



Contents lists available at SciOpen

Food Science and Human Wellness

journal homepage: <https://www.sciopen.com/journal/2097-0765>

Heyndrickxia coagulans Promotes Intestinal Calcium Absorption and Synergistically Ameliorates Bone Loss with Calcium Supplementation Efficacy and Mechanisms

Yuxuan Qiu^{a,b}, Chengcheng Zhang^{a,b}, Enduo Ma^d, Xia Zheng^d, Rong Wang^{a,b}, Qianqian Li^{a,b}, Nuo Li^{a,b}, Yichao Liu^{a,b}, Peijun Tian^{a,b}, Gang Wang^{a,b}, Shumao Cui^{a,b}, Wenwei Lu^{a,b,c}, Bo Yang^{a,b}, Xiaomei Lv^{a,b}, Jianxin Zhao^{a,b}, Wei Chen^{a,b,c}, Qixiao Zhai^{a,b*}

^a State Key Laboratory of Food Science and Resources, Jiangnan University, Wuxi, 214122, China

^b School of Food Science and Technology, Jiangnan University, Wuxi, Jiangsu, 214122, China

^c National Engineering Research Center for Functional Food, Jiangnan University, Wuxi, Jiangsu, 214122, China

^d BioBor (Guangdong) Health Technology Co., Ltd., 180 Shengli South Road, Jiangmen 529000, China

ABSTRACT: Modulating the gut microbiota has emerged as a novel strategy to address global calcium deficiency and its associated skeletal disorders, overcoming the limited bioavailability of traditional supplements. This study evaluated the efficacy of the highly tolerant, spore-forming *Heyndrickxia coagulans* (*H. coagulans*) in promoting intestinal calcium absorption. After screening 51 wild-type isolates, the selected strains significantly activated the cellular calcium transport network in a Caco-2 cell monolayer model, as evidenced by upregulation of TRPV6, VDR, PMCA1B, and NCX1 ($P < 0.05$). In a low-calcium diet-induced murine model, co-supplementation of *H. coagulans* and calcium significantly restored serum calcium levels ($P < 0.0001$) and attenuated abnormal alkaline phosphatase (ALP) activity, decreasing from 225.97 U/L to 95.00 U/L ($P < 0.0001$). Furthermore, micro-computed tomography (micro-CT) showed that the combined intervention produced greater improvements in trabecular microarchitecture than calcium supplementation alone, with bone volume fraction (BV/TV) increasing from 9.83% to 19.50% ($P < 0.001$) and trabecular number (Tb.N) rising from 1.03 to 1.94 mm⁻¹. These findings highlight the potential of *H. coagulans* as a novel probiotic adjunct to calcium supplementation for enhancing mineral bioavailability and achieving greater amelioration of bone loss than calcium supplementation alone.

Keywords: *Heyndrickxia coagulans*, Calcium absorption, Bone loss, Caco-2 cell model

1. Introduction

Calcium is the most prevalent essential mineral in humans. It serves as a cornerstone of skeletal integrity and ensures the proper functioning of the cardiovascular, nervous, and endocrine systems^[1-3]. An estimated 3.5 billion individuals worldwide are predisposed to calcium deficiency, with approximately 90% of this at-risk population concentrated in Asia and Africa^[4]. Calcium deficiency not only reduces bone strength and

*Corresponding author
zhaiqixiao@jiangnan.edu.cn

Received 18 June 2026
Received in revised form 23 July 2026
Accepted 4 September 2026

induces osteoporosis^[5] but also leads to elevated blood pressure, cardiovascular problems^[6, 7], pregnancy complications^[8-11], and various chronic metabolic diseases^[12-14]. However, traditional inorganic calcium supplements have hidden dangers such as drug interactions, interference with iron absorption^[15], and the risk of rebound after withdrawal in specific populations^[16, 17]; furthermore, their bioavailability is restricted by the saturable nature of intestinal transport, and high-dose singular supplementation exacerbates health risks such as ectopic calcification^[18].

Therefore, to overcome the bottleneck of intestinal calcium absorption, targeting the gut microbiota to enhance mineral bioavailability has emerged as a promising strategy, and probiotics with high tolerance and colonisation potential are highly promising intervention candidates^[19, 20]. By modulating immune responses and increasing the expression of calcium-binding proteins, common probiotics—including *Lactobacillus*, *Bifidobacterium*, and *Bacillus subtilis*—have recently been found to effectively boost intestinal calcium uptake and prevent bone loss^[21-25]. However, most current probiotics, such as *Lactobacillus* and *Bifidobacterium*, generally have poor heat resistance and are sensitive to environmental conditions; therefore, processing or storage conditions often reduce their survival rates and limit their applications^[26-28].

Owing to the exceptional stress resilience of its spores, *H. coagulans* exhibits formidable potential for intestinal colonisation and industrial application^[29]. Therefore, sporulation capacity was initially used as a criterion to prioritize strains with favorable industrial applicability, after which their calcium absorption-promoting properties were independently evaluated. In this study, we screened an optimal strain from 51 wild-type isolates and elucidated its targeted modulatory mechanisms on epithelial calcium transporters, such as TRPV6, utilising a Caco-2 model; furthermore, employing a low-calcium mouse model, we corroborated its *in vivo* efficacy in facilitating calcium absorption and demonstrated that its combined administration with exogenous calcium produced greater improvements in bone loss than calcium supplementation alone. This study establishes a systematic theoretical framework for developing novel microecological formulations dedicated to bone nutritional support.

2. Materials and Methods

2.1 Bacterial strain

The Culture Collection at Jiangnan University (specifically the Food Biotechnology Research Center) provided the 51 *H. coagulans* strains used in this study. S1 presents the relevant strain information.

2.2 Screening of high spore-yielding *H. coagulans*

Fifty-one strains of *H. coagulans* were cultured in MRS medium at 37 °C under aerobic conditions for 24 h, and subcultured 2-3 times. The activated strains were inoculated at an inoculum size of 2% w/w into three kinds of sporulation media respectively (Medium 1: glucose 4 g/L, yeast extract 6.16 g/L, and calcium chloride 1.25 g/L; Medium 2: wheat bran 36.91 g/L, corn steep liquor powder 13.70 g/L, and manganese sulfate monohydrate 0.84 g/L; Medium 3: wheat bran 32.12 g/L, beef extract 15.24 g/L, magnesium sulfate

heptahydrate 0.49 g/L, manganese sulfate monohydrate 0.34 g/L, and sodium dihydrogen phosphate 0.31 g/L), and cultured in shake flasks at 37 °C and 200 r/min for 48 h. The viable cell count was determined by plate counting.

The samples to be counted were treated in an 80 °C water bath for 10 min, and the spore count was determined by plate counting. Sporulation capacity was used as a pre-screening criterion for industrial applicability, and the selected strains were subsequently subjected to independent functional screening for their calcium absorption-promoting activity.

2.3 Proliferation of Caco-2 cells as influenced by varying concentrations of calcium and bacterial cells

Appropriate amounts of calcium chloride reagent were weighed to prepare calcium-containing solutions of different concentrations. After filter sterilisation, Caco-2 cells were exposed to these mixtures for 2 h. Next, we introduced the CCK-8 reagent into the wells. After a subsequent 1-h incubation, the absorbance was recorded.

After the *H. coagulans* cells were crushed by liquid nitrogen grinding, they were used to treat Caco-2 cells at concentrations of 10^6 , 10^7 , 10^8 , and 10^9 CFU/ml for 2 h. CCK-8 incubation was then allowed to proceed for 1 h before absorbance measurement^[30].

2.4 Establishment and evaluation of the Caco-2 cell model

Model construction utilized cells between passages 20 and 40. Caco-2 cells entering the logarithmic growth phase were resuspended for plating on polycarbonate Transwell filters (surface area: 1.12 cm²; pore diameter: 0.4 µm). Seeding density was maintained at roughly 1×10^5 cells/cm². The transport chamber's upper (apical) and lower (basolateral) sections received 0.5 mL and 2.5 mL of complete medium, respectively. The media exchange frequency was bidaily for the initial seven days before shifting to daily replacements for the remainder of the period. By days 20-22 of cultivation, the monolayers passed evaluation for subsequent assays.

During the cultivation of Caco-2 cells, their growth status and morphology were observed regularly using an inverted phase-contrast microscope (Olympus CKX41, Japan).

Before seeding cells onto the polycarbonate membrane of the Transwell insert, the membrane's intrinsic resistance was measured using a Millicell-ERS volt-ohm meter. During cell model establishment, Caco-2 cell monolayers grown for 3, 5, 7, 10, 14, 18, and 21 days were selected. We removed the existing culture medium and replaced it with PBS pre-warmed to 37 °C in both the AP and BL compartments. The PBS was discarded and added again, followed by regular incubation for 20 min. It was discarded again, and pre-warmed PBS was added for another 20 min of regular incubation. To assess transepithelial electrical resistance (TEER), we used a Millicell-ERS meter whose electrodes had been submerged in PBS overnight. The TEER value of the Caco-2 cell monolayer is the total resistance (microporous membrane + cell monolayer) minus the intrinsic resistance (microporous membrane), corrected by the growth area, and expressed in $\Omega \cdot \text{cm}^2$. A TEER

result greater than $400 \Omega \cdot \text{cm}^2$ served as the standard indicator for an intact, fully confluent cell layer. Higher TEER values indicate greater monolayer integrity, which typically plateaus below $1000 \Omega \cdot \text{cm}^2$ ^[31].

A purchased ALP assay kit was used to quantify enzymatic activity. After a 21-day cultivation period in the inserts, we performed two gentle washes of the cell layers with D'Hanks buffer. The plates were then incubated for 20 minutes at 37 °C.

After aspirating the solutions from both inside and outside the wells, the ALP activity on the AP and BL sides of the cell monolayer was measured according to the kit instructions.

2.5 Cellular intervention and mineral quantification assays

After culturing for 20 days in Transwell 12-well plates, the cells were divided into 6 experimental groups: a blank control (NC) and 5 *H. coagulans* intervention groups, all performed in triplicate. For each group, 0.5 mL of D'Hanks buffer containing the corresponding concentration of calcium and *H. coagulans* was added to the AL chamber of the Transwell, and 1.5 mL of D'Hanks buffer was added to the BL chamber. After incubation at 37 °C for 2 h, the buffer on the BL side was collected. The calcium content in the sample solutions was detected using ICP-MS. At the end of the experiment, the transepithelial electrical resistance of the cells in each well was measured.

2.6 Expression analysis of calcium-related transporter genes in Caco-2 cells

Caco-2 cells taken from the exponential growth phase were selected for this study. The cells were seeded into 12-well plates (1×10^5 cells/well; 1 mL of culture medium per well) and cultured for 20 days. For subsequent assays, the cells were assigned to a negative control (NC) group or one of five *H. coagulans* intervention groups (VJSXZ1M1, VYNDL1M1, CCFM1539, CCFM1540, and CCFM1541), with each condition tested in triplicate. The experimental setup involved a 2 h intervention using D'Hanks buffer containing the specified concentrations of calcium and *H. coagulans*. At the end of the treatment, the cells were harvested for RNA extraction to assess transcriptomic levels of genes encoding calcium-related transporters^[32]. To determine target gene expression levels, normalisation was performed against the internal reference gene GAPDH, and calculations were performed using the $2^{-\Delta\Delta C_t}$ comparative method.

2.7 Expression analysis of calcium-related transporters in Caco-2 cells

Cells were maintained in 12-well plates for 20 days. We then divided the cells into a negative control (NC) group or five *H. coagulans* intervention groups (VJSXZ1M1, VYNDL1M1, CCFM1539, CCFM1540, and CCFM1541), with each condition tested in triplicate. The cells were treated for 2 h with D'Hanks buffer supplemented with the indicated concentrations of calcium and *H. coagulans*. Next, the samples were harvested and directly lysed with 0.1 mL of lysis buffer containing protease inhibitors for protein extraction. The manufacturer's protocol guided the final quantification of the total protein concentration.

2.8 Preparation of oral gavage suspensions

The three *H. coagulans* strains selected from the preceding Caco-2 functional evaluation, CCFM1539, CCFM1540, and CCFM1541, were originally isolated from vegetables and stored at the Guangdong

Microbial Culture Collection Center (GDMCC). For strain activation, all *H. coagulans* were aerobically incubated in de Man, Rogosa, and Sharpe (MRS) broth (2% v/v) at 37 °C for 24 h and subcultured twice before use. The resulting activated cultures were then transferred into a sporulation medium (2% v/v). After a 24-h shaking incubation at 37 °C and 200 rpm, the incubation continued for a further 24 h at 50 °C. The culture mixture was then centrifuged at $12,000 \times g$ for 20 min to discard the supernatant. The remaining pellet was washed once with phosphate-buffered saline (PBS), blended at a 1:1 ratio with a lyophilisation protectant (skim milk: sucrose: trehalose = 13:3:3), and lyophilised to yield the bacterial powder. This *H. coagulans* bacterial powder was ultimately resuspended in PBS to establish a fixed spore concentration of 1×10^7 CFU/mL.

2.9 Mice and treatments

Male C57BL/6J mice at six weeks of age were sourced from Vital River Laboratory Animal Technology Company, located in Beijing, China. The specific grouping and treatments are described in the corresponding figure legends. We maintained the mice under standard laboratory conditions (22 ± 0.5 °C, $55 \pm 5\%$ humidity, and a 12 h light/dark cycle) with free access to water and chow. All animal procedures were approved by the Ethics Committee of Jiangnan University (JN.No20250615c0600905[360]).

Following a 7-day acclimatization period, the mice were randomly divided into six groups ($n = 6$): the normal control group (NC), the calcium-deficient model group (MC), the calcium supplementation group (Ca)、the *H. coagulans* CCFM1539 intervention group (CCFM1539+Ca)、the *H. coagulans* CCFM1540 intervention group (CCFM1540+Ca)、the *H. coagulans* CCFM1541 intervention group (CCFM1541+Ca). We maintained the NC group on a standard control diet (0.5% calcium) throughout the study. The remaining groups (MC, Ca, CCFM1539+Ca, CCFM1540+Ca, and CCFM1541+Ca) were fed a low-calcium diet containing 0.05% calcium. The two diets were completely matched in all other nutritional components.

From the second week until the end of the trial, we administered 0.2 mL of PBS daily via oral gavage to the NC and MC mice. Meanwhile, animals in the CCFM1539+Ca, CCFM1540+Ca, and CCFM1541+Ca groups received an oral calcium supplement combined with 10^7 CFU of their respective *H. coagulans* spores. Based on a previous study employing a comparable low-calcium diet-induced C57BL/6 mouse model, CaCl_2 was administered by oral gavage at a dose equivalent to 133 mg elemental Ca/kg body weight/day for 4 weeks^[33].

At the end of the 12-hour fasting period, we anesthetized the mice using carbon dioxide inhalation. Blood was drawn through the retro-orbital sinus, and we immediately sacrificed the animals by cervical dislocation. Blood samples were centrifuged at 3000 rpm for 15 minutes to isolate the serum, which was stored at

-80 °C for downstream analysis. The unilateral femur was harvested from each animal, cleared of surrounding soft tissue, and kept at -20 °C for storage.

2.10 Serum biochemical analysis

Serum calcium levels and ALP activity were quantified utilising an automated biochemical analyser.

2.11 easurement of femoral length, diameter, and weight

After removing the soft tissues, the longitudinal length and mid-diaphyseal diameter of the femurs were measured using a vernier caliper, and the femoral weight was recorded using a precision electronic balance.

2.12 Micro-computed tomography (micro-CT) analysis

Micro-CT was used to scan the distal femurs of mice in each group to generate a three-dimensional model, and relevant parameters were calculated.

2.13 Statistical analysis

Statistical analyses were performed using GraphPad Prism 9.5.1 (GraphPad Software, San Diego, CA, USA). Quantitative data are presented as the mean $\pm$ standard error of the mean (SEM). Statistical analyses were performed according to the comparisons specified by the experimental design, with the relevant treatment groups compared with their corresponding control groups. Student's *t*-tests or one-way analysis of variance (ANOVA) were used as appropriate for the original statistical comparisons. No additional multiplicity adjustment was applied in the original analyses. Data normality was further assessed using the Shapiro–Wilk test, and homogeneity of variances was evaluated using the Brown–Forsythe test. Given the relatively small sample sizes in some *in vitro* experiments, the results of formal normality testing were interpreted cautiously. No observations were excluded as outliers. A *p* value < 0.05 was considered statistically significant. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$.

3. Results

To prioritize strains with favorable industrial applicability before functional evaluation, we initially assessed the sporulation capacity of 51 *H. coagulans* strains using three different sporulation media. Based on their overall sporulation performance across the three media (S2–S4), five strains with potential industrial applicability were selected for subsequent functional evaluation: VJSXZ1M1, VYNDL1M1, CCFM1539, CCFM1540, and CCFM1541 (Table 1).

Table 1. Five strains demonstrating potential industrial feasibility

No.	Strain designation	Origin
1	VJSXZ1M1	Xuzhou, Jiangsu, Vegetables
2	VYNDL1M1	Dali, Yunnan, Vegetables
3	CCFM1539	Wuxi, Jiangsu, Vegetables
4	CCFM1540	Yichang, Hubei, Vegetables
5	CCFM1541	Laixi, Shandong, Vegetables

Following the successful screening of potential strains, we constructed an *in vitro* Caco-2 monolayer system to comprehensively assess their regulatory effects on intestinal calcium transport and absorption. The

viability of Caco-2 cells was analyzed after exposure to distinct doses of both mineral and bacterial components. After a 2-h treatment period using different calcium levels, Fig. 1a illustrates that moderate to high calcium treatments (1.25~10 mmol/L) enhanced Caco-2 cell viability. The 20 mmol/L group presented negligible changes, whereas the 40 mmol/L concentration exhibited mild cytotoxicity. Consequently, the 10 mmol/L dosage was chosen as the standard for all subsequent assays.

As depicted in Fig. 1b, following a 2-h exposure to varying concentrations of the bacterial suspension, the viability of cells in the 10^7 - 10^8 treatment groups was marginally elevated compared to the NC group, indicating an absence of cytotoxicity. Therefore, an inoculation level of 10^8 CFU/mL was adopted for downstream experimental procedures.

After determining the optimal intervention concentrations, we assessed both the integrity and functional polarization characterizing the Caco-2 monolayer. Cellular growth dynamics in Caco-2 cultures were tracked using an inverted microscope at days 2, 5, 12, and 20 of the cultivation period. During the initial culture phase, notable intercellular gaps and localised vacuolisation were observed. As cultivation progressed, the cells formed tighter intercellular connections, establishing a rudimentary monolayer by day 7. Subsequent observations on days 12 and 20 revealed compact cell junctions, indicating the morphological formation of an intact cell monolayer.

Transepithelial electrical resistance (TEER) is a crucial biophysical parameter indicative of cell monolayer integrity and exhibits a negative correlation with monolayer permeability within a specific range. It is widely accepted that transport assays are viable only when the TEER value exceeds $200 \Omega \cdot \text{cm}^2$ ^[34, 35]. As illustrated in Fig. 1c, the proliferation, confluence, and tight junction formation of Caco-2 cells were predominantly completed by the end of the second week. The TEER value increased marginally, plateauing after three weeks. Following a 21-day differentiation period, the TEER value surpassed $2500 \Omega \cdot \text{cm}^2$.

By day 21 of cultivation, measurements showed that ALP levels in the AP chamber were approximately 7 times higher than in the BL region (Figure 1d). This highly asymmetrical distribution of ALP indicates pronounced cellular polarisation, thereby confirming successful and robust cellular differentiation.

Following rigorous validation of the cell model, we used it to compare the five selected strains' abilities to facilitate calcium transport. The five selected *H. coagulans* strains were evaluated utilising the Caco-2 cell monolayer model. Figure 1e demonstrates that while *H. coagulans* VYNDL1M1 and CCFM1541 improved calcium absorption; however, these increases lacked statistical significance compared to the NC baseline. In contrast, *H. coagulans* CCFM1539 significantly enhanced calcium cellular absorption and transport compared with the blank group ($P < 0.05$). Consequently, CCFM1539 shows promising potential to facilitate intestinal calcium absorption.

To understand the targeted modulatory mechanisms responsible for this facilitated cellular absorption, we quantified the expression of calcium-related transporters, including TRPV6, VDR, PMCA1B, and NCX1. After penetrating intestinal cells via epithelial channels such as TRPV6, incoming calcium binds to calcium-binding proteins. Subsequent cytoplasmic transit brings the complex to the basolateral boundary.

Eventually, these ions shift from CABP to PMCA1B or NCX1. Such high-affinity carriers transport the mineral from enterocytes into the systemic circulation. Because VDR initiates CABP transcription and promotes PMCA1B and NCX1 expression, supplemental calcium theoretically drives a corresponding increase in these transcriptional activities^[36-38]. Transcript abundances for VDR, TRPV6, NCX1, and PMCA1B were then measured within the Caco-2 cellular model. As shown in Fig.1(f)-(i), *H. coagulans* strains 1539, 1540, and 1541 significantly enhance cellular calcium absorption.

Protein-level analyses were further conducted for all five candidate strains to complement the calcium transport and gene-expression assessments. As illustrated in Fig.1(j) and (k), all evaluated strains, except *H. coagulans* VYNDL1M1, significantly upregulated TRPV6 protein expression ($P < 0.05$). However, the protein expression of PMCA1B showed an opposite trend to its mRNA expression, suggesting potential post-transcriptional regulation.

Based on an integrated evaluation of transepithelial calcium transport and the regulation of calcium-transport-related genes and proteins, CCFM1539, CCFM1540, and CCFM1541 showed more consistent overall functional responses among the five candidate strains. These three strains were therefore selected for subsequent *in vivo* evaluation.

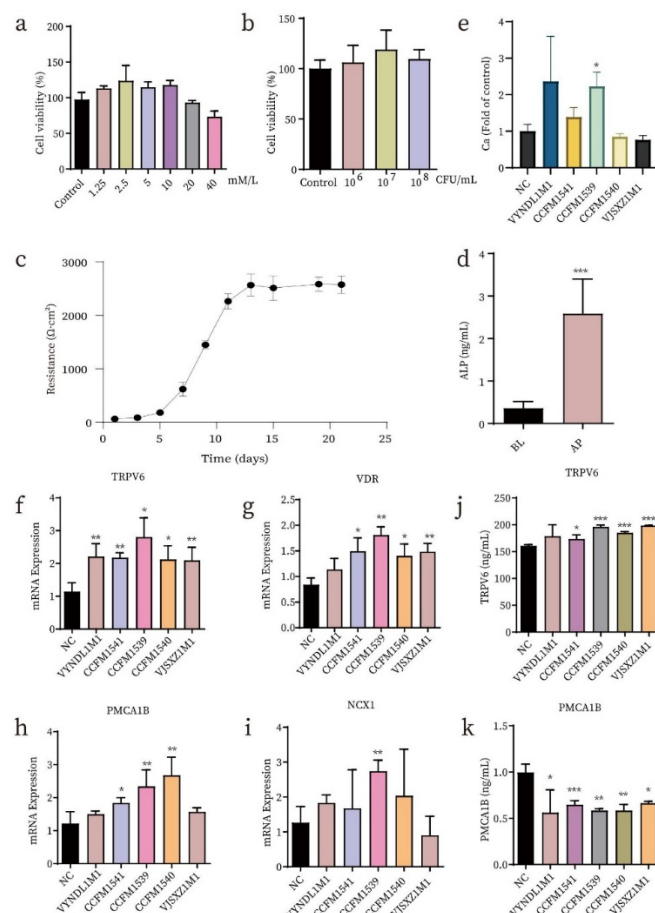


Figure 1. *H. coagulans* promotes calcium transport in the Caco-2 cell model and its underlying mechanisms (a) Effects of different concentrations of calcium on the survival rate of Caco-2 cells. (b) Effects of different concentrations of bacterial components on Caco-2 cell survival. (c) Changes in TEER values during different periods of model construction. (d) Ratio of ALP on both sides of the chamber. (e) Changes in the total calcium transport amount in the Caco-2 cell monolayer model among different groups. (f)-(i) mRNA expression levels of calcium ion transport-related genes (TRPV6, VDR, PMCA1B, NCX1); (j)-(k) Protein expression levels of TRPV6 and PMCA1B. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

To evaluate the *in vivo* efficacy of *H. coagulans*, we established a low-calcium diet-induced murine model and evaluated the efficacy of specific *H. coagulans* strains supplementation in alleviating calcium deficiency and bone loss in C57BL/6J mice (Fig. 2a). Subsequently, we assessed key serum biochemical indicators of bone metabolism. Serum calcium levels and ALP activity were quantified utilising an automated biochemical analyser.

Once dietary calcium is ingested, it is taken up by enterocytes via specific calcium-binding proteins before entering the systemic circulation. This circulating pool of calcium then accumulates within bone tissues. Any generalized calcium deficiency will inevitably lower serum calcium concentrations^[39]. As illustrated in Fig. 2b, a distinct drop in serum calcium occurred in the MC group relative to the NC controls ($P < 0.0001$), confirming that chronic exposure to a low-calcium regimen induced severe deficiency in mice. Combined treatment with calcium and individual *coagulans* strains resulted in substantial recovery from hypocalcemia in the MC animals ($P < 0.0001$), a result superior to that achieved with calcium alone. Such outcomes point to the role of *H. coagulans* in enhancing calcium uptake within the murine model.

ALP, an essential biomarker of osteogenesis secreted by osteoblasts, serves as an indicator of osteoblastic function and activity. Pathological bone conditions trigger excessive ALP secretion by osteoblasts into the bloodstream, resulting in elevated serum ALP levels^[40, 41]. Fig. 3c shows that the MC group reached a serum ALP concentration of 225.97 U/L, which is significantly higher than that of all other groups ($P < 0.0001$). This outcome indicates that lower calcium intake is associated with higher ALP levels. The administration of calcium supplemented with *H. coagulans* strains CCFM1539 (116.09 U/L), CCFM1540 (112.36 U/L), or CCFM1541 (95.00 U/L) significantly attenuated the serum ALP levels ($P < 0.0001$), achieving a more pronounced reduction than calcium supplementation alone (165.63 U/L).

Collectively, these results demonstrate that *H. coagulans* effectively promotes serum calcium absorption and restores the corresponding enzymatic activities in mice. Notably, *H. coagulans* CCFM1541 demonstrated the greatest efficacy in enhancing murine calcium absorption.

We further evaluated the physical parameters of the murine femurs. We analyzed the physical dimensions of the mouse femurs, including weight, diameter, and length, to test the ability of *H. coagulans* to boost bone calcium deposition. Fig. 2d~f show that the MC group had much lower values for femoral weight, diameter, and length than the NC controls ($P < 0.001$). Conversely, co-supplementation with calcium and *H. coagulans* significantly reversed the reductions in femoral weight, diameter, and length induced by calcium deficiency ($P < 0.01$). The extent of this recovery surpassed that observed in the Ca group, demonstrating that *H. coagulans* effectively facilitates skeletal calcium deposition in mice.

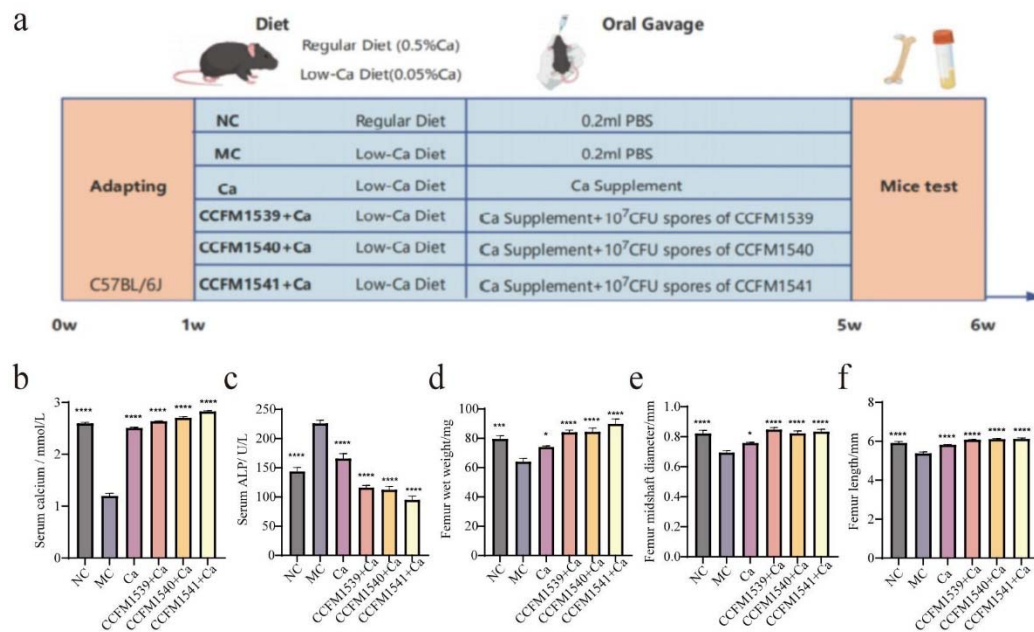


Figure 2. *H. coagulans* alleviates calcium deficiency and bone loss in C57BL/6J mice.

(a) Experiment design. (b) Serum calcium content. (c) Serum ALP content. (d) Femur weight. (e) Femur length. (f) Femur midshaft diameter. * $P < 0.05$, $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$.

We also examined the internal bone architecture using micro-CT imaging. As illustrated in Fig. 3a, the trabeculae in the distal femurs of the MC group were sparse, thinned, heterogeneously arranged, and exhibited increased separation, indicating severe deterioration of the trabecular bone architecture. Trabecular degradation in the Ca group was mitigated compared with the MC group, accompanied by a concurrent increase in bone network density; however, complete restoration to normative levels was not achieved. By contrast, the trabecular morphology in the *H. coagulans* + Ca groups exhibited a substantially more pronounced improvement relative to the Ca group. Mice in the *H. coagulans* + Ca groups displayed attenuated microstructural deterioration, characterised by increased trabecular area, thickness, and density, resulting in greater structural integrity. These findings demonstrate that, compared with calcium supplementation alone, the combined intervention with *H. coagulans* and calcium more effectively prevented bone loss and facilitated the preservation and restoration of skeletal microarchitecture.

The amount of bone tissue contained in a space is represented by the bone volume fraction (BV/TV). Increases in BV/TV are primarily driven by elevations in trabecular number (Tb.N). As depicted in Fig. 3b, the low-calcium diet significantly reduced murine BV/TV ($P < 0.001$). Relative to the MC group (9.83%), co-intervention with *H. coagulans* strains CCFM1539 (18.83%), CCFM1540 (17.04%), and CCFM1541 (19.50%) significantly restored femoral BV/TV ($P < 0.001$), exceeding the recovery observed in the Ca group (16.35%). As illustrated in Fig. 3c, the calcium-deficient diet markedly decreased femoral Tb. N to 1.03 ($P < 0.001$). While isolated calcium supplementation restored Tb. N to 1.56 ($P < 0.01$), *H. coagulans* CCFM1539, CCFM1540, and CCFM1541 further restored Tb. N values to 1.84, 1.52, and 1.94, respectively. These results indicate that the combined intervention, particularly with CCFM1539 and CCFM1541, was associated with greater improvements in bone mass-related parameters than calcium supplementation alone.

These findings align with recent micro-CT evaluations in murine models by Lim et al.^[42]. Their work showed that oral administration of specific probiotics can significantly increase BV/TV and Tb. N levels, thereby increasing bone weight and preventing bone thinning. As shown in Fig. 3d, relative to the MC group (4.15), strains *H. coagulans* CCFM1539, CCFM1540, and CCFM1541 significantly elevated femoral BS/TV to 7.18, 6.16, and 7.33, respectively ($P < 0.01$); the efficacies of CCFM1539 and CCFM1541 surpassed that of the Ca group (6.25). These results indicate that the combined intervention, particularly with CCFM1539 and CCFM1541, improved BS/TV to a greater extent than calcium supplementation alone. The ratio of bone surface area to its total volume is represented by the specific bone surface (BS/BV). Fig. 3e shows that the MC group had a much higher BS/BV value than the NC group ($P < 0.01$). This result shows that the trabeculae of the thigh bone became thinner and had more holes under calcium deficiency. Both isolated calcium supplementation and co-supplementation with *H. coagulans* significantly reversed the calcium deficiency-induced elevation in BS/BV ($P < 0.05$), suggesting that both calcium supplementation alone and the combined intervention were associated with thicker trabeculae and a more compact structural architecture in murine femurs.

Higher connectivity density (Conn. D) and a drop in trabecular separation (Tb.Sp) are key microstructural signs that the trabecular network has become tight again. Correspondingly, research by Qin et al.^[43] demonstrated that probiotic interventions significantly ameliorate these two key micro-CT parameters. Fig. 3f and Fig. 3g show that the low-calcium diet caused a clear rise in Tb.Sp values ($P < 0.05$). Compared with the MC group, the Ca and CCFM1540+Ca groups showed a downward trend in femoral Tb.Sp, though this change was not statistically significant. On the other hand, the CCFM1539+Ca and CCFM1541+Ca groups brought about a clear drop in femoral Tb.Sp ($P < 0.05$). The CCFM1541+Ca group worked the best, showing much lower numbers than the plain Ca group ($P < 0.01$). Meanwhile, we observed that femoral Conn.D was heavily reduced by the low-calcium diet ($P < 0.001$). Calcium supplementation alone increased femoral Conn.D ($P < 0.01$). Better protection was observed with the calcium-*H. coagulans* strain mixtures CCFM1539 or CCFM1541, which reached numbers distinctly higher than those in the MC group ($P < 0.001$). For the CCFM1540+Ca group, though, the numbers showed no meaningful difference when compared to the MC group. These results further indicate that the combined intervention with *H. coagulans* and calcium produced greater beneficial effects on osteogenesis and trabecular microarchitecture than calcium supplementation alone.

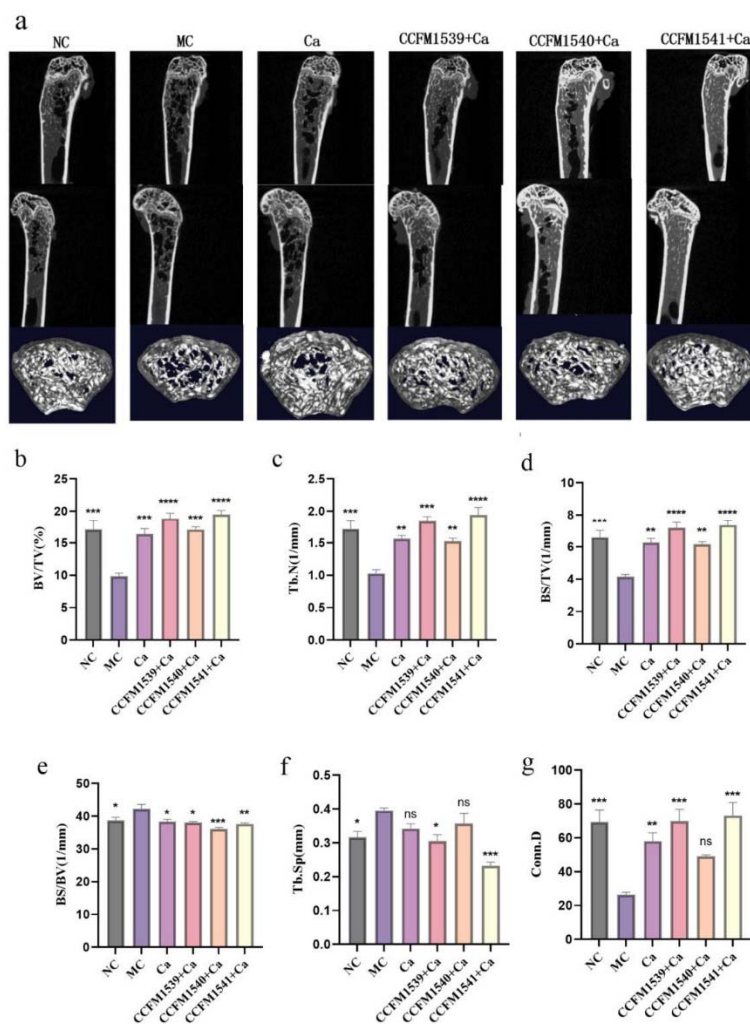


Figure 3. Micro-CT analysis of bone tissue microstructure in mice among different groups.

(a) Micro-CT scanning images of femurs from each experimental group. (b) BV/TV. (c) BS/TV. (d) Tb. N. (e) BS/BV. (f) Tb.Sp. (g) Conn.D. ns $P > 0.05$, * $P < 0.05$, $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$.

4. Discussion

The study tested whether *H. coagulans* can help the intestines better absorb calcium. Given that robust sporulation capacity is a prerequisite for the industrial application of probiotics in food processing (e.g., conferring resistance to high temperatures and gastric acid)^[29, 44], we initially screened five *H. coagulans* strains exhibiting high sporulation efficacy and industrial potential from a pool of 51 candidates. Importantly, sporulation capacity was used here as a criterion for industrial applicability rather than as a predictor of calcium absorption-promoting activity. The latter was subsequently evaluated independently using the Caco-2 monolayer model.

In vitro, we successfully built a Caco-2 cell monolayer model. Within this transport model, *H. coagulans* CCFM1539 significantly enhanced cellular calcium absorption and total transepithelial transport ($P < 0.05$). Intestinal epithelial calcium absorption is contingent upon a cascade of key transporters: calcium ions initially enter enterocytes via apical channels such as TRPV6, bind to cytosolic calcium-binding proteins, and are ultimately effluxed into the bloodstream by the higher-affinity transporters PMCA1B or NCX1 at the basolateral membrane. The transcription of these genes is activated by the vitamin D receptor (VDR)^[32, 37].

Our results show that *H. coagulans* strains 1539, 1540, and 1541 clearly increased mRNA levels of TRPV6, VDR, PMCA1B, and NCX1 in Caco-2 cells ($P < 0.05$). Notably, while these strains significantly upregulated TRPV6 protein expression ($P < 0.05$), PMCA1B protein levels showed a trend discordant with its mRNA levels. This discrepancy suggests the potential involvement of complex post-transcriptional regulatory or protein-degradation mechanisms within the probiotic-mediated intestinal calcium transport network, a phenomenon corroborated by prior integrated transcriptomic and proteomic analyses^[45]. Collectively, specific *H. coagulans* strains directly facilitate cellular calcium absorption by activating the expression of epithelial calcium channels and associated transport factors.

To further elucidate the physiological efficacies of these strains, a low-calcium diet-induced murine model was employed. Chronic consumption of a low-calcium diet leads to a marked decline in serum calcium levels; concurrently, as a compensatory response to calcium-deficiency-induced skeletal pathologies, bone-building osteoblasts release large amounts of ALP into the bloodstream. As a result, blood ALP, a biochemical marker of high bone turnover, sharply increases^[46]. Our experimental observations are highly consistent with this pathological progression. Mice in the MC group exhibited significantly diminished serum calcium levels ($P < 0.0001$) and abnormally elevated ALP concentrations (reaching 225.97 U/L, $P < 0.0001$), indicative of profound systemic calcium deficiency and disrupted bone metabolism. Compared to isolated calcium supplementation, co-administration of calcium with *H. coagulans* strains 1539, 1540, or 1541 more effectively ameliorated low serum calcium levels ($P < 0.0001$). Significantly attenuated serum ALP levels to 116.09 U/L, 112.36 U/L, and 95.00 U/L, respectively ($P < 0.0001$), outperforming the calcium-only group (165.63 U/L), with CCFM1541 exhibiting the most pronounced restorative effect. These results indicate that the combined intervention with *H. coagulans* and calcium was associated with improved systemic calcium status and normalization of ALP activity, consistent with an improvement in calcium-deficiency-induced alterations in bone metabolism.

Regarding skeletal microarchitecture and calcium deposition, chronic low-calcium diet consumption induces a comprehensive reduction in femoral weight, length, and mid-diaphyseal diameter, accompanied by severely deteriorated, sparse, and porous trabeculae as visualised via micro-CT. Recent studies and reviews show that probiotics for the gut bacteria work through the “gut-bone axis”. They can balance bone breakdown and bone growth. This means these probiotics have a real chance of repairing damaged bone structures under microscopic imaging^[47, 48]. Established guidelines delineate BV/TV and Tb. N as core parameters for assessing bone loss and structural degradation^[49]. Our findings demonstrated that co-supplementation of *H. coagulans* with calcium profoundly outperformed isolated calcium supplementation in preventing bone loss and promoting microarchitectural recovery. Specifically, *H. coagulans* strains 1539, 1540, and 1541 significantly elevated BV/TV from 9.83% to 18.83%, 17.04%, and 19.50% ($P < 0.001$) and

increased Tb. N from 1.03 to 1.84, 1.52, and 1.94 (1/mm) ($P < 0.001$)—indicative of augmented bone mass—and increased BS/TV from 4.15 to 7.18, 6.16, and 7.33 (1/mm) ($P < 0.01$). Furthermore, the

co-intervention significantly reversed the calcium-deficiency-induced elevation in BS/BV ($P < 0.05$), resulting in thicker, more compact femoral trabeculae. Notably, *H. coagulans* CCFM1539 and CCFM1541 also significantly reduced Tb. Sp ($P < 0.05$, with CCFM1541 reaching $P < 0.01$ against the Ca group) and substantially increased Conn.D ($P < 0.001$). The comprehensive improvements in these micro-CT parameters indicate that, compared with calcium supplementation alone, the combined intervention with *H. coagulans* and calcium produced greater beneficial effects on bone mass and three-dimensional trabecular microarchitecture. Nevertheless, because an *H. coagulans*-only group was not included, the independent contribution of *H. coagulans* to bone metabolism remains to be further clarified.

In conclusion, this study corroborates that *H. coagulans* strains CCFM1539, CCFM1540, and CCFM1541 enhance calcium transport efficiency *in vitro* by regulating epithelial calcium channel expression and associated protein levels. *In vivo*, the combined administration of these strains and calcium produced greater improvements in low-calcium-induced serum biochemical abnormalities and trabecular microarchitecture than calcium supplementation alone. The present study was primarily designed to screen *H. coagulans* strains with calcium absorption-promoting potential and to validate their functional efficacy, while the Caco-2 experiments provided preliminary mechanistic clues regarding the regulation of intestinal calcium transport. Although these findings support a link between enhanced intestinal calcium handling and improved bone phenotypes, the detailed molecular pathways underlying these effects *in vivo* remain to be further elucidated through additional biochemical and molecular analyses. Notably, *H. coagulans* strains 1539 and 1541 exhibited superior efficacy. Future investigations should elucidate how core metabolites (e.g., short-chain fatty acids) produced following intestinal colonisation by these specific strains regulate osteoblast and osteoclast homeostasis via the “gut-bone axis”^[50]. Our work gives a strong scientific basis and highlights useful bacterial targets. This helps use *H. coagulans* as a new dietary supplement or probiotic to improve mineral intake and prevent bone loss or osteoporosis. Future studies will build on the calcium absorption-promoting phenotype identified in the present study and further elucidate the underlying molecular mechanisms *in vivo*, with particular emphasis on the regulation of intestinal calcium transport and the potential involvement of the gut–bone axis.

5. Conclusion

In the present study, *H. coagulans* strains with favorable sporulation capacity and industrial potential were initially selected from a pool of 51 candidates and subsequently subjected to independent functional evaluation for their calcium absorption-promoting properties. Utilising both *in vitro* and *in vivo* models, we comprehensively validated their profound efficacy in facilitating intestinal calcium absorption and ameliorating skeletal microarchitecture. These findings establish a robust scientific foundation for the application of specific *H. coagulans* strains as novel dietary supplements or probiotic formulations to optimise mineral absorption and preserve skeletal health.

Declaration of Interest Statement

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.

Acknowledgements

This work was supported by the National Key Technologies Research and Development Program [No. 2024YFA1307100]; supported by the (3) the National Key Research and Development Program of China (2024YFD2100901); and supported by the the Special Fund for Science and Technology Program of Jiangsu Province BM2022019 and Fundamental Research Funds for the Central Universities JUSRP202607003; Supercomputing facilities were provided by the Computing Center of Xi'an and the High-Performance Computing Platform of the School of Biotechnology, Jiangnan University. MOE Medical Basic Research Innovation Center for Gut Microbiota and Chronic Diseases, School of medicine, Jiangnan university.

References

- [1] G. Cormick, J.M. Belizán, Calcium intake and health, *Nutrients* 11 (2019) 1606. <https://doi.org/10.3390/nu11071606>.
- [2] P.M. Coates, J.M. Betz, M.R. Blackman, G.M. Cragg, M. Levine, J. Moss, J.D. White (Eds.), *Encyclopedia of Dietary Supplements*, 2nd ed., CRC Press, Boca Raton, FL, 2010. <https://doi.org/10.1201/b14669>.
- [3] J. Shlisky, R. Mandlik, S. Askari, et al., Calcium deficiency worldwide: prevalence of inadequate intakes and associated health outcomes, *Ann. N.Y. Acad. Sci.* 1512 (2022) 10–28. <https://doi.org/10.1111/nyas.14758>.
- [4] D.B. Kumssa, E.J.M. Joy, E.L. Ander, et al., Dietary calcium and zinc deficiency risks are decreasing but remain prevalent, *Sci. Rep.* 5 (2015) 10974. <https://doi.org/10.1038/srep10974>.
- [5] Institute of Medicine, *Dietary Reference Intakes for Calcium and Vitamin D*, National Academies Press, Washington, DC, 2011. <https://doi.org/10.17226/13050>.
- [6] G. Cormick, A. Ciapponi, M.L. Cafferata, et al., Calcium supplementation for prevention of primary hypertension, *Cochrane Database Syst. Rev.* 1 (2022) CD010037. <https://doi.org/10.1002/14651858.CD010037.pub4>.
- [7] L.A.J. van Mierlo, L.R. Arends, M.T. Streppel, et al., Blood pressure response to calcium supplementation: a meta-analysis of randomized controlled trials, *J. Hum. Hypertens.* 20 (2006) 571–580. <https://doi.org/10.1038/sj.jhh.1002038>.
- [8] G.J. Hofmeyr, T.A. Lawrie, Á.N. Atallah, L. Duley, Calcium supplementation during pregnancy for preventing hypertensive disorders and related problems, *Cochrane Database Syst. Rev.* 8 (2010) CD001059. <https://doi.org/10.1002/14651858.CD001059.pub3>.
- [9] G.J. Hofmeyr, A.P. Betrán, M. Singata-Madliki, et al., Prepregnancy and early pregnancy calcium supplementation among women at high risk of pre-eclampsia: a multicentre, double-blind, randomised, placebo-controlled trial, *Lancet* 393 (2019) 330–339. [https://doi.org/10.1016/S0140-6736\(18\)31818-X](https://doi.org/10.1016/S0140-6736(18)31818-X).
- [10] E. Bergel, J.M. Belizán, A deficient maternal calcium intake during pregnancy increases blood pressure of the offspring in adult rats, *BJOG* 109 (2002) 540–545. <https://doi.org/10.1111/j.1471-0528.2002.01155.x>.
- [11] E. Bergel, L. Gibbons, M.G. Rasines, et al., Maternal calcium supplementation during pregnancy and dental caries of children at 12 years of age: follow-up of a randomized controlled trial, *Acta Obstet. Gynecol. Scand.* 89 (2010) 1396–1402. <https://doi.org/10.3109/00016349.2010.518228>.
- [12] I.J. Onakpoya, R. Perry, J. Zhang, E. Ernst, Efficacy of calcium supplementation for management of overweight and obesity: systematic review of randomized clinical trials, *Nutr. Rev.* 69 (2011) 335–343. <https://doi.org/10.1111/j.1753-4887.2011.00397.x>.
- [13] C. Chen, S. Ge, S. Li, et al., The effects of dietary calcium supplements alone or with vitamin D on cholesterol metabolism: a meta-analysis of randomized controlled trials, *J. Cardiovasc. Nurs.* 32 (2017) 496–506. <https://doi.org/10.1097/JCN.0000000000000379>.

- [14] S. Bonovas, G. Fiorino, T. Lytras, et al., Calcium supplementation for the prevention of colorectal adenomas: a systematic review and meta-analysis of randomized controlled trials, *World J. Gastroenterol.* 22 (2016) 4594–4603. <https://doi.org/10.3748/wjg.v22.i18.4594>.
- [15] J.D. Cook, S.A. Dassenko, P. Whittaker, Calcium supplementation: effect on iron absorption, *Am. J. Clin. Nutr.* 53 (1991) 106–111. <https://doi.org/10.1093/ajcn/53.1.106>.
- [16] L.M.A. Jarjou, M.A. Laskey, Y. Sawo, et al., Effect of calcium supplementation in pregnancy on maternal bone outcomes in women with a low calcium intake, *Am. J. Clin. Nutr.* 92 (2010) 450–457. <https://doi.org/10.3945/ajcn.2010.29217>.
- [17] L.M.A. Jarjou, Y. Sawo, G.R. Goldberg, et al., Unexpected long-term effects of calcium supplementation in pregnancy on maternal bone outcomes in women with a low calcium intake: a follow-up study, *Am. J. Clin. Nutr.* 98 (2013) 723–730. <https://doi.org/10.3945/ajcn.113.061630>.
- [18] K. Li, X.-F. Wang, D.-Y. Li, et al., The good, the bad, and the ugly of calcium supplementation: a review of calcium intake on human health, *Clin. Interv. Aging* 13 (2018) 2443–2452. <https://doi.org/10.2147/CIA.S157523>.
- [19] C.M. Whisner, L.F. Castillo, Prebiotics, bone and mineral metabolism, *Calcif. Tissue Int.* 102 (2018) 443–479. <https://doi.org/10.1007/s00223-017-0339-3>.
- [20] R. Pacifici, Bone remodeling and the microbiome, *Cold Spring Harb. Perspect. Med.* 8 (2018) a031203. <https://doi.org/10.1101/cshperspect.a031203>.
- [21] I.A. Harahap, M. Moszak, M. Czlapka-Matyasik, et al., Effects of daily probiotic supplementation with *Lactobacillus acidophilus* on calcium status, bone metabolism biomarkers, and bone mineral density in postmenopausal women: a controlled and randomized clinical study, *Front. Nutr.* 11 (2024) 1401920. <https://doi.org/10.3389/fnut.2024.1401920>.
- [22] A.G. Nilsson, D. Sundh, F. Bäckhed, M. Lorentzon, *Lactobacillus reuteri* reduces bone loss in older women with low bone mineral density: a randomized, placebo-controlled, double-blind, clinical trial, *J. Intern. Med.* 284 (2018) 307–317. <https://doi.org/10.1111/joim.12805>.
- [23] T. Takimoto, M. Hatanaka, T. Hoshino, et al., Effect of *Bacillus subtilis* C-3102 on bone mineral density in healthy postmenopausal Japanese women: a randomized, placebo-controlled, double-blind clinical trial, *Biosci. Microbiota Food Health* 37 (2018) 87–96. <https://doi.org/10.12938/bmfh.18-006>.
- [24] C. Ohlsson, C. Engdahl, F. Fåk, et al., Probiotics protect mice from ovariectomy-induced cortical bone loss, *PLoS One* 9 (2014) e92368. <https://doi.org/10.1371/journal.pone.0092368>.
- [25] R. Rizzoli, E. Biver, Are probiotics the new calcium and vitamin D for bone health?, *Curr. Osteoporos. Rep.* 18 (2020) 273–284. <https://doi.org/10.1007/s11914-020-00591-6>.
- [26] M. Gueimonde, B. Sánchez, Enhancing probiotic stability in industrial processes, *Microb. Ecol. Health Dis.* 23 (2012) 18562. <https://doi.org/10.3402/mehd.v23i0.18562>.
- [27] K. Vivek, S. Mishra, R.C. Pradhan, et al., A comprehensive review on microencapsulation of probiotics: technology, carriers and current trends, *Appl. Food Res.* 3 (2023) 100248. <https://doi.org/10.1016/j.afres.2022.100248>.
- [28] A. Terpou, A. Papadaki, I.K. Lappa, et al., Probiotics in food systems: significance and emerging strategies towards improved viability and delivery of enhanced beneficial value, *Nutrients* 11 (2019) 1591. <https://doi.org/10.3390/nu11071591>.
- [29] J. Payne, D. Bellmer, R. Jadeja, P.M. Muriana, The potential of *Bacillus* species as probiotics in the food industry: a review, *Foods* 13 (2024) 2444. <https://doi.org/10.3390/foods13152444>.
- [30] C. Chu, H. Ru, Y. Chen, et al., Gallic acid attenuates LPS-induced inflammation in Caco-2 cells by suppressing the activation of the NF- κ B/MAPK signaling pathway: gallic acid alleviates the inflammation in Caco-2 cells, *Acta Biochim. Biophys. Sin.* 56 (2024) 905–915. <https://doi.org/10.3724/abbs.2024008>.
- [31] Z. Wang, R. Ma, Z. Jia, et al., Investigating on the influence mechanism of sausage of sea bass on calcium absorption and transport based on Caco-2 cell monolayer model, *Front. Nutr.* 9 (2022) 1046945. <https://doi.org/10.3389/fnut.2022.1046945>.
- [32] J. Xiang, R. Guo, C. Wan, et al., Regulation of intestinal epithelial calcium transport proteins by stanniocalcin-1 in Caco2 cells, *Int. J. Mol. Sci.* 17 (2016) 1095. <https://doi.org/10.3390/ijms17071095>.

- [33] L. Chen, S. Lin, X. He, et al., A calcium-loaded complex based on Antarctic krill protein and supplemented with pectin promotes calcium absorption and bone health, *Int. J. Biol. Macromol.* 306 (2025) 141634. <https://doi.org/10.1016/j.ijbiomac.2025.141634>.
- [34] I. Hubatsch, E.G.E. Ragnarsson, P. Artursson, Determination of drug permeability and prediction of drug absorption in Caco-2 monolayers, *Nat. Protoc.* 2 (2007) 2111–2119. <https://doi.org/10.1038/nprot.2007.303>.
- [35] P. Artursson, K. Palm, K. Luthman, Caco-2 monolayers in experimental and theoretical predictions of drug transport, *Adv. Drug Deliv. Rev.* 64 (2012) 280–289. <https://doi.org/10.1016/j.addr.2012.09.005>.
- [36] A.L. Deluque, H. Dimke, R.T. Alexander, Biology of calcium homeostasis regulation in intestine and kidney, *Nephrol. Dial. Transplant.* 40 (2025) 435–445. <https://doi.org/10.1093/ndt/gfae204>.
- [37] V. Khattar, L. Wang, J.-B. Peng, Calcium selective channel TRPV6: structure, function, and implications in health and disease, *Gene* 817 (2022) 146192. <https://doi.org/10.1016/j.gene.2022.146192>.
- [38] G.D. de Barboza, S. Guizzardi, N.T. de Talamoni, Molecular aspects of intestinal calcium absorption, *World J. Gastroenterol.* 21 (2015) 7142–7154. <https://doi.org/10.3748/wjg.v21.i23.7142>.
- [39] M. Peacock, Calcium metabolism in health and disease, *Clin. J. Am. Soc. Nephrol.* 5 (Suppl. 1) (2010) S23–S30. <https://doi.org/10.2215/CJN.05910809>.
- [40] S. Vimalraj, Alkaline phosphatase: structure, expression and its function in bone mineralization, *Gene* 754 (2020) 144855. <https://doi.org/10.1016/j.gene.2020.144855>.
- [41] T.-R. Kuo, C.-H. Chen, Bone biomarker for the clinical assessment of osteoporosis: recent developments and future perspectives, *Biomark. Res.* 5 (2017) 18. <https://doi.org/10.1186/s40364-017-0097-4>.
- [42] Y. Lim, O.-J. Park, C. Park, et al., Oral intake of heat-killed *Lactiplantibacillus plantarum* alleviates bone loss in an ovariectomized mouse model similarly to live *L. plantarum*, *J. Microbiol. Biotechnol.* 36 (2026) e2510013. <https://doi.org/10.4014/jmb.2510.10013>.
- [43] X. Qin, Y.-E. Lian, H. Tang, et al., *Lactobacillus rhamnosus* GG mitigates bone loss induced by mechanical unloading via regulation of the gut-bone axis, *Front. Nutr.* 12 (2026) 1734220. <https://doi.org/10.3389/fnut.2025.1734220>.
- [44] A. Poshadri, H.W. Deshpande, U.M. Khodke, S.D. Katke, *Bacillus coagulans* and its spore as potential probiotics in the production of novel shelf-stable foods, *Curr. Res. Nutr. Food Sci.* 10 (2022) 858–870. <https://doi.org/10.12944/CRNFSJ.10.3.4>.
- [45] C. Vogel, E.M. Marcotte, Insights into the regulation of protein abundance from proteomic and transcriptomic analyses, *Nat. Rev. Genet.* 13 (2012) 227–232. <https://doi.org/10.1038/nrg3185>.
- [46] M.H.-C. Sheng, K.-H.W. Lau, R. Lakhan, et al., Unique regenerative mechanism to replace bone lost during dietary bone depletion in weanling mice, *Endocrinology* 158 (2017) 714–729. <https://doi.org/10.1210/en.2016-1379>.
- [47] L. Sapra, C. Saini, P.K. Mishra, et al., *Bacillus coagulans* ameliorates inflammatory bone loss in post-menopausal osteoporosis via modulating the ‘gut-immune-bone’ axis, *Gut Microbes* 17 (2025) 2492378. <https://doi.org/10.1080/19490976.2025.2492378>.
- [48] L. Wang, S. Chen, X. Cai, et al., The influence of immune regulation mediated by intestinal microbiota on postmenopausal osteoporosis and intervention strategies, *Front. Endocrinol.* 16 (2026) 1720484. <https://doi.org/10.3389/fendo.2025.1720484>.
- [49] M.L. Bouxsein, S.K. Boyd, B.A. Christiansen, et al., Guidelines for assessment of bone microstructure in rodents using micro-computed tomography, *J. Bone Miner. Res.* 25 (2010) 1468–1486. <https://doi.org/10.1002/jbmr.141>.
- [50] B. Feng, J. Lu, Y. Han, et al., The role of short-chain fatty acids in the regulation of osteoporosis: new perspectives from gut microbiota to bone health: a review, *Medicine* 103 (2024) e39471. <https://doi.org/10.1097/MD.00000000000039471>.