant's anionic and nonionic properties, surfactants have different effects on intestinal mucus rheology. An anionic surfactant, sodium dodecyl sulfate, can make mucus more viscous and elastic, whereas a non-ionic surfactant, Tween 80, can decrease these qualities [119]. (2) Electrostatic interactions increase the retention of positively charged biomaterials in intestinal mucus over negatively or neutrally charged biomaterials [120]. The interaction between mucus and biomaterials can be further enhanced by hydrophobic interactions and hydrogen bonds. Therefore, biomaterials that are negatively or neutrally hydrophilic, such as polyethylene glycol or its derivatives, have been extensively used for mucus penetration [121]. Probiotics are also subject to harsh chemical environments, including stomach acid, antibiotics, etc. These external gastrointestinal conditions can be addressed through characterized biomaterials for enhancing the viability and therapeutic effect of probiotics. (3) Probiotics can be protected from chemical factors such as antibiotics and stomach acids by nano-level protective shells. For example, Pan et al. [122] have demonstrated that bacteria are protected against antibiotics by nanoarmor, a coating made of tannic acids and ferric ions (Fig. 3 A&B). Using such a platform, the potency of probiotics can be enhanced and antibiotics' negative effects can be avoided. (4) Biomaterials can improve probiotics' resistance to gastric acid as well as improving their adhesion to intestinal mucus. The cysteine on the surface of probiotics can be disulfide-bonded with thiolated polymers (thiopolymers). Through the exchange reaction of sulfhydryl or disulfide bonds, thiopolymers can also form disulfide bonds with the mucogel layer [123,124]. Hence, thiopolymers can be used as a bridge connecting probiotics and mucus to achieve long-term adhesion of probiotics to mucus. Using thiolated oxidized konjac glucomannan (sOKGM) microspheres, Liu et al. [125] developed a mucoadhesive oral delivery system for probiotics, which significantly improved gastric acid resistance as well as intestinal adhesion and colonization, thereby optimizing the probiotic function. (Fig. 3 C&D). (5) Hydrogel microspheres with probiotics-loaded therapy have been considered an effective and safe strategy to promote the low survival rate under harsh

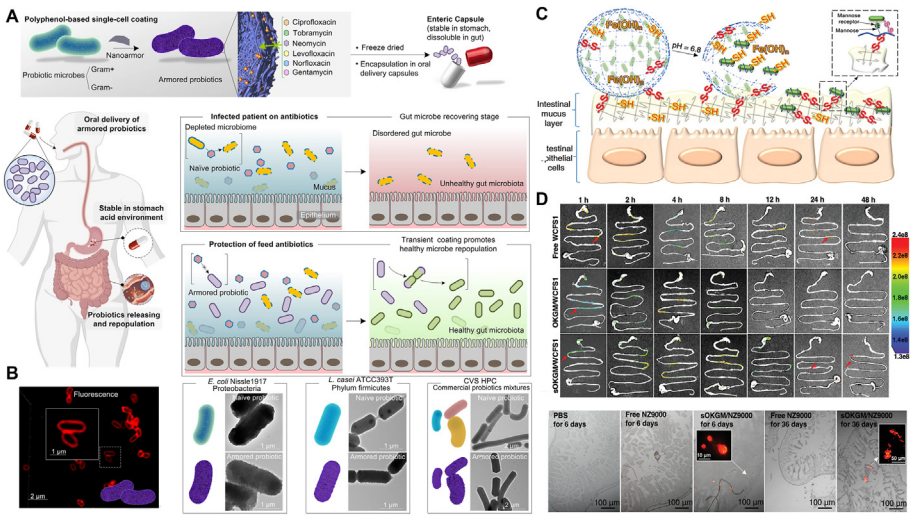

Fig. 3. Throughout the harsh gastrointestinal environment, probiotics are armored against chemical barriers such as low pH, antibiotics, and constantly changing mucus. A) Probiotics are protected in the gastrointestinal tract from antibiotics by a polyphenol-based single-cell coating (nanoarmor) designed for oral administration. By protecting probiotics from antibiotics, the nanoarmor facilitates the repopulation of healthy microbes. B) CLSM (confocal laser scanning microscopy) images of armored EcN. Nanoarmor, and the TEM (transmission electron microscopy) images of naïve or armored EcN, L. casei and CVS HPC. Reprinted with permissions from Ref. [122], Copyright 2022, nature publishing group. C) sOKGM microspheres were endowed with gastric acid resistance and pH responsive characteristics and served as a bridge between probiotics and mucus, thereby improving their mucoadhesion through disulfide bonds as well as mannose-probiotic receptor binding. D) The in vivo imaging system and fluorescence signals of the NZ9000/sOKGM were used to assess enhanced colonization and proliferation of sOKGM/Probiotics. Reprinted with permissions from Ref. [125], Copyright 2020, Wiley-VCH.

stomach conditions. For example, Wang et al. [126] proposed a novel NO-responsive poly-γ-glutamic acid (γ-PGA) hydrogel microcapsule (NRPM) strategy based on a droplet microfluidic technology platform, which was a promising approach for improving the efficacy of orally administered probiotics in patients with colonic inflammatory bowel disease (IBD) (Fig. 4A–E). As shown above, probiotics were encapsulated in NRPMs, which were endowed with the ability of gastric acid resistance and smart targeting to withstand chemical barrier and treat IBD. It is well known that biomaterials targeting intestinal chemical barriers have been applied to various disease, such as IBD, arthritis, and cancer, however, strategies for targeting OP remain to be further explored and studied.

4.2. *Living probiotic biomaterials for biological barriers*

The intestinal biological barrier is composed of bacteria, fungi, viruses and other organisms. Forming the external barrier of the body, it consists of a complex interconnected system. Through biological competition, it can prevent harmful bacteria from damaging the host, but it can also inhibit beneficial bacteria from performing their functions. Reprogramming the GM has been achieved through various methods, including transplanting the fecal microbiota, enteral nutrition, using probiotics, prebiotics, and postbiotic supplements, etc. Probiotic-carrying biomaterials can be designed against biological barriers, resulting in improved competitiveness of probiotics and reversing gut dysbiosis in diseases. The composition and structure of the gut microbiota can be modulated by oral administration of nanomaterials, such as TiO_2 nanoparticles [127]. Biomaterials based on polysaccharides can modulate the diversity and abundance of GM. It has been shown that chitosan intake improved menopausal symptoms in estrogen-deficient rats by improving gut microbiota diversity and composition [128]. Polyphenols can also be

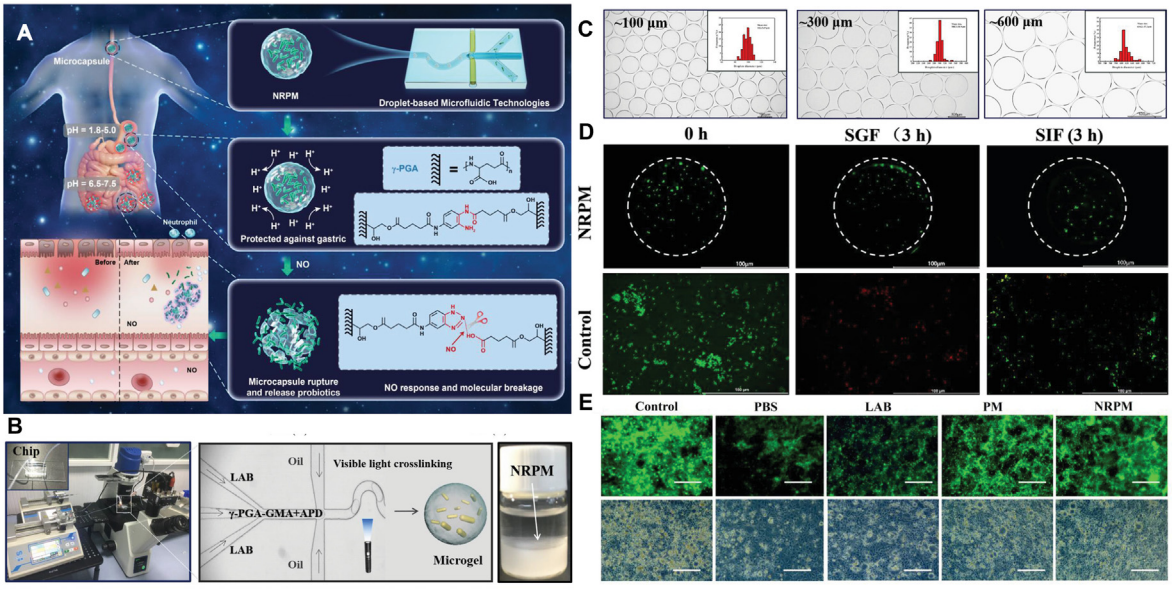

Fig. 4. Probiotics encapsulated in NRPMs with gastric acid resistance and smart targeting to withstand chemical barrier and treat ulcerative colitis. A) NRPMs targeting the gastrointestinal tract provides improved protection against lactic acid bacteria (LAB). B) Microcapsules encapsulating LAB are based upon droplet microfluidic technology. C) Microfluidic encapsulation allows the manufacture of hydrogel microspheres of varying diameters (100–600 μm). D) Stress conditions in the gut could be effectively prevented by NRPMs. E) The epithelial barrier function of Caco-2 monolayers was improved by NRPMs. Reprinted with permissions from Ref. [126], Copyright 2022, Wiley-VCH.

engineered as biomaterials involved in the regulation of gut microbiota. In rats, curcumin, a plant-derived diketone compound, partially reversed the distribution, structure, and diversity of GM induced by estrogen deficiency [129]. Bacterial metabolites have been shown to manipulate GM to protect against colitis. In order to modulate GM, Yang et al. [130] have developed a novel synergistic prebiotic/postbiotic delivery microcapsule (IPA@MC), which significantly increased the overall abundance and richness of bacteria that produce SCFAs. (Fig. 5 A&B). Similarly, through combining probiotics and prebiotics, Zheng et al. [131] developed prebiotics-encapsulated probiotic spores (spores-dex) as a safe microbiota-modulating material. (Fig. 5 C&D). Combining highly safe probiotics and prebiotics with functionally rich ingredients can be an effective way to treat gastrointestinal disorders. In mice fed with oligofructose-inulin mixture, Kleessen et al. [132] found that probiotics (including Bifidobacterium and Bacteroides) increased and the intestinal mucosal barrier was stabilized by GM. In order to regulate the GM efficiently and precisely, Yang et al. [133] developed a bionic regulator (CaWO4@YCW) that suppressed the abnormal proliferation of *E. coli* and boosted probiotics growth (Fig. 6A–D). CaWO4@YCW modulates the dysregulated microbiome by breaking the eco-niche of pathogenic bacteria to demonstrate remarkable therapeutic benefits by eliminating pathogenic bacteria while increasing probiotics abundance synergistically. There has been little attention paid to biomaterials that modulate GM and metabolites to affect OP. As described in Table 1, there are many probiotics that can promote osteogenesis, but it remains to be investigated that how biomaterials can enhance the metabolism and reproduction of probiotics, change the biological structure of the intestinal community, and promote osteogenesis and inhibit bone loss.

4.3. Living probiotic biomaterials for immune barriers

As the largest immune organ in the body, the gut contains a large number of intestinal resident immune cells, including microfold cells (M cells), T cells, macrophages, and dendritic cells [134]. Multiple aspects of human health can be improved by modulating the GM via oral delivery of living probiotics. The therapeutic effects of probiotics can be amplified through a dietary probiotic-based engineering strategy as a safe and straightforward approach to in situ modulation of the gut microbiome. The binding of specific ligands to receptors allows ingested biomaterials to influence the function of different types of immune cells, thereby treating a variety of different diseases. (1) Biomaterials interfering with DCs can affect intestinal antigen presentation. PLGA-based nanoparticles and chitosan are both effective carriers for antigen delivery to DCs to induce immune responses, making them suitable for mucosal vaccine [135]. (2) Biomaterials can be used to stimulate and utilize M cells, which have poor mucus coverage and are responsible for antigen transport and induction of mucosal immunity. Glucan particles (GPs) derived from Baker's yeast cell wall are rich in β-1,3-d glucan and can act as ligands to interact with M cell membrane receptors. Therefore, M cells can be used to deliver biomacromolecules such as siRNA, DNA, peptides, and even bacteria through GPs [136,137]. For example, Lin et al. [137] delivered live probiotics into Peyer's patches to modulate gut microbiota via mucosal immunity (Fig. 7 A&B). A β-glucan embedding in the yeast membrane camouflages the probiotics, promoting phagocytosis by intestinal epithelial M cells. In response to mucosal immune stimulation, the gut microbial community can be positively modulated, maintaining gut homeostasis and providing defenses against external influences. (3) The gut T-cell immunity can also be influenced by biomaterials. For example, Han et al. [138] developed a colon-retentive oral gel using inulin, a fiber widely consumed in diets. By modulating GM in situ, oral inulin gel induces systemic memory T-cell responses, enhances antitumor effects of immune checkpoint inhibitors, and induces proliferation of commensal microbes in the gut that stimulate T-cell immunity (Fig. 7 C&D). (4) Macrophages are gatekeepers of immune homeostasis in the gut, and biomaterials can help regulate the gates on and off. Through galactose receptor-mediated endocytosis, tea leaves-derived natural nanotherapeutics can be specifically internalized by macrophages [139]. Oral ingestion of this "green" nanotherapeutics enhances diversity and abundance of the GM, effectively suppresses bowel inflammatory responses, and restores disrupted colonic barriers. For another example, colon-targeted adhesive core-shell hydrogel microspheres were designed and fabricated to congregate and induce differentiation of specific type 2 macrophages for regulating the gut immune system in the GM (Fig. 8A–D) [140]. The immune system is considered one of the most critical factors reshaping the GM through crosstalk with the microbiome. Through intestinal immune dysregulation, GM dysregulation can trigger excessive osteoclast activation and promote OP, especially postmenopausal OP caused by estrogen deficiency. It has become

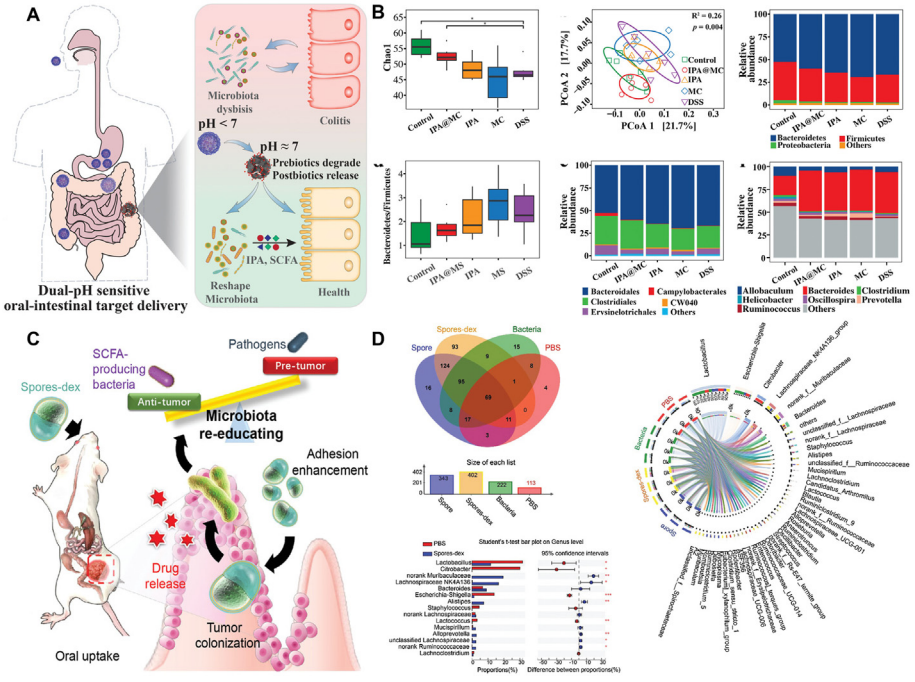

Fig. 5. Intestinal biological barriers can be broken through the manipulation of GM by probiotics and metabolites. A) Dual-pH-sensitive prebiotic and postbiotic microcapsules with a core-shell structure for oral-intestinal target therapy. B) The effects of IPA@MC on GM modulation in colitis mice are illustrated through Chao1 richness analysis, principal coordinate analysis, Bacteroidetes-firmicutes ratio, and species distribution. Reprinted with permissions from Ref. [130], Copyright 2022, Wiley-VCH. C) Probiotics encapsulated in prebiotics regulate GM by converting them from a pro-tumor to an anti-tumor type to suppress colon cancer. D) Venn diagrams and circular plot descriptions showing the interactions between strains and the abundance changes of different genera of the intestine microbial community. Reprinted with permissions from Ref. [131], Copyright 2020, Wiley-VCH.

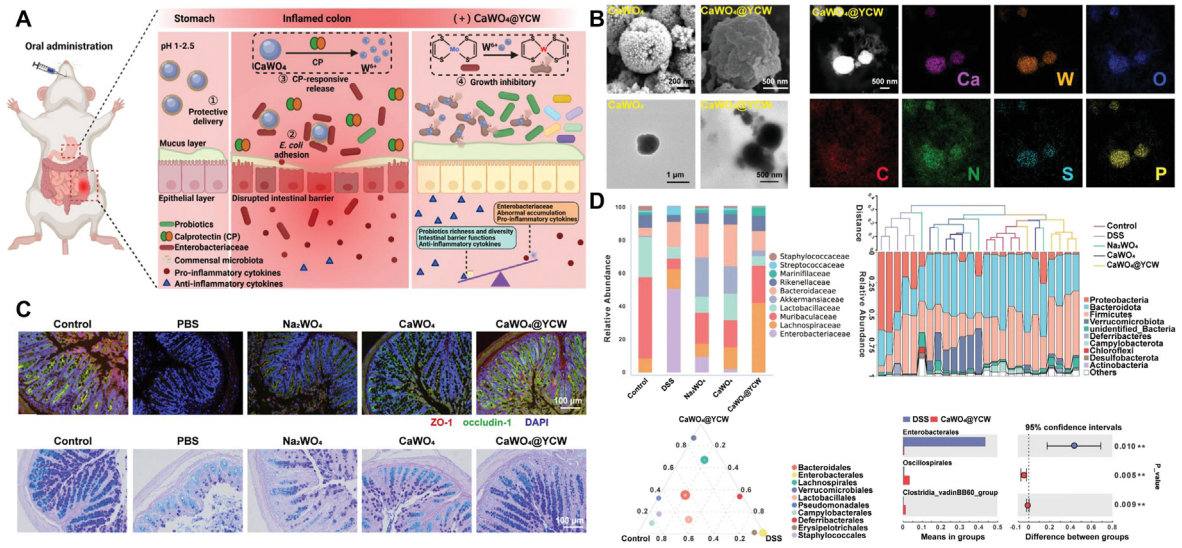

Fig. 6. A bionic regulator (CaWO4@YCW) modulates the dysregulated microbiome by breaking the eco-niche of pathogenic bacteria. A) CaWO4@YCW reprogramed GM to demonstrate remarkable therapeutic benefits by eliminating pathogenic bacteria while increasing probiotics abundance synergistically. B) CaWO4@YCW synthesis and characterization as illustrated by SEM images, TEM images, and element mapping images. C) CaWO4@YCW restored barrier function and suppressed intestinal inflammation as illustrated by immunofluorescence staining (ZO-1 and occludin-1) and AB/PAS staining. D) CaWO4@YCW restored the microbiome dysbiosis at the family, order, and species levels. Reprinted with permissions from Ref. [133], Copyright 2022, Wiley-VCH.

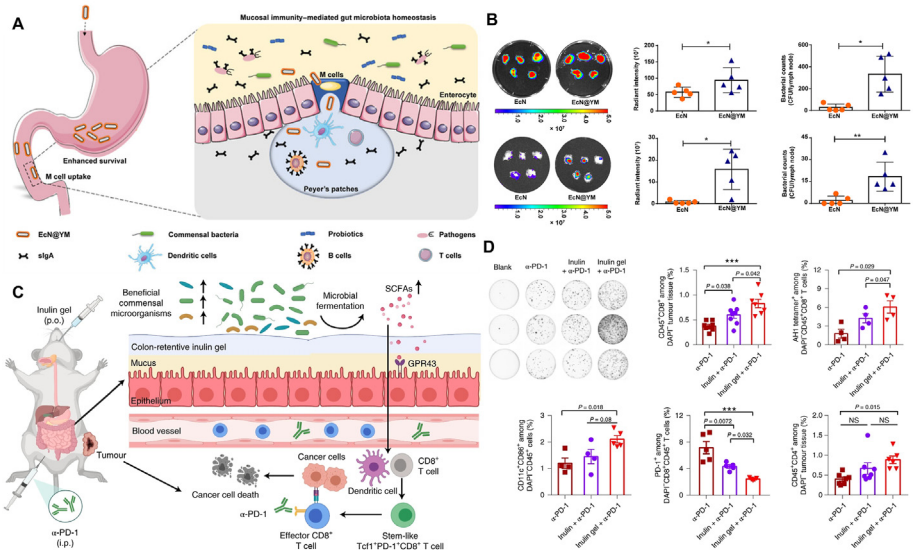

Fig. 7. Faced with intestinal immune barrier, combining probiotics with biomaterials efficiently promotes probiotics to reshape immune homeostasis for treating diseases. A) Mucosal immunity modulates the GM by oral delivery of probiotics (yeast membrane-coated EcN, EcN@YM) into Peyer's patches through M cells. B) Mucosal immune responses elicited by EcN@YM, illustrated by IVIS images, radiant fluorescence intensity, and bacterial counts. Reprinted with permissons from Ref. [137], Copyright 2021, American Association for the Advancement of Science. C) Inulin gel modulates the GM in situ to improve cancer immunotherapy. D) Treatment with oral inulin gel potently enhances α-PD-1's therapeutic efficacy, proved by the frequencies analysis of various immune cells including $CD8^+$ T cells, $CD11c^+CD86^+$ dendritic cells, $PD\text{-}1^+CD8^+$ and $CD4^+$ T cells, etc. Reprinted with permissons from Ref. [138], Copyright 2021, Springer.

increasingly popular among researchers to combine biomaterials with probiotics to regulate intestinal immunity. However, most of their researches focus on tumor immunity, while biomaterials engineering strategies from intestinal immunity to bone immunity are not explored as thoroughly.

4.4. *Living probiotic biomaterials for mechanical barriers*

Through manipulating the opening and closing of tight junction-related proteins (TJs) or controlling intracellular transport, biomaterials can facilitate the passage of biomolecules through the gut's mechanical barrier for synergistic therapy. (1) Chitosan and its derivatives are capable of reversibly opening TJs between adjacent intestinal cells due to their positively charged properties and their effect on integrin receptors (Fig. 9 A&B). Through a PKC-dependent signaling pathway, positively charged chitosan induces redistribution of TJs by interacting with negatively charged ZO-1 and Claudin-4 between intestinal cells [141–143]. (2) Extracellular calcium ions are required for intercellular interactions and maintenance of TJs, while calcium chelators can remove them to open TJs between intestinal epithelial cells. Calcium chelators include nitrophenyl egtazic acid, ethylene glycol tetraacetic acid (EGTA) and ethylenediaminetetraacetic acid, etc. [144]. Chuang et al. [145] fabricated chitosan/poly(γ-glutamic acid)–EGTA conjugate (CS/γPGA–EGTA) nanoparticles (NPs) to chelate Ca^{2+} ions in a controlled manner, which led to a reversible opening of TJs and realized controllable epithelial barrier function (Fig. 9 C&D). (3) Polycations, such as polyethyleneimine and polylysine, can also trigger reversible opening of TJs in an energy-dependent manner. Changes in the cell surface charge density and different structural conformations of polymers are thought to be responsible for the underlying mechanism [146, 147]. Polymers with high molecular weight exerted greater effects on TJs at the same concentration than polymers with low molecular weight. (4) Several types of metal nanoparticles (iron oxide, zinc and alumina, etc.) can trigger the opening of TJs by downregulating Claudin or Occludin,

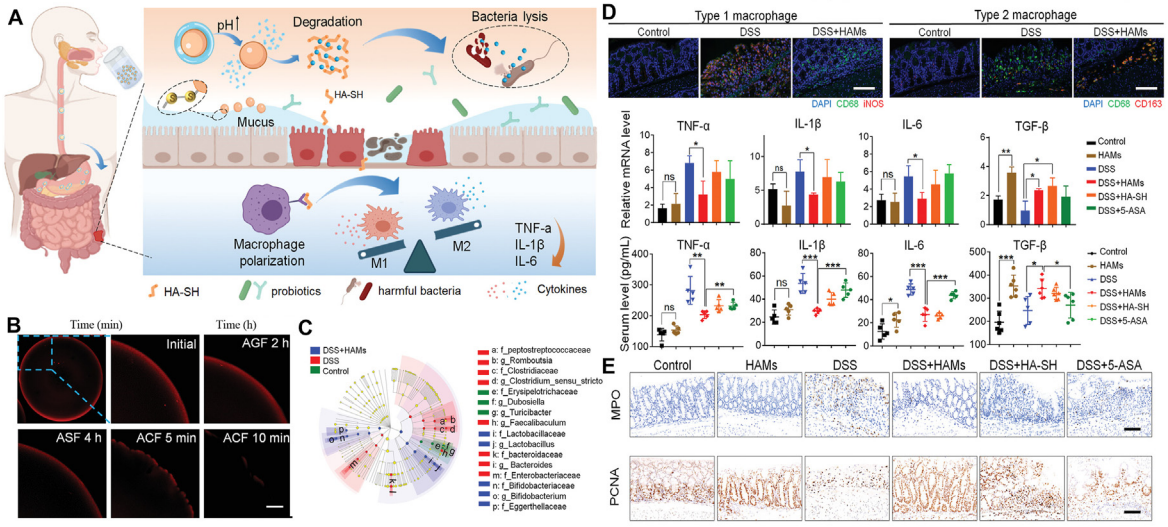

Fig. 8. Oral adhesive hydrogel microspheres targeted at the colon for regulating gut flora and immunity. A) HA-SH-Ag/Alginate-Ca microspheres (HAMs) are orally administered, accumulated in inflamed colon mucosa and modulated gut immunity through M2 macrophage differentiation. B) HAMs degraded with time in artificial gastric and intestine fluids. C) HAMs altered the richness and abundance of GM, illustrated by a cladogram. D) HAMs promoted M2 macrophage differentiation, increased tissue repair-associated TGF-β, and decreased pro-inflammatory cytokines, including IL-6, IL-1β, and TNF-α. E) HAMs inhibit intestinal inflammation (decreased myeloperoxidase) and promote tissue repair (increased proliferating cell nuclear antigen). Reprinted with permissions from Ref. [140], Copyright 2021, Wiley-VCH.

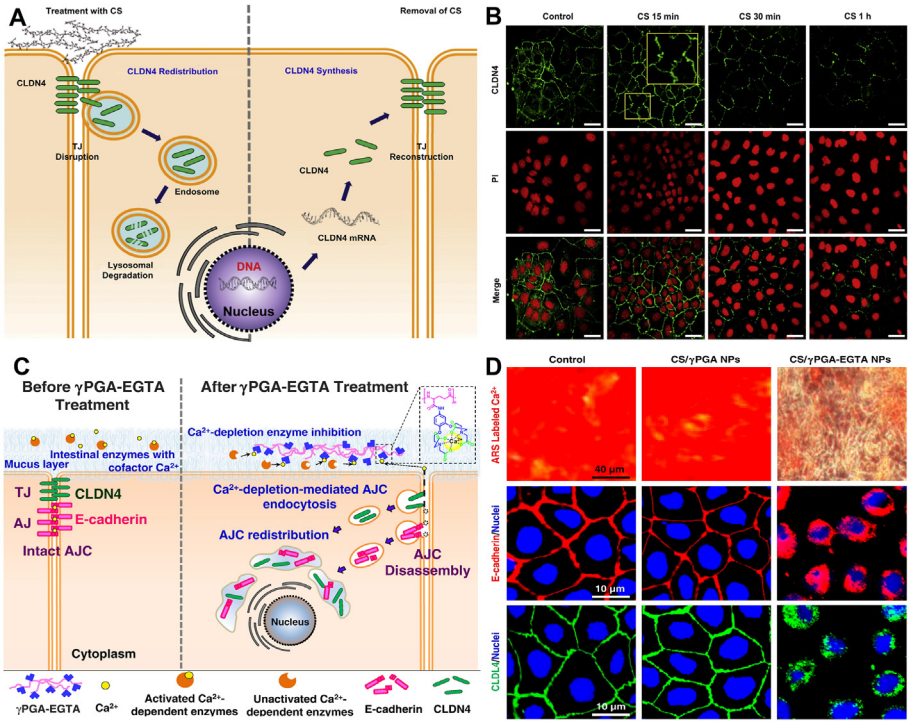

Fig. 9. TJs between intestinal cells are manipulated to alter the integrity of the intestinal mechanical barrier. A) Reversible TJs opening mediated by CS. B) CLDN4 transmembrane protein redistribution by CS to reversibly open epithelial TJs. Reprinted with permissions from Ref. [142], Copyright 2011, Elsevier B.V. C) Intestinal proteases are inhibited and paracellular permeation is enhanced by γPGA-EGTA's specific deprivation of extracellular Ca^{2+} from the intestinal lumen. D) Intestinal cell-cell junctions maintenance relies on extracellular Ca^{2+} levels and EGTA-conjugated NPs changed the translocation of E-cadherin and CLDN4. Reprinted with permissions from Ref. [145], Copyright 2013, Elsevier B.V.

inducing oxidative stress, or triggering inflammatory responses [148, 149]. Silica nanoparticles, for instance, can open TJs and induce reversible intestinal permeability through their interaction with epithelial integrins [150]. Nanoparticles can also be used to penetrate cells, for example, Au nanorods were integrated with interference oligonucleotides to fabricate the spherical nucleic acids, which promote cell internalization efficiency into chondrocytes [151]. Through the interaction between nanoparticles and integrin receptors, the enzyme MLCK is activated, phosphorylating myosin in the cytoskeleton and promoting the redistribution of TJs [152]. Physiologically, proteins of the JAMs and claudin families seal off intestinal epithelial cells, preventing bacteria and bacterial products from penetrating throughout the cell walls and activating the immune system. While in the disease state of OP, especially that induced by estrogen deficiency, transcriptional levels of some claudin family TJs (such as claudin 2, 3, and 15) and JAM3 are reduced, resulting in a decrease in intestinal mechanical barrier integrity [153, 154]. The resulting increased bacterial translocation triggers local and systemic immune responses, leading to increased osteoclastic factors and the formation of OP. Ingestion of probiotics can increase the expression of claudin and JAMs, restoring normal intestinal barrier function,

thereby preventing OP occurrence and development [71]. In order to modulate the intestinal mechanical barrier, engineering biomaterials that synergize with probiotics may be effective strategies. In the field of OP and GM, however, there is still a lack of relevant research.

5. Overview and outlook

Global aging has made OP a public health problem that has serious effects on individuals, families, and society as a whole. However, due to the high cost and side effects of current drugs, many OP patients fail to adhere to the treatment. It is urgent that new strategies that are safe, effective, and inexpensive for the treatment of OP should be developed. In this review, we first reviewed the physiological basis of bone metabolism related to OP, and concluded that GM changes bone metabolism and participates in the formation of OP by affecting intestinal barrier, host metabolites, immune system and endocrine system. Then, we discussed that GM was expected to be a potential therapeutic strategy for treating OP, but there were limitations. Furthermore, we discussed how a smart biomaterial system for providing probiotics to the gut could be constructed based on the chemical barrier, biological barrier, immune barrier, and mechanical barrier. In light of the limitations of current drugs and the therapeutic potential of probiotics for treating OP, it is worthwhile for medical researchers to consider and explore oral probiotic bioactive smart materials as new strategies for treating OP. Hence, we provide the following outlook:

(1) GM serves as a biological link between the external environment and bone health, as well as a communication bridge between the skeletal system and other systems such as the digestive system, endocrine system, and immune system. GM-targeted therapies provide a new therapeutic option for treating bone metabolic diseases like OP.
(2) The regulatory effects of GM on bone metabolism were found to be heterogeneous in animal studies, which may be attributed to differences in animal strains, rearing environments, and physiological and pathological states used in different studies. It is imperative to further understand the complicated relationship between the GM and bone metabolism under different physiological and pathological conditions.
(3) Despite differences in GM community structure, the metagenome of metabolic pathways is stable among individuals. Elucidating the specific regulatory effects of GM on bone homeostasis and analyzing the underlying mechanisms through metagenomic or metabolomic approaches will help to gain a clearer understanding of the mechanisms by which GM affects bone growth, bone turnover, and bone mechanical strength.
(4) As the understanding of the link between GM and OP has grown, the manipulation of GM to treat OP has aroused great interest among researchers. Diversified applications tailored to each individual may be safer and more effective alternatives. Therefore, identifying and isolating strains or specific metabolites with positive osteogenetic effects, and constructing individualized GM therapies based on physiological and pathological characteristics may provide new individualized therapeutic strategies for the treatment of OP.
(5) With a focus on the intestinal chemical barrier, the design of smart biomaterials combined with probiotics may be an effective strategy for OP treatment, which can enhance the materials' resistance to digestive juices, ability to adhere to mucosa, and capacity to colonize the gut.
(6) Aiming at the intestinal biological barrier, the target intestinal flora can be up-regulated by developing biomaterials that are capable of regulating the proportion, structure, and distribution of GM. Combined with probiotics that promote bone formation, this strategy is expected to improve OP in the long run.
(7) Designing biomaterials that can alter intestinal resident immune cells to reshape intestinal immune homeostasis is expected to achieve ideal OP treatment effects by regulating the intestinal immune barrier. It is expected that this strategy combined with probiotics will up-regulate anti-osteoporotic immune factors and down-regulate pre-osteoporotic immune factors.
(8) With the goal of regulating the intestinal mechanical barrier, designing biomaterials that regulate the switch of TJs and combining them with probiotics that can regulate them, is expected to improve the mechanical barrier in OP, leading to the development of a new treatment strategy for OP.
(9) It is anticipated that further innovative thinking should be stimulated to focus on the intestinal barriers, including chemical barrier, biological barrier, immune barrier, and mechanical barrier, as key targets for delivery of probiotics and treatment of OP.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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