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Combined effects of a low-dose multi-target supplement (CaHMB, CBP, and HA) on delaying musculoskeletal aging

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Age-related musculoskeletal diseases underscore the importance of comprehensive dietary interventions. The individual benefits of calcium β-hydroxy-β-methylbutyrate (CaHMB), colostrum basic protein (CBP), and hyaluronic acid (HA) on the musculoskeletal system have been well documented; however, their combined effects remain unclear. This study aims to investigate the effects of combined supplementation with CaHMB, CBP, and HA on age-related musculoskeletal degeneration and to explore the underlying mechanisms, with a particular focus on the muscle–bone axis. Twelve-month-old male C57BL/6J mice received a 6-month dietary intervention. The combination (COM) group was supplemented with CaHMB, CBP, and HA at substantially reduced doses (37.5%, 20%, and 14%, respectively). The COM group showed higher lean mass (18%) and a greater muscle cross-sectional area (35%) compared to the OLD group, with significantly enhanced grip strength by 41% and exercise performances. The COM group presented less decline in whole-body bone mineral density (5%) than the OLD group. The bone microstructure was improved by increasing the bone volume fraction and trabecular thickness/number, while a decrease in trabecular separation was observed in the COM group. Musculoskeletal improvements either matched or surpassed the benefits of individual components. Mechanistically, interventions modulated the key mediators of the muscle–bone axis, significantly upregulating beneficial myokines (irisin and IGF-1) and osteokine osteocalcin, while downregulating negative regulators (myostatin and sclerostin). These changes were correlated with the observed phenotypic enhancements. The low-dose combination of CaHMB, CBP, and HA provides comprehensive benefits against age-related muscle and bone loss, likely by modulating the muscle–bone axis, and outperforms individual components. Our findings support its potential as a multi-targeted nutritional strategy for the aging population.

1. Introduction

The global population is undergoing irreversible aging, with the process exhibiting an accelerating trend. According to the World Health Organization (WHO), in 2020, there were about 1 billion people aged 60 or older worldwide, accounting for 13.5% of the global population (7.8 billion). This figure is expected to rise to nearly 2.1 billion by 2050.[1] The health of the aging population has become a significant public health concern in the world. Aging involves progressive cellular degeneration driven by accumulated inflammation, oxidative stress, and apoptosis,[2,3] which leads to a series of physiological declines, including impaired nutrient metabolism that increases malnutrition risk, causes deleterious changes in body composition, and results in the functional deterioration of key systems such as bone, muscle, joint, cognition, and immunity.[4–6] Therefore, the elderly face a high burden of chronic diseases and prolonged disability, frequently leading to multimorbidity.[7] Among these comorbid conditions, age-related musculoskeletal diseases, such as sarcopenia, osteoporosis and osteoarthritis, are frequently observed together.[6]

Sarcopenia, characterized by the progressive loss of muscle mass and strength with aging,[8] has a reported global prevalence ranging between 10% and 27%.[9] Osteoporosis is defined

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by low bone mineral density (BMD) and deterioration of bone microstructure,[10] with an estimated worldwide prevalence of 19.7%.[11] Osteoarthritis is a progressive and degenerative joint disease characterized by articular cartilage degeneration, bone remodelling and osteophyte formation.[12] The global prevalence of osteoarthritis is approximately 7.6% and knee osteoarthritis, as the most common form, exhibits a prevalence of about 43% in individuals aged 40 years and older.[13] The musculoskeletal system reaches its peak function around age 30. After that, bone and muscle gradually decline, with this loss accelerating rapidly after age 45–50.[14–16] Sarcopenia, osteoporosis and osteoarthritis are independently and synergistically associated with an increased risk of adverse health outcomes, including frailty, fractures, mobility disability, and mortality.[6,13] Beyond the well-known biomechanical, neuroendocrine, and nutritional interactions,[17] the musculoskeletal system also engages in molecular crosstalk through the secretion and reception of various cytokines, a network of interactions collectively termed the "muscle–bone axis".[18] Myokines, secreted by skeletal muscles, such as irisin,[19] insulin like growth factor-1 (IGF-1)[20] and myostatin (MSTN)[21] can positively or negatively regulate bone health through autocrine, paracrine, and endocrine modes.[22] Conversely, bone-derived osteokines like osteocalcin (OCN)[23] and sclerostin (SOST)[24] signal back to influence muscle mass and function.[22] Accordingly, given the inseparable link between muscle, bone and joint, integrated management of sarcopenia, osteoporosis and osteoarthritis is imperative for the elderly.

β-Hydroxy β-methylbutyrate (HMB) is a natural metabolite of the essential amino acid leucine.[25] Its commonly supplemented form is calcium HMB (CaHMB), the calcium salt of HMB. Extensive research, spanning from animal models to human clinical trials, has established the potential of HMB to positively modulate muscle mass and function.[25–27] The primary mechanism of HMB involves stimulating muscle protein synthesis and inhibiting degradation.[28,29] Colostrum basic protein (CBP) is a small bioactive protein extracted from colostrum, with a molecular weight ranging from 1 to 30 kDa.[30] Although clinical evidence of CBP in humans remains limited, several animal experiments have shown that CBP could promote bone growth and increase bone strength through the direct regulation of osteoblast and osteoclast activity.[30,31] Hyaluronic acid (HA), also known as hyaluronan, is a high-molecular-weight linear glycosaminoglycan. It is naturally and widely distributed throughout the human body, including in joints, skin, and the vitreous body of the eye.[32] Oral HA supplementation has been widely studied for its potential benefits in multiple areas, including joint, skin and gastrointestinal health.[33–36] Specifically, its role in osteoarthritis management is notable for enhancing muscle strength, reducing pain and stiffness, and modulating inflammation.[37–39] Based on the broad beneficial effects of CaHMB, CBP, and HA on the musculoskeletal system, they are currently approved for use in a variety of food categories in many countries, primarily including sports nutrition foods, dairy products and foods for special medical purposes.[40–43]

Currently, most of the interventions primarily target sarcopenia, osteoporosis and osteoarthritis in isolation, often overlooking the critical interconnection within the musculoskeletal system. Moreover, despite the well-established effects of the individual administration of CaHMB, CBP, and HA on muscle, bone and joint, their combined effects remain largely unexplored. An important and unexplored aspect of this gap is a low-dose combination, formulated from human food regulation limits, that would represent a critical translational step to bridge nutritional science and practical application.

Therefore, this study aimed to evaluate the efficacy of a combined CaHMB, CBP, and HA supplement on delaying degenerative changes in muscle, bone, and joints as mice naturally progress to early old age and to explore the underlying molecular mechanisms involving the muscle–bone axis.

2.   Materials and methods

2.1.   Animals and experimental design

Male C57BL/6J mice aged 2 or 12-months were provided by Vital River Laboratory Animal Technology Co., Ltd (Beijing, China) and were housed in a pathogen-free facility (12 h day/night cycle, 20–26 °C, relative air humidity 40–60%) with *ad libitum* access to food and water. A 6-month dietary intervention was administered. 2-month-old mice (the young control group, YOU) received no intervention and were fed the standard AIN93G diet (Jiangsu Medicine, China) throughout the study, reaching 8-months of age at the end of the experiment. 12-month-old mice were randomly assigned to five groups and received the following dietary interventions for 6-months until euthanasia at 18-months of age: (1) the old control group (OLD), fed with the standard AIN93G diet; (2) the CaHMB group (HMB), fed with AIN93G containing 2400 mg kg^{-1} CaHMB;[44] (3) the CBP group (CBP), fed with AIN93G containing 150 mg kg^{-1} CBP;[45] (4) the HA group (HA), fed with AIN93G containing 40 mg kg^{-1} HA;[35] and (5) the combination group (COM), fed with AIN93G supplemented with 900 mg kg^{-1} CaHMB, 30 mg kg^{-1} CBP, and 5.6 mg kg^{-1} HA. CaHMB was provided by Jiangyin Tsi Pharmaceutical Co., Ltd (Jiangsu, China), CBP was obtained from Seperex Nutritionals® Ltd (Otago, New Zealand), and HA was obtained from Shandong Bloomage Hyinc Biopharm Co. Ltd (Shandong, China).

The dose in the COM group was set at the animal-feed equivalent of the highest level permitted in human food by relevant Chinese national regulations.[41–43] Specifically, the regulations stipulate the following maximum addition limits in dairy products: CaHMB ≤ 3 g per 100 g, CBP ≤ 100 mg per 100 g, and HA ≤ 20 mg per 100 g. Therefore, taking a recommended daily human intake of 50 g of milk powder as an example, this would provide 1.5 g of CaHMB. The daily intake of humans is thus 1.5 g per 60 kg body weight (BW). According to the Meeh–Rubner formula, k = 9.1 for dose translation from a human to a mouse (based on the body surface area), and the equivalent mouse dose was calculated to be 225 mg per kg

BW. This dose was subsequently converted to the dietary concentration based on the average mouse BW and food consumption. The doses for the three single-intervention groups were determined by referencing efficacious doses reported in the previous literature.[35,44,45]

The BW and food intake were recorded weekly. After 6-months of intervention, the mice were fasted for 10–12 h, anesthetized with pentobarbital sodium, and blood samples were collected. Serum was obtained by centrifugation at $1000g$ for 15 min and stored at -80 °C until analysis. The tibias, femurs, knee joints and hind limb muscles of all mice were dissected. The entire hind limb bones and 5 mm³ of quadriceps muscle (QA) samples were selected and fixed in 4% paraformaldehyde (PFA, Servicebio, China) for 24 h. All animal protocols were approved by the ethics committee of the School of Public Health, Sun Yat-sen University (permit no. 2024-066). Experimental design and group assignment are presented in Fig. 1.

2.2. Body composition measurement

At the end of the dietary intervention, body composition was examined. The mice were anesthetized by pentobarbital sodium and placed in a prone position on a dual-energy X-ray absorptiometer (DXA, Discovery W; Hologic Inc., USA). The mode of "small animal" was applied to record the body composition, including lean mass, fat mass and whole-body bone mineral density (BMD).

2.3. Behaviour experiment

The grip strength was measured using a grasp meter (DS2-50N, China) every two weeks during the intervention. In brief, after acclimation, the mice were placed on a metal grid with all four limbs, and then their tails were pulled horizontally with a steadily increasing force until the mice released the grip. The peak force was recorded. The measurement was repeated four times per mouse, and the result was calculated as the average of the two highest values.

The exercise capacity of mice was evaluated using a treadmill (XR-PT-10B, Shanghai Xinrun Information Technology Co., Ltd, China) after 6-month intervention. Before the formal test, the mice underwent a 3-day acclimation. The formal test consisted of a 15-minute run, starting at 15 m min^{-1} and increasing to 25 m min^{-1} over 2 min. The current intensity for electrical stimulation was set as 0.5 mA. The distance and the time of the running test were recorded.

2.4. Micro-CT

The left femur and tibia samples ($N = 5$ per group) collected from the mice were scanned by micro-CT (VNC-102, PINGSENG Healthcare (Kunshan) Inc., China), and the resulting images were reconstructed using Recon software. A standardized region of interest (ROI) 2 mm beneath the femoral and tibial growth plates was defined and analysed uniformly across all samples using Avatar software. The analysis covered the bone volume (BV/TV) fraction, BMD of femur and tibia, trabecular bone thickness (Tb·Th), trabecular bone separation (Tb·Sp), and trabecular bone number (Tb·N).

2.5. Histological analysis

The right QA samples ($N = 8$ per group), right femur and tibia samples ($N = 5$ per group) and right knee joint ($N = 5$ per
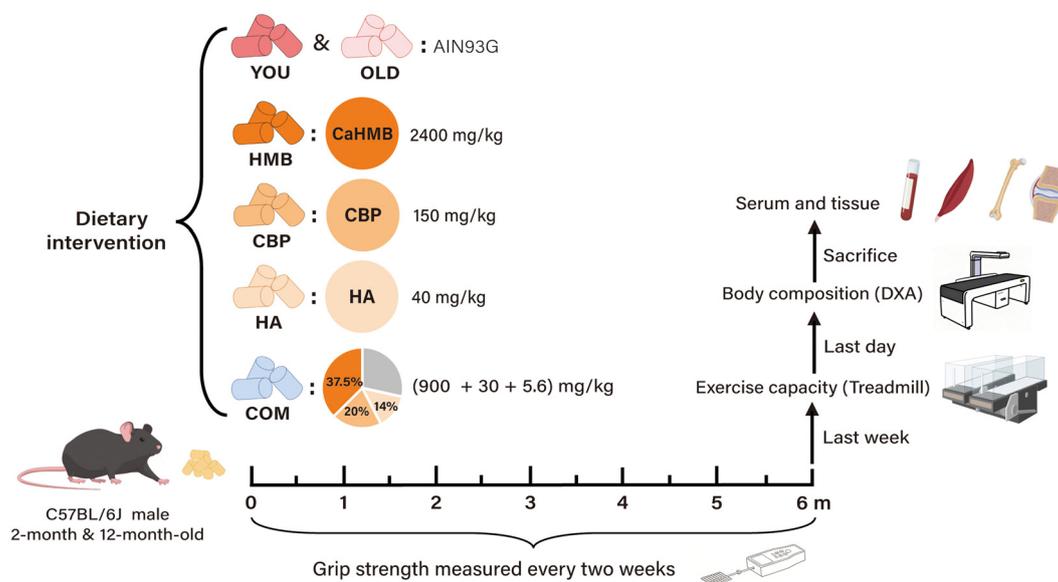
Fig. 1 Experimental design and group assignment. Male C57BL/6J mice were divided into six groups. Young (YOU, 2-month-old) and old (OLD, 12-month-old) control groups received the standard AIN93G diet. Four intervention groups (all 12-month-old) were fed AIN93G supplemented with CaHMB, CBP, and HA, or a combination (COM). The study lasted 6-months. Abbreviations: CBP, the colostrum basic protein group; COM, the combination group; DXA, dual-energy X-ray absorptiometer; HA, the hyaluronic acid group; HMB, the calcium β-hydroxy β-methylbutyrate group; OLD, the old control group; YOU, the young control group.

group) were harvested. Briefly, 5 mm³ QAs were fixed with 4% PFA for 24 h, paraffin-embedded, and sectioned at 4 μm for hematoxylin and eosin (H&E) staining (Servicebio, China). Four random fields from each skeletal muscle cross-section were photographed under an optical microscope equipped with a digital camera (ECLIPSE Ci-L, Nikon, Tokyo, Japan). The cross-sectional area (CSA) was evaluated using ImageJ 2.16.0 software.

For the femur and tibia samples, following 24 h fixation in 4% PFA, the bone tissues were decalcified in 0.5 M EDTA (Servicebio, China) and paraffin-embedded. Tissues were sectioned at 8 μm and stained with H&E and tartrate-resistant acid phosphatase (TRAP) (Servicebio, China). To quantify osteoclasts, three random fields were analysed per sample using ImageJ to determine the osteoclast number per unit bone surface (OC·N/BS) and the osteoclast surface per unit bone surface (OC·S/BS).

Knee joints were processed identically to bone samples (fixation, decalcification, and embedding). The samples were sliced into 8-μm-thick sections and subjected to H&E and Safranin O and Fast Green staining (Servicebio, China). The medial tibial cartilage of the knee was examined microscopically, photographed, and scored using the Osteoarthritis Research Society International (OARSI) system for cartilage degeneration.[46,47]

2.6. ELISA

The levels of OCN (SEKM-0301, Solarbio, Beijing, China), bone-specific alkaline phosphatase (BALP) (ELK2689, ELK Biotechnology, Wuhan, China) and C-telopeptide of type I collagen (CTX-I) (ELK2223, ELK Biotechnology, Wuhan, China) in the serum, irisin (PI470, Beyotime, China), MSTN (SEKM-0158, Solarbio, Beijing, China) and IGF-1 (EK0378, BOSTER, Wuhan, China) in the QA tissues and SOST (EK1179, BOSTER, Wuhan, China) in the femur tissues were measured using respective ELISA kits according to the manufacturers' instructions. All standard curves had an $R^2 > 0.99$.

2.7. Statistical analysis

The data were expressed as the mean ± standard deviation (SD). Statistical analysis was performed using SPSS 27.0 statistical software (IBM Corp., Armonk, New York, USA). Student's t test and one-way analysis of variance (ANOVA), followed by least-significant difference (LSD) and Spearman correlation analysis were used in this study. Adobe Illustrator 29.6 and GraphPad Prism 10.0 were used to draw the graphs. The p-value < 0.05 was considered statistically significant.

3. Results

3.1. Muscle and bone loss in aged mice

The BW increased progressively in all groups during the intervention period (Fig. 2A), with no significant differences observed among groups at the endpoint (Fig. 2B). Average weekly food intake did not differ among groups, which was

25.9 g per mouse per week throughout the intervention (Fig. 2C).

Marked musculoskeletal deterioration was observed in the OLD group. Compared to the YOU group, the OLD group exhibited a significant decrease in the lean mass percentage (LM%) and muscle fibre CSA (Fig. 2D and H). Muscle function was also significantly impaired in the OLD group including the decrease of grip strength and the decrease of running distance and the time on the treadmill (Fig. 2E, F and Fig. S1A). Moreover, the whole-body BMD, BV/TV%, Tb·Th, and Tb·N of lower limb bones significantly reduced and the Tb·Sp significantly increased in the OLD group (Fig. 2I–M and Fig. S1B–D and F–H). Three-dimensional (3D) reconstruction and H&E staining further confirmed substantial trabecular bone loss in the aged mice (Fig. 2Q and Fig. S1E, I and J). Enhanced bone resorption in the OLD group was supported by the elevated CTX-I level and increased osteoclast numbers (Fig. 2O, P, and R and Fig. S1K and J–M), along with a decrease in the BALP level (Fig. 2N).

Notably, no overt knee osteoarthritis-related pathological changes were observed in the aged mice. In the OLD group, both the medial tibial plateau (MTP) and the medial femoral condyle (MFC) exhibited smooth articular surfaces without observable cartilage defects or significant synovitis-related changes, and the cartilage showed continuous red in Safranin O staining, similar to the YOU group (Fig. 2T). Furthermore, no significant differences were found between the OLD and YOU groups in the OARSI scores or MTP thickness (Fig. 2S and Fig. S1N).

Collectively, the naturally aged mice (18-months old after intervention) developed pronounced age-related muscle and bone loss accompanied by a functional decline, whereas spontaneous degenerative changes in the knee joint were not observed.

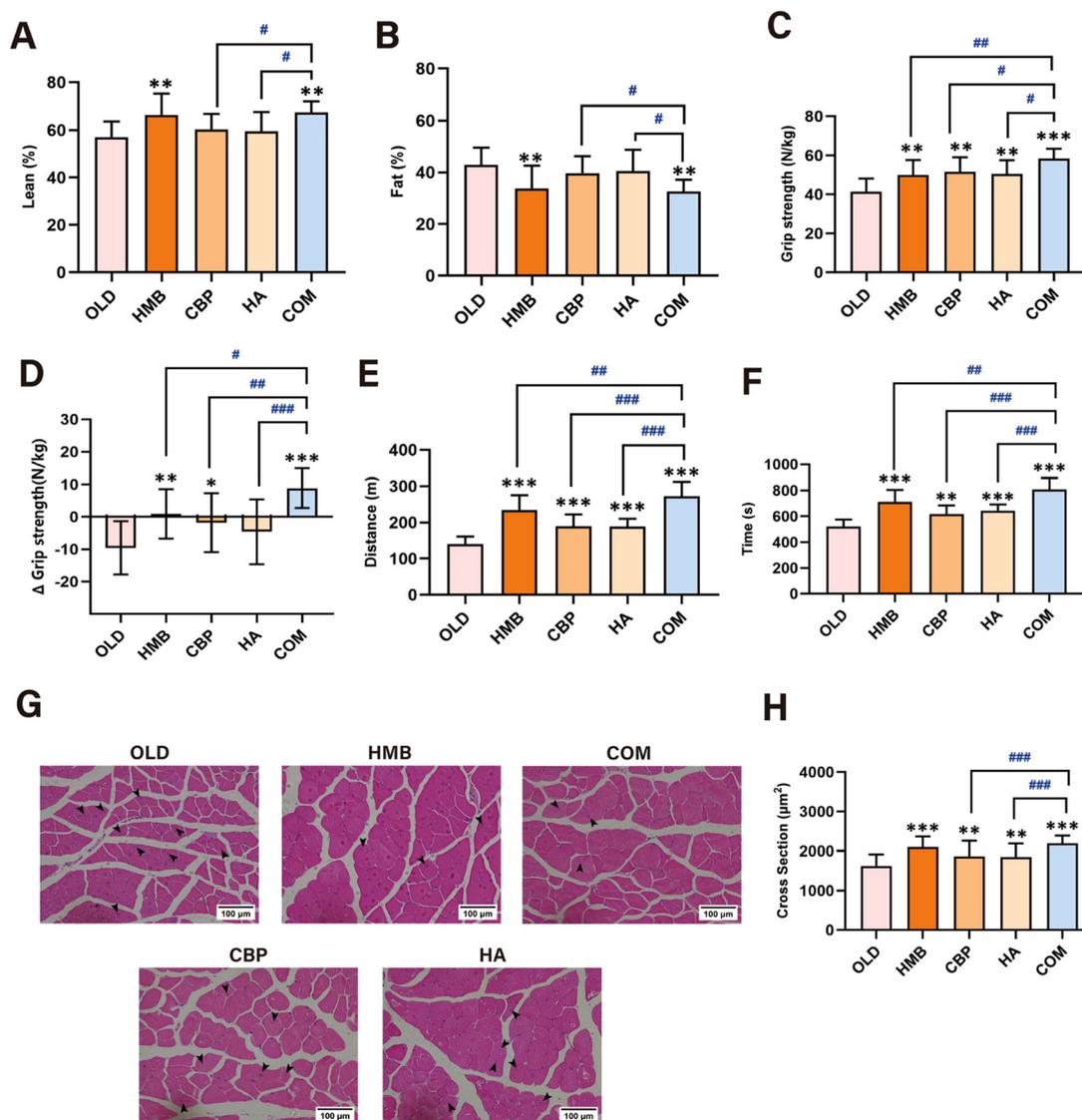### 3.2. Intervention preserves muscle mass and function in aged mice

Following the 6-month intervention, the body composition was assessed by DXA. The HMB and COM groups effectively maintained LM% (COM vs. OLD: 67.4 ± 4.6% vs. 57.0 ± 6.6%, p = 0.002) and decreased FM% (COM vs. OLD: 32.6 ± 4.6% vs. 43.0 ± 6.6%, p = 0.002), and the efficacy of the COM group was significantly superior to both the CBP and HA groups (p < 0.05) (Fig. 3A and B). To further assess the muscle morphology, right QAs were subjected to H&E staining. All intervention groups prevented the decline of muscle fibre CSA in the aged mice. Specifically, the COM group showed the highest increase (1.35-fold), followed by the HMB group (1.29-fold), the CBP group (1.15-fold), and the HA group (1.14-fold). The COM group matched the effect of the HMB group (p = 0.213) and significantly exceeded the CBP and HA groups (p < 0.001) in preserving the muscle fibre CSA (Fig. 3G and H). A higher prevalence of nucleus centralization (NC) was observed in the OLD group, and both the COM and HMB groups could reduce the prevalence of NC, despite no significant difference between the two groups (Fig. 3G).
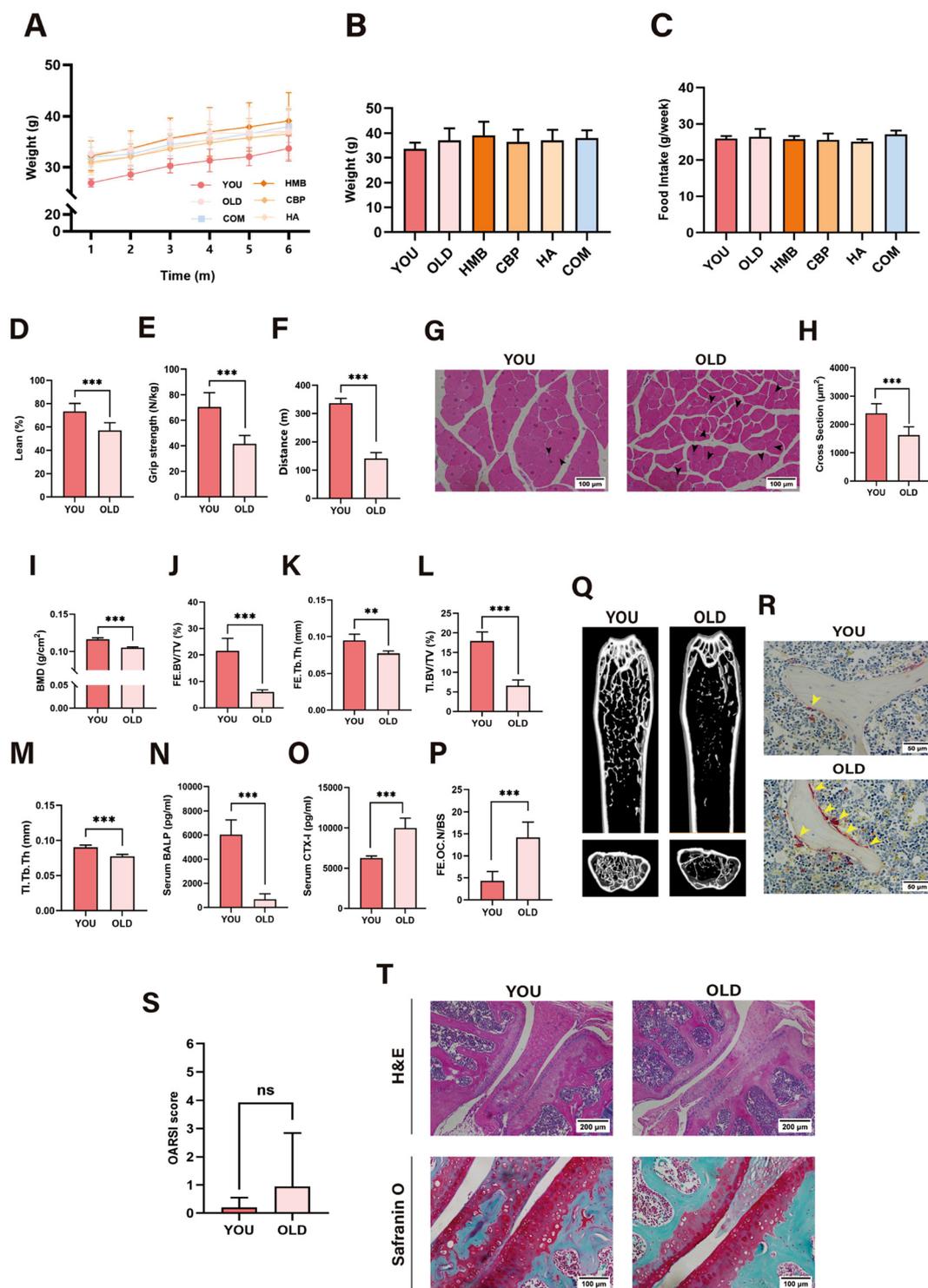
Fig. 2 Muscle and bone loss in aged mice. (A) BW changes during the intervention. (B) Endpoint BW. (C) Average weekly food intake per mouse. (D) Lean mass%. (E) Endpoint grip strength. (F) Treadmill running distance. ($N = 10$–12 for A–F and I). (G) H&E staining of right QAs. The black arrows indicate the centralized nucleus. (H) CSA of right QAs. ($N = 8$ for G and H). (I) Whole-body BMD. (J) Femur BV/TV%. (K) Femur Tb·Th. (L) Tibia BV/TV%. (M) Tibia Tb·Th. ($N = 4$–5 for J–M and P–T). (N) Serum BALP level. (O) Serum CTX-I level. ($N = 6$ for N and O). (P) Femur OC·N/BS. (Q) 3D reconstruction images of the left femoral ROI. (R) TRAP staining of the right femur. The yellow arrows indicate osteoclasts. (S) OARSI score. (T) H&E staining and Safranin O and fast green staining of the right knee joint. The data are presented as the mean $\pm$ SD. ** $p < 0.01$ and *** $p < 0.001$. ns = no significance. Abbreviations: BALP, bone-specific alkaline phosphatase; BMD, bone mineral density; BV/TV, bone volume fraction; BW, body weight; CSA, cross-sectional area; CTX-I, C-telopeptide of type I collagen; FE, femur; H&E, hematoxylin and eosin; MTP, medial tibial plateau; OLD, the old control group; OC·N/BS, osteoclast number per unit bone surface; OARSI, Osteoarthritis Research Society International; QA, quadriceps muscle; ROI, region of interest; Tb·Th, trabecular bone thickness; TI, tibia; TRAP, tartrate-resistant acid phosphatase; YOU, the young control group.

Fig. 3 Intervention preserves muscle mass and function in aged mice. (A) Lean mass%. (B) Fat mass%. (C) Endpoint grip strength. (D) Change in grip strength. (E) Treadmill running distance. (F) Treadmill running time. (N = 10–12 for A–F) (G) H&E staining of right QAs. The black arrows indicate the centralized nucleus. (H) CSA of right QAs. (N = 8 for G and H). The data are presented as the mean $\pm$ SD. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ (compared with the OLD group). # $p < 0.05$, ## $p < 0.01$, and ### $p < 0.001$ (compared with the COM group). Abbreviations: CBP, the colostrum basic protein group; CSA, cross-sectional area; COM, the combination group; HA, the hyaluronic acid group; H&E, hematoxylin and eosin; HMB, the calcium β-hydroxy β-methylbutyrate group; OLD, the old control group; QA, quadriceps muscle.

Beyond improving the muscle mass, the intervention also exerted beneficial effects on muscle function, including grip strength and exercise performance. The aged mice in all intervention groups not only showed improved grip strength (Fig. 3C and D) but also exhibited extended running distance and time (Fig. 3E and F). Furthermore, these improvements in the COM group were superior to the three individual component groups.

In summary, our findings demonstrated that both CaHMB alone and the combination effectively preserved muscle mass and all interventions enhanced muscle function in aged mice. Moreover, the combination significantly outperformed both CBP and HA alone.

3.3. Intervention mitigates bone loss by improving bone metabolism in aged mice

DXA analysis revealed all interventions preserved the whole-body BMD among groups, with the COM group showing the most significant improvement (COM vs. OLD: 0.1101 ± 0.0013 vs. 0.1049 ± 0.0011 g cm^{-2}, $p < 0.001$). Notably, while the efficacy of the COM group was comparable to the CBP group ($p = 0.096$), it was significantly superior to both the HMB and HA groups ($p < 0.05$) (Fig. 4A).

Micro-CT analysis of the femoral microstructure indicated that both CBP and COM groups significantly improved the
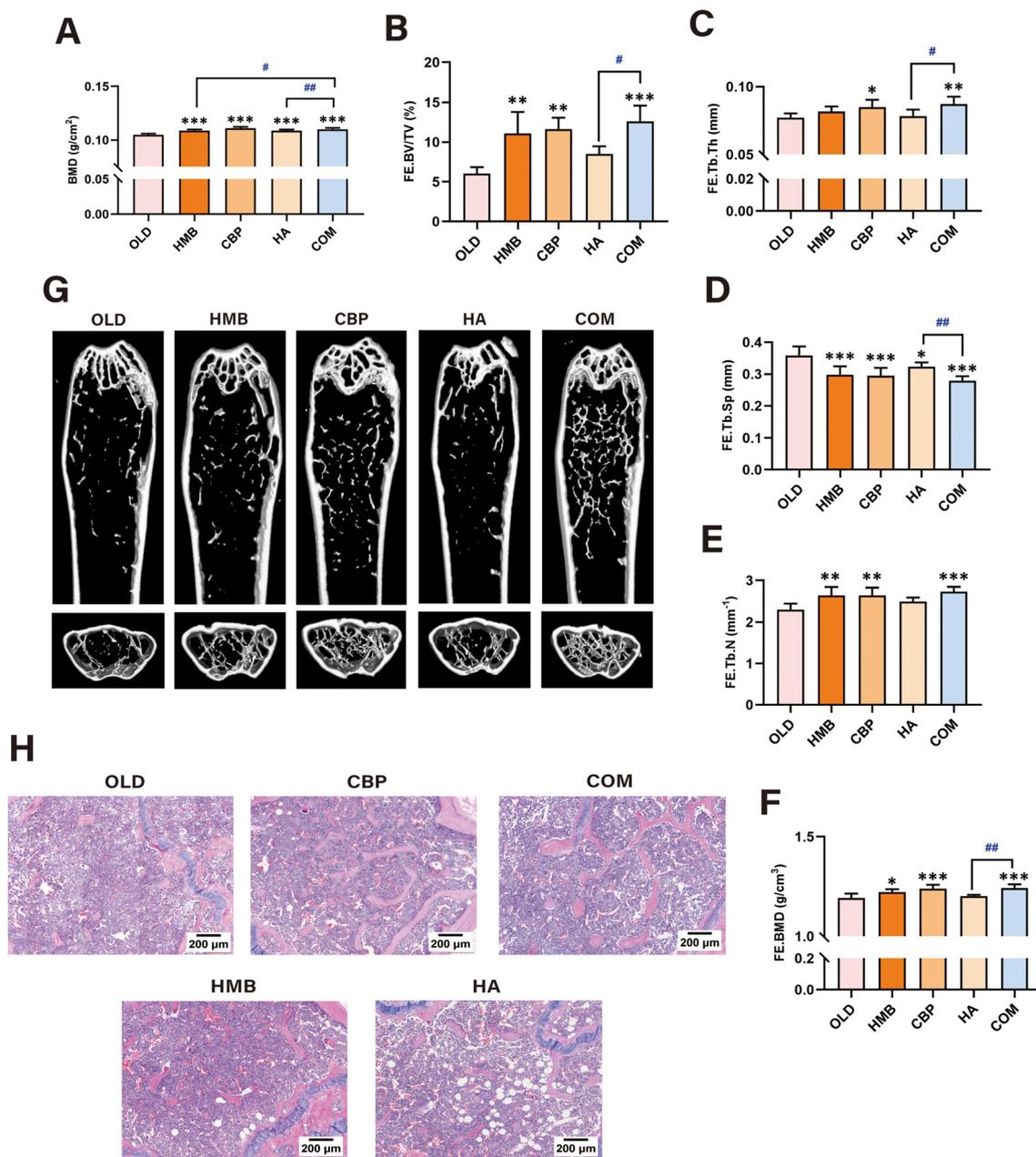
Fig. 4 Intervention mitigates bone loss in the whole body and femur of aged mice. (A) Whole-body BMD. (N = 10–12 for A). (B) Femur BV/TV%. (C) Femur Tb·Th. (D) Femur Tb·Sp. (E) Femur Tb·N. (F) Femur BMD. (G) 3D reconstruction images of the left femoral ROI. (H) H&E staining of the right femur. (N = 4–5 for B–H). The data are presented as the mean ± SD. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ (compared with the OLD group). # $p < 0.05$ and ## $p < 0.01$ (compared with the COM group). Abbreviations: BMD, bone mineral density; BV/TV, bone volume fraction; CBP, the colostrum basic protein group; COM, the combination group; FE, femur; HA, the hyaluronic acid group; H&E, hematoxylin and eosin; HMB, the calcium β-hydroxy β-methylbutyrate group; OLD, the old control group; ROI, region of interest; Tb·N, trabecular bone number; Tb·Sp, trabecular bone separation; Tb·Th, trabecular bone thickness.

femur (FE) microstructure in the aged animals, increasing the BV/TV% (COM: +109% and CBP: +93%), FE·BMD (COM: +4% and CBP: +4%), Tb·Th (COM: +13% and CBP: +10%), and Tb·N (COM: +19% and CBP: +15%) and decreasing Tb·Sp (COM: −22% and CBP: −18%), even though no statistically significant differences were found between the two groups across all parameters. What's more, apart from the Tb·N, COM group demon-

strated significantly greater efficacy than the HA group in all parameters (Fig. 4B–G). 3D reconstruction images and H&E staining of FE showed age-related bone loss in the trabecular bone area, which was mitigated in both the CBP and COM groups (Fig. 4G and H).

Similarly, analysis of the tibial microstructure by micro-CT indicated that the CBP and COM groups markedly ameliorated

tibia (TI) degeneration, with an increase in BV/TV% (COM: +87% and CBP: +100%), TI·BMD (COM: +3% and CBP: +4%), Tb·Th (COM: +7% and CBP: +6%), and Tb·N (COM: +21% and CBP: +17%) and a decrease in Tb·Sp (COM: −24% and CBP: −20%); however, the effectiveness of the two groups was statistically comparable. Additionally, the COM group outperformed the HA group in improving the TI·BMD and also showed a better effect than the HMB group in increasing Tb·Th (Fig. 5A–F). Similarly, tibial 3D reconstruction images and H&E staining demonstrated that both CBP and COM interventions attenuated the age-related loss of trabecular bone (Fig. 5F and G).

For the assessment of bone metabolism, we measured the serum levels of the bone turnover markers CTX-I and BALP and performed TRAP staining. Both COM and CBP groups reduced the CTX-I levels (COM: 7435 ± 627.9 and CBP: 6948 ± 266.5 pg ml^{-1}) and increased the BALP levels (COM: 3607 ± 1389 and CBP: 2811 ± 927.1 pg ml^{-1}) in aged mice, with no statistically significant difference observed between the two groups. However, the effect of the COM group was significantly superior to both the HMB and HA groups (Fig. 6A and B). TRAP staining and the following quantitative analysis demonstrated that COM and CBP were similarly effective in suppressing the overall osteoclast activity, reducing both OC·N/BS and OC·S/BS in the FE and TI (Fig. 6C–H). Moreover, COM was significantly more potent than the HMB and HA groups in inhibiting the OC·N/BS of FE (Fig. 6C) and surpassed the HA group in both the OC·N/BS and OC·S/BS of TI (Fig. 6E and F).

In conclusion, our results indicate that CBP alone and the combination attenuated bone loss by reducing bone resorption and improving bone metabolic homeostasis, thereby preserving bone mass and microstructure. Furthermore, the combination resulted in significantly greater improvements than either HMB or HA alone.

3.4. Molecular regulation of the muscle–bone axis

Given the superior musculoskeletal benefits observed with the combination treatment at relatively lower doses, we next investigated potential molecular mechanisms underlying its effects by examining key myokines and osteokines involved in muscle–bone crosstalk.

Among myokines, positive factors such as irisin and IGF-1 promote bone formation and growth.[20,48] In contrast, MSTN acts as a negative regulator, not only inhibiting muscle mass but also potentially accelerating bone loss[21] (Fig. 7F). Our results showed that the levels of irisin and IGF-1 were significantly decreased, whereas the level of MSTN was significantly increased in the OLD group (Fig. 7A–C). Treatment with COM or HMB significantly elevated the levels of irisin (COM: 2338 ± 874.4 and HMB: 2190 ± 421.4 pg ml^{-1}) and IGF-1 (COM: 469.8 ± 118.3 and HMB: 439.4 ± 42.4 pg ml^{-1}) and suppressed the level of MSTN (COM: 696.2 ± 186.0, HMB: 722.9 ± 229.2 pg ml^{-1}), with no significant difference between them. However, the COM group had significantly higher irisin levels than both CBP and HA groups and showed greater efficacy than the HA

group in modulating IGF-1 and down-regulating MSTN (Fig. 7A–C).

Regarding osteokines, OCN enhances muscle regeneration and performance,[23] while SOST not only inhibits osteoblast activity in bone but also impairs muscle function[24] (Fig. 7F). The results revealed a significantly lower OCN level and a significantly higher SOST level in the OLD group (Fig. 7D and E). The COM and CBP groups comparably increased the OCN level (COM: 275.9 ± 79.4 and CBP: 307.8 ± 134.2 ng ml^{-1}) and decreased the SOST level (COM: 3515 ± 549.7 and CBP: 3479 ± 1100 pg ml^{-1}). COM outperformed the HMB and HA groups in lowering the SOST level and showed a stronger effect than the HA group in increasing the OCN level (Fig. 7D and E).

Spearman correlation analysis revealed that irisin, IGF-1 and OCN levels were positively correlated with muscle strength, lean mass, exercise performance, and bone microarchitectural parameters, while MSTN and SOST exhibited inverse correlations (Fig. 7G). These findings support the involvement of key myokines and osteokines in regulating musculoskeletal benefits through the muscle–bone axis.

Collectively, these results suggest that the combination of CaHMB, CBP and HA exerted the enhanced effects through an indirect mechanism: by concurrently boosting muscle and bone health and performance, the combination modulated the bidirectional signalling of the muscle–bone axis, leading to benefits beyond individual components.

4. Discussion

In this study, 12-month-old mice received a 6-month dietary intervention and were evaluated at 18-months of age (approximately 55–65 human years). This model allowed us to explore whether a long-term intervention across the middle and early aged status could exert effects on delaying the age-related musculoskeletal decline. We found that while individual components (CaHMB, CBP, and HA) conferred selective benefits on either muscle or bone, their low-dose combination produced broader musculoskeletal improvements, preserving both muscle mass and bone microstructure. Notably, these enhanced effects were potentially mediated by the modulation of key molecules of the muscle–bone axis.

The early aged mice in this model showed clear degenerative losses in the muscle and bone but did not develop significant histological alterations in knee osteoarthritis under our experimental conditions. This outcome contrasts with some reports of high spontaneous osteoarthritis incidence in aged C57BL/6J mice (up to 80% at the age of 18-months).[49,50] This might be attributed to factors including housing conditions, physical activity levels, genetic/epigenetic backgrounds, or inherent individual resistance to osteoarthritis. Furthermore, this natural aging model has not been widely standardized or adopted, and most studies in the field have relied on surgical or chemically induced osteoarthritis models or spontaneous models with specific genotypes to ensure more predictable and severe joint pathology.[51–53] Additionally, aging is highly
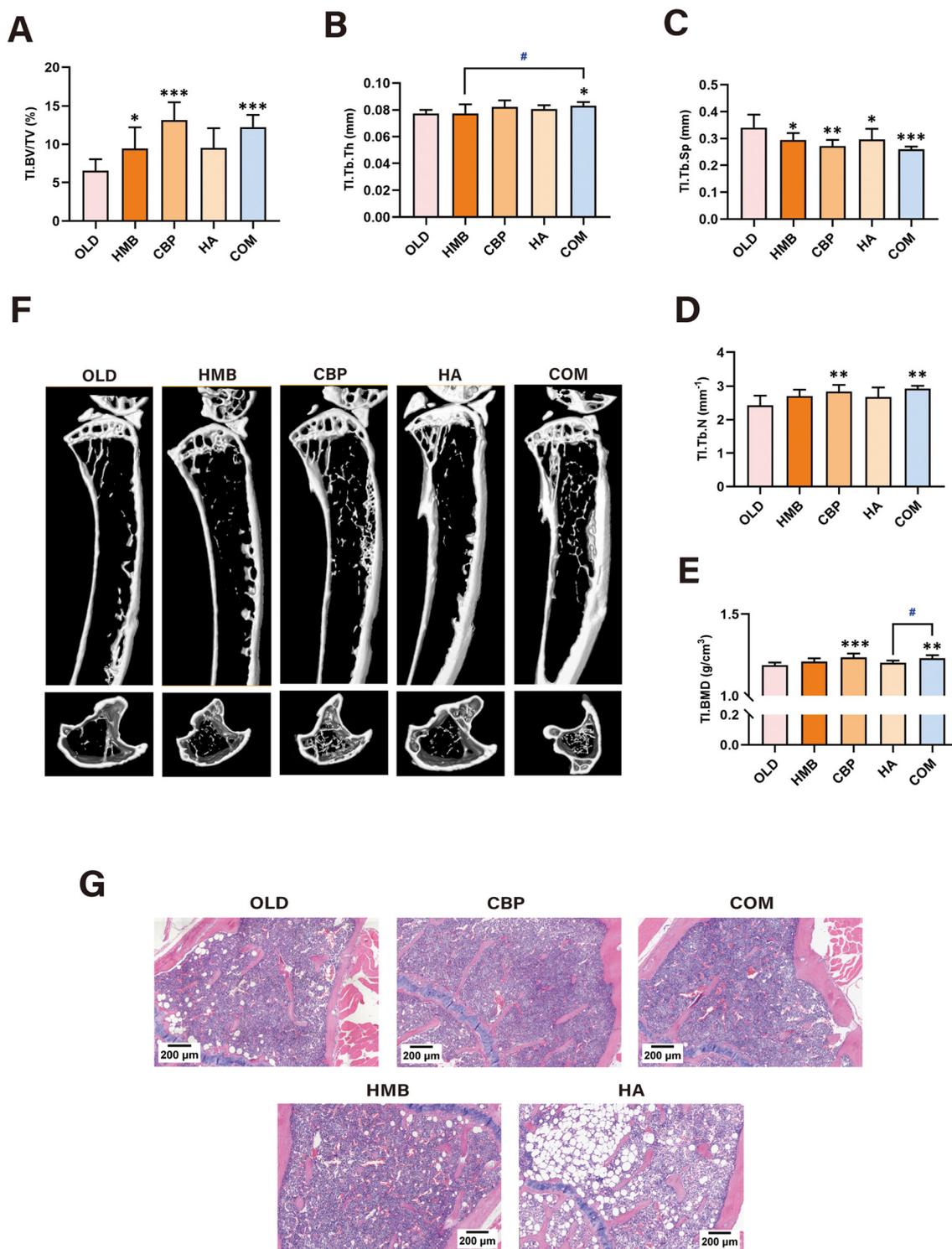
Fig. 5 Intervention mitigates bone loss in the tibia of aged mice. (A) Tibia BV/TV%. (B) Tibia Tb·Th. (C) Tibia Tb·Sp. (D) Tibia Tb·N. (E) Tibia BMD. (F) 3D reconstruction images of the left tibial ROI. (G) H&E staining of the right tibia. ($N = 4-5$ for A–G). The data are presented as the mean $\pm$ SD. $*\ p < 0.05$, $**\ p < 0.01$, and $***\ p < 0.001$ (compared with the OLD group). $\#\ p < 0.05$ (compared with the COM group). Abbreviations: BMD, bone mineral density; BV/TV, bone volume fraction; CBP, the colostrum basic protein group; COM, the combination group; HA, the hyaluronic acid group; H&E, hematoxylin and eosin; HMB, the calcium β-hydroxy β-methylbutyrate group; OLD, the old control group; ROI, region of interest; Tb·N, trabecular bone number; Tb·Sp, trabecular bone separation; Tb·Th, trabecular bone thickness; TI, tibia.
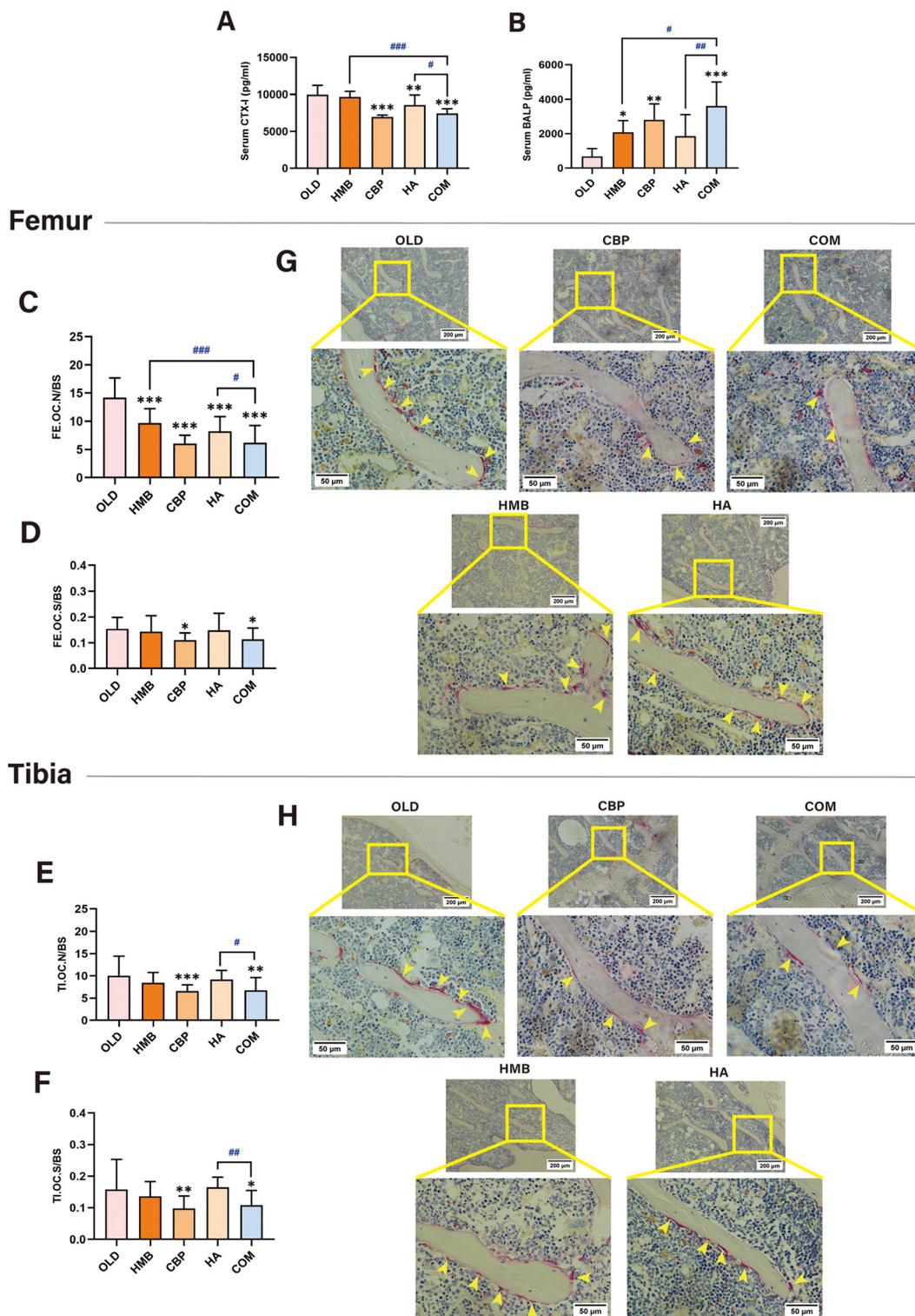
Fig. 6 Intervention improves bone metabolism in aged mice. (A) Serum CTX-I level. (B) Serum BALP level. (N = 6 for A and B). (C) Femur OC·N/BS. (D) Femur OC·S/BS. (E) Tibia OC·N/BS. (F) Tibia OC·S/BS. (G) TRAP staining of the right femur. The yellow arrows indicate osteoclasts. (H) TRAP staining of the right tibia. The yellow arrows indicate osteoclasts. (N = 4–5 for C–H). The data are presented as the mean ± SD. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ (compared with the OLD group). # $p < 0.05$, ## $p < 0.01$, and ### $p < 0.001$ (compared with the COM group). Abbreviations: BALP, bone-specific alkaline phosphatase; CBP, the colostrum basic protein group; COM, the combination group; CTX-I, C-telopeptide of type I collagen; FE, femur; HA, the hyaluronic acid group; HMB, the calcium β-hydroxy β-methylbutyrate group; OLD, the old control group; OC·N/BS, osteoclast number per unit bone surface; OC·S/BS, osteoclast surface per unit bone surface; TI, tibia; TRAP, tartrate-resistant acid phosphatase.
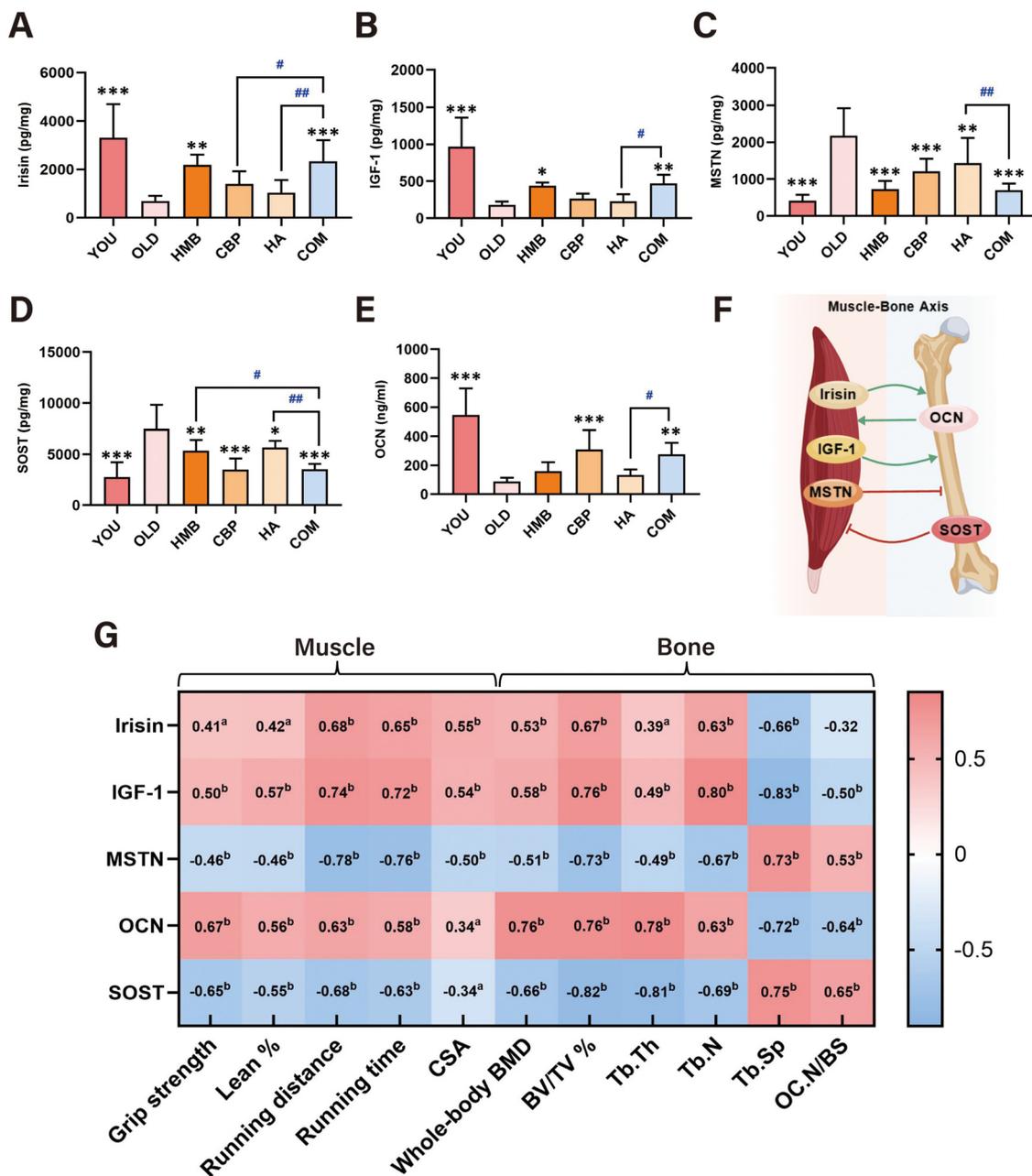
Fig. 7 Molecular regulation of the muscle–bone axis. (A) Tissue irisin level. (B) Tissue IGF-1 level. (C) Tissue MSTN level. (D) Tissue SOST level. (E) Serum OCN level. ($N = 6$ for A–E). (F) Key myokines and osteokines in the muscle–bone axis. (G) Correlation heatmap between the key mediators of the muscle–bone axis with musculoskeletal phenotypic parameters. The data are presented as the mean ± SD. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ (compared with the OLD group). # $p < 0.05$ and ## $p < 0.01$ (compared with the COM group). [a] $p < 0.05$ and [b] $p < 0.01$ (Spearman's correlation significance). Abbreviations: BMD, bone mineral density; BV/TV, bone volume fraction; CBP, the colostrum basic protein group; COM, the combination group; CSA, cross-sectional area; HA, the hyaluronic acid group; HMB, the calcium β-hydroxy β-methylbutyrate group; IGF-1, insulin like growth factor-1; MSTN, myostatin; OCN, osteocalcin; OC·N/BS, osteoclast number per unit bone surface; OLD, the old control group; SOST, sclerostin; Tb·N, trabecular bone number; Tb·Sp, trabecular bone separation; Tb·Th, trabecular bone thickness; YOU, the young control group.

heterogeneous, which differentially impacts the progression of degenerative changes across tissues, and the pace of aging varies across muscle, bone, and joint.[13,54,55] Therefore, it is possible that in our model, significant aging-related deterioration had already been observed in muscle and bone, while

the knee joint remained in a relatively preserved state, highlighting a potential heterogeneity in tissue aging.

Consistent with the existing literature, the individual components in our study demonstrated tissue-specific benefits. The HMB groups showed improvements in muscle mass and

function in aged animals (Fig. 3), aligning with prior evidence that CaHMB enhances muscle health in aged or sarcopenia-model animals and is supported by human randomized controlled trials (RCT) in the elderly.[56–58] These effects were largely attributed to CaHMB's ability to stimulate muscle protein synthesis (e.g., via the mammalian target of the rapamycin/ribosomal protein S6 kinase (mTOR/p70S6K1) pathway),[59,60] inhibit protein degradation (e.g., the suppression of the ubiquitin–proteasome pathway),[61–64] and promote myocyte proliferation.[65] On the other hand, the CBP group effectively maintained bone mass and improved bone metabolism in aged mice (Fig. 4–6), consistent with previous reports that CBP promotes bone health, likely through stimulating osteoblast activity[30,31] and modulating calcium metabolism.[45] However, research on CBP remains relatively limited, especially human intervention studies. These findings reinforce the targeted, yet limited, efficacy of single-nutrient approaches.

Notably, existing evidence points to the modest efficacy of single interventions in comprehensively addressing musculoskeletal aging, as each component primarily benefits a specific tissue, and findings across human studies were often inconsistent.[66–68] In contrast, multi-target nutritional strategies have shown more promising results.[69,70] A growing number of RCTs indicate that a range of different supplement strategies, employing various combinations of protein (e.g., whey), leucine or CaHMB, vitamin D, calcium, chondroitin sulfate, glucosamine, polyunsaturated fatty acids and other micronutrients, synergistically promote musculoskeletal health in older adults.[71–77] Our study aligns with and extends this concept: while CaHMB and CBP exerted protective effects on muscle and bone, respectively, the low-dose combination achieved comparable or superior benefits across both tissues, despite containing each component at a substantially reduced dose (37.5%, 20%, and 14% of their respective monotherapy levels for CaHMB, CBP, and HA) (Fig. 3–6). This can be explained by a multi-target strategy, in which the combination simultaneously addresses the musculoskeletal system: CaHMB supports muscle health,[26] CBP enhances bone metabolism,[45] and HA contributes to joint and inflammatory modulation.[78] This approach more closely reflects the physiological interconnectivity of the musculoskeletal system as a functional unit. This combined effect is probably mediated through the modulation of key myokines and osteokines of the muscle–bone axis.

As established in previous research, aging reduces the irisin, IGF-1, and OCN levels,[79,80] while exercise can stimulate their expression and suppress the levels of MSTN and SOST.[66,81] Consistent with this pattern, our ELISA assay results confirmed that aged mice exhibited significantly decreased levels of irisin, IGF-1, and OCN, alongside increased levels of MSTN and SOST, which was reversed by COM intervention (Fig. 7A–E). Notably, our correlation analysis further linked these COM-induced changes in myokines and osteokines to the specific improvements in muscle mass, function, and bone microstructure (Fig. 7G), consistent with findings from observational human studies.[82–86] This association further

supports the role of muscle–bone crosstalk in mediating the efficacy of the interventions.

On the one hand, the COM group significantly elevated the levels of beneficial myokines irisin and IGF-1 while suppressing the level of the negative regulator MSTN (Fig. 7A–C). As reported in the literature, irisin promotes bone formation by stimulating osteoblast activity through pathways such as Wnt/β-catenin,[87] mitogen-activated protein kinase/extracellular signal-regulated protein kinase (MAPK/ERK),[88] and adenosine monophosphate protein kinase (AMPK),[89] while also inhibiting osteoclast formation through the receptor activator of the nuclear factor-κB ligand (RANKL)/the nuclear factor of the activated T cell (NFAT) c1 pathway.[48,90] IGF-1 enhances osteoblast survival, proliferation, and differentiation,[20,91,92] primarily via the Ras/MAPK and phosphoinositide 3′-kinase (PI3K)/Akt pathways.[20] Conversely, MSTN inhibits muscle growth and promotes bone resorption[93,94] by suppressing Wnt/β-catenin signaling[95] and enhancing RANKL-mediated osteoclast activity.[21,96] Beyond the myokines we discussed in this study, other muscle-derived factors like interleukin-6 (IL-6), fibroblast growth factor21 (FGF21), β-aminoisobutyric acid (BAIBA) and so on also played important roles in bone homeostasis.[97–99] On the other hand, COM increased the level of osteokine OCN and decreased the level of SOST (Fig. 7D and E). OCN contributes to bone mineralization,[100] regulates muscle function[101] and promotes muscle cell proliferation and differentiation via p38MAPK and PI3K/Akt signaling,[23] whereas SOST negatively regulates bone formation[102] and might impair muscle mass[82,103,104] and regeneration by inhibiting Wnt/β-catenin signaling.[105] Osteokines such as transforming growth factor-β (TGF-β), FGF23, and RANKL are also important regulators of muscle metabolism and function.[106–108]

In addition to classical signalling molecules, extracellular vesicles (EVs) have emerged as a crucial communication medium in the muscle–bone axis,[109,110] carrying bioactive cargos—including microRNAs (miRNAs)—that mediate efficient bidirectional crosstalk between muscle and bone.[111] For instance, muscle-derived miR-27a-3p enhances osteoblast differentiation by activating β-catenin signaling,[112] while miR-34a, which increases with age, accelerates bone marrow stem cell (BMSC) senescence by suppressing sirtuin 1 (SIRT1).[113] Similarly, bone tissue also expresses a variety of miRNAs. Aged BMSCs downregulate miR-24,[111] which is a promoter of myogenic differentiation,[114] and upregulate miR-15b and miR-99b,[111] which inhibit muscle cell differentiation[115] and regulate muscle mass via the mTOR/Akt pathway.[116] Thus, future studies could further explore whether the combination supplement influences EV-mediated communication, particularly through the regulation of specific miRNAs involved in muscle–bone crosstalk.

While the existing literature predominantly focuses on single components or isolated musculoskeletal systems, our study provides the first systematic evaluation of the combined intervention of CaHMB, CBP, and HA. Our findings demonstrate that this combination has more comprehensive and multiple musculoskeletal system benefits at lower doses,

potentially through regulating key signalling molecules (*e.g.*, irisin, IGF-1, MSTN, OCN, and SOST) of the muscle–bone axis. Therefore, the low-dose, multi-target supplement presents enhanced potential for real-world generalization, providing direct evidence to support this nutritional strategy. However, several limitations of this study should also be acknowledged: (1) the study used only male mice. Given the crucial role of estrogen in maintaining bone density and muscle mass, and the higher prevalence of osteoporosis and sarcopenia in post-menopausal women, our results may not be directly generalizable to females. (2) The naturally aged model did not develop significant spontaneous osteoarthritis pathology in the knee joint, limiting the assessment of the intervention's effects on joint degeneration. (3) The experimental design included only the three single-component groups and the full combination group, without pairwise combination groups (*e.g.*, CaHMB + CBP, CaHMB + HA, and CBP + HA). This limits our ability to identify which specific binary interactions might be driving the observed combined effects; therefore, a more precise mechanistic interpretation and optimal formulation refinement might be overlooked. (4) Our mechanistic investigation is correlative and lacks depth. The exploration of the muscle–bone axis was limited to a few proteins *via* ELISA and we did not assess other key mediators like miRNAs. Furthermore, without western blotting and quantitative real-time PCR data, changes in the expression and regulation of key functional molecules in muscle and bone tissue remain unexamined, narrowing the pathway-specific conclusions. Future studies should utilize animals of both sexes and other established age-related musculoskeletal disease models. Additionally, targeted molecular techniques and *in vitro* co-culture systems should be employed to clarify the precise signalling mechanisms. Finally, long-term, large-scale randomized controlled trials in elderly populations with musculoskeletal decline should be conducted to determine the clinical effects of the combination of CaHMB, CBP and HA.

5. Conclusions

This study suggested that CaHMB improved muscle mass and function in naturally aged animals, while CBP alleviated bone loss by improving bone metabolism. Furthermore, the combination of CaHMB, CBP, and HA had multiple musculoskeletal benefits at relatively lower doses compared to individual components. Specifically, the combination could maintain muscle mass, enhance grip strength and exercise performance, and reduce bone resorption, thereby mitigating bone loss. The possible mechanism involved the regulation of critical signalling molecules of the muscle–bone axis, including irisin, IGF-1, MSTN, OCN, and SOST. Our findings highlight the potential effects of CaHMB, CBP, and HA supplementation on delaying age-related progression of muscle and bone loss, and their combined application appears to provide comprehensive and multiple benefits. This provides new research perspectives and potential intervention targets for preventing and improv-

ing age-related musculoskeletal diseases, thereby contributing to the extension of health span.

Author contributions

Haoqi Chen: writing – original draft, methodology, investigation, and conceptualization. Junhong Peng: investigation. Shanshan Guo: methodology and investigation. Ting Chen: investigation. Si Chen: methodology, writing – review & editing, and conceptualization. Xinyuan Jin: investigation. Wenge Huang: methodology and investigation. Chao Xu: methodology and investigation. Mengchu Li: methodology and investigation. Mengxing Xie: investigation. Mengtao Yang: methodology and investigation. Jinzhu Pang: investigation and conceptualization. Huilian Zhu: writing – review & editing, funding acquisition, and conceptualization. All authors have read and agreed to the published version of the manuscript.

Conflicts of interest

There are no conflicts to declare.

Data availability

The data used to support the findings of this study are included within the article and are available from the corresponding author upon request.

Supplementary information is available. See DOI: https://doi.org/10.1039/d6fo00352d.

Acknowledgements

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