





# Influence of Daidzein, Tempeh, and Probiotics on Phosphorus Levels in the Postmenopausal Rat Model

Iskandar Azmy Harahap<sup>1,2,\*</sup>, Katarzyna Skrypnik<sup>1</sup>, Paweł Kołodziejki<sup>3</sup>,  
Joanna Suliburska<sup>1,\*</sup>

<sup>1</sup>Department of Human Nutrition and Dietetics, Faculty of Food Science and Nutrition, Poznan University of Life Sciences, 60-624 Poznan, Poland

<sup>2</sup>Research Organization for Agricultural and Food, National Research and Innovation Agency, 16911 Bogor, Indonesia

<sup>3</sup>Department of Animal Physiology, Biochemistry and Biostructure, Faculty of Veterinary Medicine and Animal Science, Poznan University of Life Sciences, 60-637 Poznan, Poland

\*Correspondence: [iskandar.azmy.harahap@brin.go.id](mailto:iskandar.azmy.harahap@brin.go.id) (Iskandar Azmy Harahap); [joanna.suliburska@up.poznan.pl](mailto:joanna.suliburska@up.poznan.pl) (Joanna Suliburska)

Academic Editor: Torsten Bohn

Submitted: 18 September 2025 Revised: 6 November 2025 Accepted: 14 November 2025 Published: 25 December 2025

## Abstract

**Background:** Phosphorus is an essential component of bone and energy metabolism; however, the mechanism involved in the regulation of phosphorus under postmenopausal conditions remains insufficiently understood. Interestingly, the disruption of estrogen status can alter mineral homeostasis. Meanwhile, phytoestrogens and probiotics may offer a non-pharmacological strategy to support mineral homeostasis; nonetheless, evidence of their impact on phosphorus distribution remains limited. Thus, this study aimed to evaluate the effect of daidzein, tempeh, and *Lactobacillus acidophilus* on phosphorus distribution in ovariectomized (OVX) rats. **Methods:** A total of 64 female Wistar rats were allocated to sham-operated (n = 8) or OVX (n = 56) groups. Following a three-week calcium-deficient diet, the OVX rats were randomized to seven dietary interventions for six weeks: control (O), alendronate (OB), daidzein (10 mg/kg diet/day; OD), tempeh (250 g/kg diet/day; OT), probiotic *L. acidophilus* ( $1 \times 10^{10}$  CFU/day; OL), daidzein + probiotic (ODL), or tempeh + probiotic (OTL). Food intake, body mass, and phosphorus concentrations in feces, the femur, kidney, pancreas, spleen, heart, and liver were analyzed. **Results:** OVX groups (O,  $366 \pm 14$  g;  $p = 0.01$ ; OD,  $361 \pm 18$  g;  $p = 0.02$ ; OL,  $358 \pm 29$  g;  $p = 0.04$ ) gained significantly more body mass than sham controls ( $311 \pm 30$  g). Dietary interventions with daidzein, tempeh, and *L. acidophilus* enhanced phosphorus deposition in the femur, kidney, and pancreas compared with the OVX controls. The OT group showed the highest femoral phosphorus levels ( $178,111 \pm 32,628$  mg/kg d.w.;  $p = 0.02$ ), while the ODL group revealed elevated phosphorus levels in the kidney ( $12,966 \pm 462$  mg/kg d.w.;  $p = 0.01$ ). The OD ( $914 \pm 115$  mg/kg d.w.;  $p < 0.001$ ), ODL ( $913 \pm 39$  mg/kg d.w.;  $p < 0.001$ ), and OTL ( $926 \pm 70$  mg/kg d.w.;  $p < 0.001$ ) groups showed significantly increased levels of pancreatic phosphorus. A positive correlation was noted between the pancreatic and femoral phosphorus levels ( $r = 0.33$ ;  $p = 0.02$ ). Alendronate treatment had no significant effect. **Conclusions:** Dietary daidzein, tempeh, and probiotics modulate phosphorus distribution in postmenopausal rats. These results support the translational potential of dietary bioactives to improve phosphorus metabolism in postmenopausal women.

**Keywords:** minerals metabolism; phytoestrogens; probiotics; postmenopausal; women's health

## 1. Introduction

Menopause is characterized by a marked decline in circulating estrogen, profoundly affecting mineral metabolism and skeletal health [1]. Estrogen deficiency disrupts the balance between bone formation and resorption, leading to increased bone turnover, reduced bone mineral density, and heightened susceptibility to osteoporosis and fractures [2]. While calcium homeostasis has been extensively investigated [3], less is known about the regulation of phosphorus, a critical mineral for bone structure and systemic metabolic processes. Phosphorus contributes to hydroxyapatite formation in bone and participates in adenosine triphosphate (ATP) synthesis, enzymatic reactions, and intracellular signaling [4]. Its systemic levels are tightly controlled through intestinal absorption, renal excretion, and hormonal regulation, primarily via fibroblast growth factor 23 (FGF23), parathyroid hormone (PTH),

and vitamin D [5]. Despite its physiological importance, phosphorus metabolism under postmenopausal conditions remains poorly characterized, particularly in relation to dietary interventions targeting mineral homeostasis. Previous studies, including our work on probiotic and isoflavone combinations, have demonstrated significant effects on calcium status and bone metabolism biomarkers [6,7]; however, tissue phosphorus deposition and its regulation under estrogen-deficient conditions have received little attention.

Dietary interventions offer a promising strategy to modulate mineral metabolism in postmenopausal populations. Isoflavones, particularly daidzein, are phytoestrogens abundant in soy and fermented soy products such as tempeh [8]. Daidzein exists as both glycoside and aglycone forms, with the latter exhibiting higher bioavailability [9]. Preclinical and clinical studies indicate that daidzein can modulate bone turnover and enhance calcium

absorption, partially compensating for estrogen deficiency [10,11]. However, its direct influence on phosphorus homeostasis has not been systematically investigated. Fermented soy products, including tempeh, offer additional benefits: fermentation enhances isoflavone bioavailability, generates bioactive peptides, and improves mineral absorption by reducing antinutritional factors such as phytates [8]. These properties may facilitate phosphorus absorption and systemic utilization, supporting skeletal and metabolic health in estrogen-deficient states. Similarly, probiotics such as *Lactobacillus acidophilus* can modulate gut microbiota composition and activity, liberating minerals from dietary complexes through enzymatic processes such as phytase-mediated hydrolysis [12,13]. Despite these mechanistic insights, empirical data on the effects of isoflavones and probiotics on phosphorus distribution in postmenopausal models remain scarce.

Collectively, while calcium metabolism has been extensively explored, phosphorus homeostasis in postmenopausal conditions is understudied [14], and its modulation by dietary bioactives remains largely unknown. In addition, direct comparisons of dietary strategies with pharmacological interventions, such as alendronate, which inhibit osteoclast-mediated bone resorption, are lacking. Addressing these gaps is crucial for understanding how diet-based interventions may influence systemic phosphorus regulation in estrogen-deficient states.

The present study aimed to systematically evaluate the effects of three dietary interventions—pure daidzein, fermented soy product (tempeh), and probiotic *L. acidophilus*—on phosphorus homeostasis in ovariectomized rats as a model of postmenopausal bone loss. These interventions were chosen to represent distinct but complementary dietary strategies: daidzein as a phytoestrogen influencing mineral metabolism, tempeh as a naturally fermented isoflavone source, and *L. acidophilus* as a probiotic potentially enhancing nutrient bioavailability. It was hypothesized that daidzein and tempeh enhance phosphorus deposition in bone and metabolically active organs, reflecting improved mineral bioavailability and systemic distribution, while probiotics link to phosphorus availability through gut microbiota-mediated mechanisms. Furthermore, combinations of daidzein, tempeh, and probiotics may produce synergistic effects, and these patterns of phosphorus modulation were anticipated to differ from those observed with alendronate treatment. This investigation provided a rationale for exploring both single-component and combined dietary strategies to address critical knowledge gaps in postmenopausal phosphorus metabolism, with potential translational relevance for nutritional approaches to skeletal and metabolic health.

## 2. Materials and Methods

### 2.1 Animals and Diet Composition

Three-month-old female Wistar rats were selected as the animal model and obtained from the Nencki Institute of Experimental Biology (Warsaw, Poland). Animals were maintained on a purified diet based on the AIN-93M formulation, supplied by Zoolab (Sędziszów, Poland). Wistar rats were selected due to their established sensitivity to estrogen deficiency and their reproducibility as a model for postmenopausal metabolic alterations [15]. The primary dietary ingredients consisted of soybeans of the Augusta cultivar, sourced from the Poznań University of Life Sciences (Poznań, Poland), while purified daidzein was provided by Gentaur Molecular Products BVBA (Kampenhout, Belgium). Calcium citrate tetrahydrate, incorporated as the mineral source in the basal diet, was purchased from Warchem Sp. z o.o. (Warsaw, Poland). Tempeh flour was prepared using *Rhizopus oligosporus* NRRL 2710, a strain obtained from the Agricultural Research Service Culture Collection (Peoria, IL, USA) [16]. The probiotic strain *Lactobacillus acidophilus* DSM 20079 was supplied by the Leibniz Institute DSMZ (Braunschweig, Germany) and prepared according to a method described in a previous study [17].

### 2.2 Housing Conditions

The rats were housed in pairs in stainless steel cages with enamel-coated bases, equipped with wire-mesh tops to allow adequate ventilation. Each cage contained a polycarbonate drinking bottle, regularly refilled with fresh water, and stainless-steel feeders to provide consistent access to experimental diets. The choice of stainless-steel cages minimized contamination risks and facilitated routine cleaning and disinfection, ensuring a hygienic and controlled environment. Environmental parameters were strictly regulated, with the animal room maintained at  $21 \pm 2$  °C, relative humidity of 55–65%, and a 12:12 h light/dark cycle. These conditions were selected to provide a stable and stress-reduced environment, as fluctuations in temperature or humidity are known to influence metabolic and hormonal responses in rodents. Pair housing was deliberately chosen to reduce the stress associated with social isolation while preventing overcrowding, which can otherwise increase aggression and competition. Animals were provided with standard bedding material to absorb moisture and reduce ammonia build-up, as well as nesting material to promote natural behaviors and improve welfare. Cages were inspected daily to monitor food and water availability, hygiene, and overall animal health. Body weight was recorded weekly using a calibrated scale (RADWAG PS 750.X2, Radom, Poland), and daily inspections were performed to assess health status and behavior. Careful handling protocols were followed to minimize stress during the study.

### 2.3 Ovariectomy and Recovery

A total of 64 female Wistar rats were included in this study. After a one-week acclimatization period, the animals were randomly assigned into two experimental groups: a sham-operated control group ( $n = 8$ ) and an ovariectomized (OVX) group ( $n = 56$ ). Bilateral ovariectomy was performed in the OVX group under general anesthesia using a ketamine/medetomidine combination (Cepetor KH, CP-Pharma Handelsgesellschaft mbH, Burgdorf, Germany), administered by an experienced veterinary surgeon under aseptic conditions. The sham-operated animals underwent identical surgical procedures, with the exception that the ovaries were left intact. Immediately following surgery, all rats were placed on heated recovery pads maintained at 30 °C and continuously observed until full consciousness was regained. Postoperative monitoring was performed daily for seven consecutive days, including assessments of general behavior, surgical wound integrity, food and water intake, and body weight. Analgesic support was provided when required, and no adverse events or losses occurred during this period. Throughout the recovery phase, all animals received a semi-synthetic AIN-93M diet and had unrestricted access to fresh drinking water. This recovery protocol was designed to ensure animal welfare, minimize postoperative stress, and provide stable baseline conditions before dietary interventions.

### 2.4 Randomization and Calcium Deficit (Stage 1)

After a one-week postoperative recovery period, the rats were weighed, and the ovariectomized cohort was randomly assigned to experimental subgroups to ensure comparable baseline body weights across groups. Randomization was conducted to establish similar weight distributions at the start of the dietary intervention. Animals were randomly assigned to treatment groups by an independent researcher not involved in outcome assessment. Due to the laboratory setting and fixed biochemical procedures, blinding during assay measurement was not feasible, but sample handling followed a standardized protocol to minimize bias. To standardize baseline mineral status and simulate conditions that exacerbate post-menopausal bone loss, a calcium-deficient diet was administered for three weeks before the main dietary interventions. This short-term calcium restriction has been used previously to induce a reproducible state of reduced calcium availability and was selected based on published evidence indicating that three weeks is sufficient to produce measurable calcium depletion in rodents [18]. During this period, food intake was recorded at the cage level and body weights were measured weekly to monitor tolerance and ensure animal welfare. The basal diet contained calcium at 5.06 g/kg [7] and phosphorus at 1.10 g/kg, yielding a calcium-to-phosphorus ratio of approximately 4.6:1 on a mass basis ( $\approx 3.56:1$  molar ratio). This dietary ratio was considered in the interpretation of phosphorus endpoints, as the balance between calcium and phosphorus crit-

ically influences intestinal absorption and systemic mineral homeostasis.

### 2.5 Experimental Groups and Diet Allocation (Stage 2)

After the three-week calcium-deficient induction period, all animals were reassigned to their respective experimental diets for a six-week intervention phase. The sham-operated group (S;  $n = 8$ ) was returned to the standard AIN-93M maintenance diet containing adequate calcium, serving as the physiological reference. The ovariectomized rats ( $n = 56$ ) were then randomized into seven dietary intervention groups ( $n = 8$  per group), with randomization stratified by baseline body weight to minimize allocation bias. The experimental groups were as follows: ovariectomized controls (O,  $n = 8$ ) received the standard AIN-93M diet; alendronate treatment (OB,  $n = 8$ ) consisted of AIN-93M supplemented with a therapeutic dose of alendronate; daidzein supplementation (OD,  $n = 8$ ) received AIN-93M enriched with daidzein at 10 mg/kg diet; tempeh supplementation (OT,  $n = 8$ ) received AIN-93M containing tempeh flour at 250 g/kg diet; probiotic treatment (OL,  $n = 8$ ) received AIN-93M with a daily administration of *L. acidophilus* at  $1 \times 10^{10}$  CFU/day; combined daidzein and probiotic treatment (ODL,  $n = 8$ ) received AIN-93M with daidzein at 10 mg/kg diet together with *L. acidophilus* ( $1 \times 10^{10}$  CFU/day); and combined tempeh and probiotic treatment (OTL,  $n = 8$ ) received AIN-93M supplemented with tempeh flour at 250 g/kg diet plus *L. acidophilus* ( $1 \times 10^{10}$  CFU/day). This six-week intervention period was selected based on previous nutritional studies in rodent osteoporosis models, which demonstrated that such a duration is sufficient to elicit measurable changes in phosphorus metabolism and related biomarkers. Table 1 summarizes the allocation of animals into the respective experimental groups, including the group codes, number of rats per group, and the specific dietary interventions applied during the six-week feeding period. The experimental diets were formulated based on the AIN-93M standard, with modifications according to each intervention (alendronate, daidzein, tempeh, and/or *L. acidophilus*). Each diet was prepared in powdered form, stored at 4 °C, protected from light, and used within four weeks of preparation. The complete composition of all diets (g/kg diet), including analyzed macronutrients and minerals (protein, fat, fiber, ash, calcium, phosphorus, phytic acid, and metabolizable energy), are available at the REPOd data repository (<https://doi.org/10.18150/QHJUSA>).

### 2.6 Rationale for Dosage Selection

The dosages of all interventions were selected based on established experimental practice and translational considerations. Alendronate was included as a pharmacological reference group at a dose of 3 mg/kg body weight, administered once weekly. This dose was chosen because alendronate is a well-characterized anti-resorptive agent for

**Table 1. Dietary formulas used during stage 2.**

Group	Number of rats	Description	Composition	Dosage
S	8	Sham	AIN-93M	-
O	8	OVX	AIN-93M	-
OB	8	OVX	AIN-93M + alendronate	3 mg/kg body weight
OD	8	OVX	AIN-93M + daidzein	10 mg/kg of diet
OT	8	OVX	AIN-93M + tempeh flour	250 g/kg of diet
OL	8	OVX	AIN-93M + <i>L. acidophilus</i>	$1 \times 10^{10}$ CFU/day
ODL	8	OVX	AIN-93M + daidzein + <i>L. acidophilus</i>	10 mg/kg of diet + $1 \times 10^{10}$ CFU/day
OTL	8	OVX	AIN-93M + tempeh flour + <i>L. acidophilus</i>	250 g/kg of diet + $1 \times 10^{10}$ CFU/day

S, Sham group receiving AIN-93M; O, Ovariectomized (OVX) group receiving AIN-93M; OB, OVX group receiving AIN-93M with alendronate; OD, OVX group receiving AIN-93M with daidzein; OT, OVX group receiving AIN-93M with tempeh; OL, OVX group receiving AIN-93M with probiotics; ODL, OVX group receiving AIN-93M with daidzein and probiotics; OTL, OVX group receiving AIN-93M with tempeh and probiotics.

postmenopausal osteoporosis, and its efficacy has been consistently demonstrated in ovariectomized rat models, particularly with respect to bone remodeling and fracture healing [19]. To ensure consistent exposure, dosing was recorded for each animal in dosing logs maintained throughout the study. Standard AIN-93M diet served as the vehicle for the compound. Body weight was measured weekly to adjust the dose accordingly. No pharmacodynamic markers were assessed in this study; however, alendronate's established effects on bone tissue serve as a reference for expected skeletal outcomes.

The doses of daidzein and tempeh were established according to the isoflavone profile quantified in the tempeh preparation applied in this experiment. Analytical assessment showed that 250 g of tempeh provided approximately 10 mg of daidzein equivalents. Based on this composition, the experimental diet was formulated to contain 250 g/kg of tempeh powder, which corresponds to an estimated 10 mg/kg of daidzein. For consistency across treatments, the diet supplemented with pure daidzein was adjusted to deliver the same concentration (10 mg/kg diet) in its aglycone form. This strategy allowed a controlled comparison between isoflavones supplied as conjugated glycosides in tempeh and as the isolated aglycone in pure form. Since glycoside conjugates require microbial hydrolysis to release bioactive aglycones during digestion [20], the distinction between these forms was an important consideration in evaluating bioavailability and biological effects. The chosen dosages are consistent with previously reported dietary intakes shown to benefit bone health in ovariectomized rodent models [21,22].

For the probiotic group, *L. acidophilus* DSM 20079 was administered at  $1 \times 10^{10}$  CFU/day. This dose was based on earlier findings where  $1 \times 10^9$  CFU/day influenced osteoclastogenic activity in ovariectomized mice [23], while human studies demonstrated greater efficacy at  $1 \times 10^{10}$  CFU/day compared with lower intakes [24]. Therefore, the selected level reflects both preclinical and clinical evidence for effective modulation of mineral metabolism and skeletal outcomes.

## 2.7 Study Termination

At the conclusion of the six-week dietary intervention, the rats underwent a 4–6 hour fasting period to minimize the influence of recent food intake on body weight and biochemical measurements. Animals were then humanely euthanized by decapitation, a rapid and widely accepted method that minimizes pain and distress. Immediately following euthanasia, relevant tissues and organs, including feces, spleen, femur, heart, liver, kidney, and pancreas, were collected for subsequent phosphorus analysis. Throughout the study, no deaths, exclusions, or adverse events occurred, and all experimental procedures were performed in compliance with national and institutional guidelines for animal care.

## 2.8 Phosphorus Levels Measurement

Phosphorus concentrations in experimental diets and feces samples were determined by dry ashing. Specifically, 2 g of diet and 1 g of feces were placed in porcelain crucibles and incinerated in a muffle furnace at 450 °C until complete mineralization was achieved. The resulting ash was dissolved in 1 N ultrapure nitric acid (Merck, Kenilworth, NJ, USA) for subsequent analysis.

For tissue samples—including feces (mg/kg), spleen (mg/kg), femur (mg/kg), heart (mg/kg), liver (mg/kg), kidney (mg/kg), pancreas (mg/kg)—approximately 0.5–1 g of each tissue was digested using 65% (w/w) ultrapure nitric acid (Merck, Kenilworth, NJ, USA) in a microwave digestion system (Speedwave Xpert, Berghof, Eningen, Germany). After digestion, the samples were diluted appropriately with deionized water to achieve phosphorus concentrations within the working range of the colorimetric assay.

Phosphorus quantification was performed using the ammonium molybdate–vanadate colorimetric method, as described previously by Zubek *et al.* (2015) [25], with minor modifications. In brief, the molybdenum–vanadate reagent was prepared by mixing an ammonium heptamolybdate tetrahydrate (Sigma-Aldrich, St. Louis, MO, USA) solution with ammonium metavanadate



(Sigma-Aldrich, St. Louis, MO, USA) in nitric acid, and then diluting the mixture to volume with deionized water. Standard phosphorus solutions (25–300 µg/mL) were prepared from a 1 mg/mL KH<sub>2</sub>PO<sub>4</sub> (Merck, Darmstadt, Germany) stock solution for calibration purposes. For each sample, 2.5 mL of the prepared solution was mixed with 2.5 mL of the molybdenum-vanadate reagent and incubated at room temperature (~20 °C) for at least 10 minutes. Absorbance was measured at 430 nm using a UV–Vis spectrophotometer (Nanocolor UV/VIS, Macherey-Nagel MN, Germany), and phosphorus concentrations were calculated by interpolation from the standard calibration curve. The calibration curve demonstrated strong linearity, with a correlation coefficient ( $R^2$ ) of 0.99. Each measurement was duplicate, and assay precision was confirmed with relative standard deviation (RSD) values below 5%. No matrix interference or blank signal irregularities were observed. Outlier screening was conducted using a  $\pm 2$  SD threshold from the group means and excluded data points were limited to less than 5% of the dataset. The larger variance observed in spleen samples likely reflects biological heterogeneity rather than analytical variability.

## 2.9 Statistical Analysis

All continuous data were tested for normality using the Shapiro–Wilk test and for homogeneity of variance using Levene’s test. Differences between groups were assessed using one-way ANOVA with Tukey’s Honestly Significant Difference (HSD) post hoc test for pairwise comparisons. When assumptions were not met, non-parametric alternatives were applied. Potential outliers were identified a priori as values exceeding  $\pm 2$  standard deviations from the group mean and were excluded prior to analysis using Microsoft Excel (Version 365, Microsoft Corporation, Redmond, WA, USA). Outlier removal affected no more than one data point per group and did not alter the overall statistical trends. Statistical significance was defined as  $p < 0.05$ . Daily phosphorus intake and Ca:P ratios were similar across groups, and therefore no additional covariate adjustment was applied. Analyses were conducted on complete datasets, with the full per-group sample sizes maintained (sham,  $n = 8$ ; each OVX group,  $n = 8$ ). The selection of eight animals per group was based on prior studies demonstrating that this sample size provides sufficient statistical power to detect meaningful physiological and metabolic differences [26,27]. All confidence intervals (CIs) are expressed as intervention group minus control group. Positive CI values indicate higher mean levels in the intervention group compared to the control groups (S and O). All analyses were performed using SPSS software, version 22 (IBM Corp., Armonk, NY, USA) on a Windows platform.

## 3. Results

### 3.1 Phosphorus Content in Diet, Daily Food Intake, and Daily Dietary Phosphorus Intake

Table 2 illustrates the comparison of dietary phosphorus content and intake among the experimental groups. No significant differences were observed between the S and O control groups regarding phosphorus content, daily food consumption, and daily phosphorus intake, confirming comparable baseline conditions. Among the intervention groups, the OL group showed a significantly higher dietary phosphorus content compared with the O group (95% CI: 77.5 to 285;  $p < 0.001$ ), corresponding to an approximate 17% higher phosphorus content. While overall food intake remained consistent across all groups, this increase resulted in a significantly higher daily phosphorus intake for the OL group (95% CI: 0.50 to 5.32;  $p = 0.01$ ), approximately 15% greater than the O group. Compared with the ODL group, the OL group also exhibited significantly higher dietary phosphorus content and intake (95% CI: 2.51 to 7.32;  $p < 0.001$ ), with OL showing approximately 24% higher phosphorus in the diet and a corresponding 28% greater daily phosphorus intake.

### 3.2 Effect on Body Mass After the Six-Week Intervention With Various Modified Diets

Table 3 summarizes the effects of dietary interventions on body mass after the six-week experimental period. Among the control groups, the O group exhibited a significantly higher body mass compared with the S group (95% CI: 8.05 to 103;  $p = 0.01$ ). No significant differences were observed among the treated OVX groups; however, when compared with the S group, both the OD (95% CI: 4.12 to 95.9;  $p = 0.02$ ) and OL (95% CI: 0.87 to 92.6;  $p = 0.04$ ) groups displayed significantly higher body mass values.

### 3.3 Effect on Phosphorus Levels in Feces and Tissues After the Six-Week Intervention With Various Modified Diets

Fig. 1 summarizes phosphorus levels across feces and tissue samples. No significant differences in phosphorus levels were observed between the S and O control groups in any of the analyzed tissues. Significant differences in phosphorus levels were observed among different organs in the treated groups. In femoral tissue, the OT group exhibited a significantly higher phosphorus level compared with the O group (95% CI: 3.90 to 66.0;  $p = 0.01$ ), corresponding to an increase of roughly 25%. In the kidney, phosphorus levels of the ODL group were elevated compared to the S group (95% CI: 157 to 1874;  $p = 0.01$ ), though differences compared to the O group did not reach statistical significance. In the pancreas, phosphorus levels were significantly higher in the OD (95% CI: 42.8 to 335;  $p < 0.001$ ), ODL (95% CI: 36.4 to 338;  $p = 0.01$ ), and OTL (95% CI: 55.4 to 347;  $p < 0.001$ ) groups compared with the O group, reflecting an approximate 25–30% increase.

**Table 2. Phosphorus content in diet, daily food intake, and daily dietary phosphorus intake throughout the six-week intervention with various modified diets.**

Group	Parameter/unit	Mean $\pm$ SD	Q1	Q3	IQR
S	Phosphorus (mg/kg diet d.w.)	1098 $\pm$ 51.5 <sup>ab</sup>	7.7	9.0	1.4
O		1098 $\pm$ 51.5 <sup>ab</sup>	8.0	9.3	1.4
OB		1185 $\pm$ 46.6 <sup>bc</sup>	8.0	8.3	0.4
OD		1060 $\pm$ 47.3 <sup>a</sup>	8.0	9.0	1.1
OT		1198 $\pm$ 10.6 <sup>bc</sup>	7.7	8.0	0.3
OL		1279 $\pm$ 24.6 <sup>c</sup>	7.8	8.1	0.3
ODL		1035 $\pm$ 15.3 <sup>a</sup>	7.7	8.7	1.0
OTL		1121 $\pm$ 11.1 <sup>ab</sup>	8.0	8.4	0.5
S	Food Intake (mg/day)	16,754 $\pm$ 1100	16,007	16,939	933
O		17,782 $\pm$ 1138	17,074	18,000	925
OB		17,265 $\pm$ 963	17,092	17,383	291
OD		17,982 $\pm$ 1198	17,338	18,684	1346
OT		16,802 $\pm$ 903	16,120	17,164	1045
OL		17,538 $\pm$ 1311	16,516	18,034	1518
ODL		16,931 $\pm$ 1098	16,208	17,038	830
OTL		16,668 $\pm$ 1381	16,159	16,846	687
S	Phosphorus Intake (mg/day)	18.4 $\pm$ 1.2 <sup>ab</sup>	17.6	18.6	1.0
O		19.5 $\pm$ 1.3 <sup>ab</sup>	18.8	19.8	1.0
OB		20.5 $\pm$ 1.1 <sup>bc</sup>	20.3	20.6	0.4
OD		19.1 $\pm$ 1.3 <sup>ab</sup>	18.4	19.8	1.4
OT		20.1 $\pm$ 1.1 <sup>bc</sup>	19.3	20.6	1.3
OL		22.4 $\pm$ 1.7 <sup>c</sup>	21.1	23.1	1.9
ODL		17.5 $\pm$ 1.1 <sup>a</sup>	16.8	17.6	0.9
OTL		18.7 $\pm$ 1.6 <sup>ab</sup>	18.1	18.9	0.8

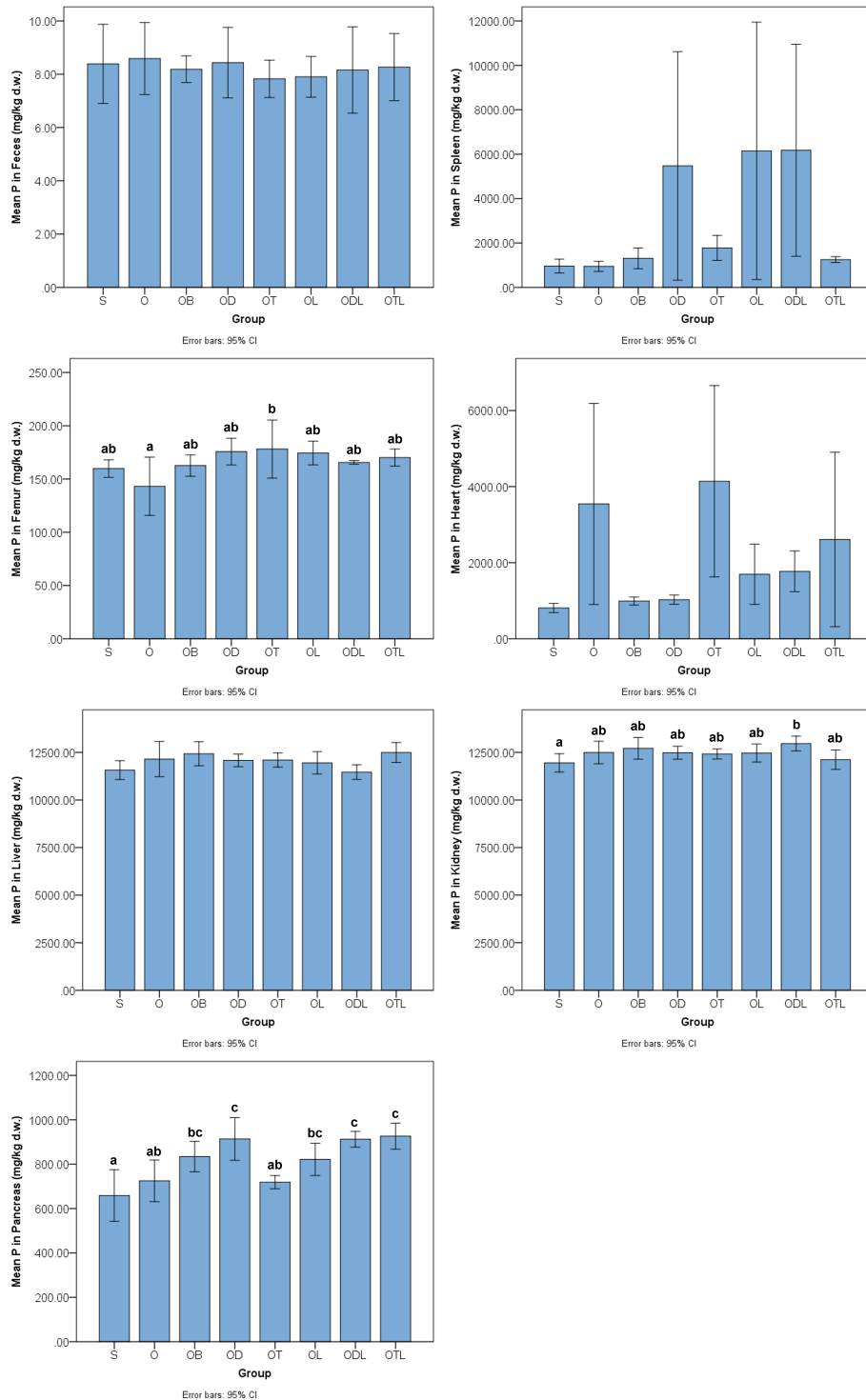
S, Sham group receiving AIN-93M; O, Ovariectomized (OVX) group receiving AIN-93M; OB, OVX group receiving AIN-93M with alendronate; OD, OVX group receiving AIN-93M with daidzein; OT, OVX group receiving AIN-93M with tempeh; OL, OVX group receiving AIN-93M with probiotics; ODL, OVX group receiving AIN-93M with daidzein and probiotics; OTL, OVX group receiving AIN-93M with tempeh and probiotics. Statistical analysis was conducted using ANOVA, followed by Tukey's post hoc test to determine significant differences between groups. Data are expressed as mean  $\pm$  standard deviation (SD), first quartile (Q1), third quartile (Q3), and interquartile range (IQR) to illustrate both central tendency and variability in data distribution among experimental groups. Values are reported based on dry weight (d.w.) of the phosphorus content in the diet. Distinct letters (a, b, c) indicate statistically significant differences ( $p < 0.05$ ) among dietary treatments.

### 3.4 Correlation of Phosphorus Levels Across Feces and Tissues

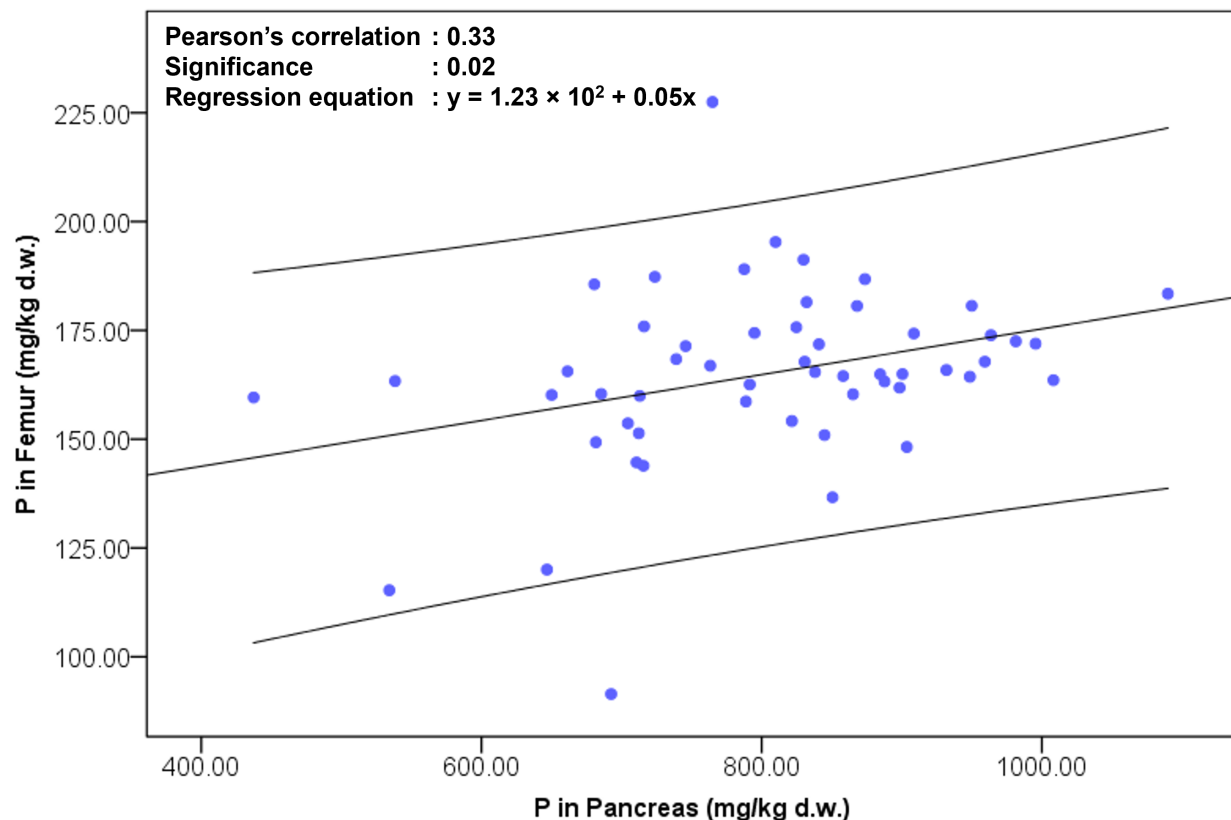
Table 4 presents Pearson's correlation coefficients between feces and tissue phosphorus concentrations after six weeks of dietary intervention. No significant correlations were detected among most evaluated tissues (feces, spleen, femur, heart, liver, kidney, and pancreas). However, as illustrated in Fig. 2, a positive correlation was observed between pancreatic and femoral phosphorus levels, with a correlation coefficient of 0.33 and a significance level of 0.02. This finding indicates that higher phosphorus accumulation in the pancreas was associated with greater deposition in bone.

## 4. Discussion

This study was designed to investigate the effects of dietary interventions, specifically daidzein, tempeh, and *L. acidophilus*, on phosphorus metabolism in a postmenopausal rat model. Our findings indicate that the dietary interventions yielded differential effects on phosphorus levels among the examined tissues. Notably, the femur, kidney, and pancreas exhibited significant increases in phosphorus deposition following treatment. Furthermore, a significant positive correlation was observed between pancreatic and femoral phosphorus levels, suggesting a potential systemic link in mineral handling under dietary modulation. This study provides novel insights into the ability of



**Fig. 1. Phosphorus levels in feces and tissues after the six-week intervention with various modified diets.** Data are presented as bar plots representing mean values  $\pm$  95% confidence intervals ( $n = 8$  rats per group). S, Sham rats receiving AIN-93M; O, Ovariectomized (OVX) rats receiving AIN-93M; OB, OVX rats receiving AIN-93M with alendronate; OD, OVX rats receiving AIN-93M with daidzein; OT, OVX rats receiving AIN-93M with tempeh; OL, OVX rats receiving AIN-93M with probiotics; ODL, OVX rats receiving AIN-93M with daidzein and probiotics; OTL, OVX rats receiving AIN-93M with tempeh and probiotics. Statistical analysis was conducted using ANOVA, followed by Tukey's post hoc test to determine significant differences between groups. Values are reported based on dry weight (d.w.) of the phosphorus content in specimens. Distinct letters (a, b, c) indicate statistically significant differences ( $p < 0.05$ ) among dietary treatments.



**Fig. 2. Pearson's correlation between phosphorus (P) concentrations in the femur and pancreas following a six-week dietary intervention.** Each data point represents an individual sample (blue circles). The x-axis shows P concentration in the pancreas (mg/kg dry weight), and the y-axis shows P concentration in the femur (mg/kg dry weight). A linear regression line with 95% confidence intervals is fitted to the data. The regression equation is  $y = 1.23 \times 10^2 + 0.05x$ . Pearson's correlation coefficient was  $r = 0.33$ , with statistical significance  $p = 0.02$ .

phytoestrogen- and probiotic-based interventions to modulate systemic phosphorus metabolism in ovariectomized animals.

The primary finding of this study (Fig. 1) is that the femur, kidney, and pancreas were the most responsive tissues to dietary treatments with daidzein, tempeh, and *L. acidophilus*. These outcomes are consistent with previous research on calcium metabolism, where modified diets were shown to alter mineral distribution in serum and bone tissues. Specifically, previous studies demonstrated that dietary interventions such as tempeh and probiotics reduced serum calcium levels while enhancing femoral calcium deposition [6], suggesting a shift in mineral allocation from circulation to bone tissue. The current findings extend this concept to phosphorus, indicating that bioactive dietary components not only modulate calcium handling but also promote phosphorus deposition in skeletal and metabolically active organs.

The parallel responses of calcium and phosphorus may be partly explained by shared transport and regulatory mechanisms. Earlier investigations showed that dietary treatments altered the expression of calcium transporters,

including TRPV5 (transient receptor potential vanilloid 5) and TRPV6 (transient receptor potential vanilloid 6), in the small intestine, with reduced expression in some groups and compensatory upregulation in others [7]. Although these transporters are specific to calcium, their modulation could indirectly influence phosphorus availability, given the interdependence of calcium–phosphorus absorption and homeostasis. Furthermore, the observed increase in feces calcium excretion in some treatment groups corresponds with the present lack of significant feces–tissue phosphorus correlations, underscoring that mineral redistribution in response to bioactive compounds may be tissue-specific rather than systemic.

Building on these observations, these results demonstrate that daidzein, tempeh, and *L. acidophilus* act in concert to influence calcium and phosphorus metabolism, thereby contributing to mineral equilibrium in the postmenopausal state. The marked increases in phosphorus deposition within the femur, kidney, and pancreas point to tissue-specific responsiveness, suggesting that both skeletal and metabolic compartments participate in dietary regulation of mineral balance. This coordinated response high-



**Table 3. Body mass after the six-week intervention with various modified diets.**

Group	Body mass (g)			
	Mean $\pm$ SD	Q1	Q3	IQR
S	311 $\pm$ 30.3 <sup>a</sup>	298	331	33.0
O	366 $\pm$ 14.0 <sup>b</sup>	351	374	23.0
OB	356 $\pm$ 31.0 <sup>ab</sup>	329	376	46.5
OD	361 $\pm$ 18.4 <sup>b</sup>	350	372	22.5
OT	354 $\pm$ 32.4 <sup>ab</sup>	335	366	30.8
OL	358 $\pm$ 29.3 <sup>b</sup>	338	377	39.5
ODL	345 $\pm$ 36.1 <sup>ab</sup>	321	367	45.3
OTL	350 $\pm$ 32.7 <sup>ab</sup>	327	364	36.3

S, Sham rats receiving AIN-93M; O, Ovariectomized (OVX) rats receiving AIN-93M; OB, OVX rats receiving AIN-93M with alendronate; OD, OVX rats receiving AIN-93M with daidzein; OT, OVX rats receiving AIN-93M with tempeh; OL, OVX rats receiving AIN-93M with probiotics; ODL, OVX rats receiving AIN-93M with daidzein and probiotics; OTL, OVX rats receiving AIN-93M with tempeh and probiotics. Statistical analysis was conducted using ANOVA, followed by Tukey's post hoc test to determine significant differences between groups. Data are expressed as mean  $\pm$  standard deviation (SD), first quartile (Q1), third quartile (Q3), and interquartile range (IQR) to illustrate both central tendency and variability in data distribution among experimental groups. Distinct letters (a, b) indicate statistically significant differences ( $p < 0.05$ ) among dietary treatments.

lights a broader systemic interplay, in which dietary bioactives modulate not only bone mineralization but also extra-skeletal phosphorus handling. These findings extend the understanding of postmenopausal mineral metabolism and underscore the translational potential of dietary strategies for improving mineral regulation and skeletal health.

In the femoral bone, adequate phosphorus availability, in combination with calcium, is critical for hydroxyapatite formation and bone mineralization [28], and the observed improvements may reflect enhanced bone matrix synthesis under conditions of estrogen deficiency [29]. In the kidney, estrogen deficiency can impair phosphate excretion and renal handling, mediated via changes in FGF23 and PTH signaling [30]. In the pancreas, phosphate is essential for ATP synthesis, enzyme activation, and insulin secretion, suggesting that these dietary interventions could impact metabolic regulation in estrogen-deficient states [31].

These findings demonstrate that dietary interventions with daidzein, tempeh, and *L. acidophilus* distinctly influenced phosphorus metabolism in a postmenopausal rat model. Administration of pure daidzein increased pancreatic phosphorus content, which may be attributed to phytoestrogenic modulation of mineral transporters [32] and

**Table 4. Pearson correlation coefficients between feces and tissue phosphorus concentrations following a six-week dietary intervention.**

Pair	Correlation coefficient	Significance
Feces–Spleen	−0.36	0.07
Feces–Femur	−0.05	0.80
Feces–Heart	0.09	0.65
Feces–Liver	−0.05	0.80
Feces–Kidney	0.26	0.17
Feces–Pancreas	−0.26	0.18
Spleen–Femur	0.19	0.18
Spleen–Heart	−0.12	0.40
Spleen–Liver	−0.17	0.21
Spleen–Kidney	0.12	0.39
Spleen–Pancreas	0.20	0.14
Femur–Heart	−0.26	0.06
Femur–Liver	−0.21	0.12
Femur–Kidney	0.10	0.48
Heart–Liver	0.10	0.45
Heart–Kidney	−0.03	0.85
Heart–Pancreas	−0.20	0.14
Liver–Kidney	−0.15	0.25
Liver–Pancreas	0.09	0.49
Kidney–Pancreas	0.18	0.16

enhanced intestinal absorption [33]. Importantly, daidzein was provided in its aglycone form, which is more bioavailable than the glycoside-conjugated isoflavones naturally present in tempeh [34]. Variations in chemical form may contribute to differences in how phosphorus is metabolized and distributed across organs.

Diets containing tempeh significantly associated with femoral phosphorus content compared to OVX controls. This effect may result from the combined action of bioactive isoflavones, fermentation-derived peptides, and improved mineral bioaccessibility [35]. Fermentation processes are known to enhance the solubility and absorption of calcium and phosphorus [35], which may explain the observed increases in bone mineral deposition.

Differences between daidzein alone and tempeh highlight the importance of the food matrix. Glycoside hydrolysis by intestinal microbiota in tempeh diets releases aglycone forms [8], while fermentation-derived peptides may further facilitate mineral uptake [36]. Although both interventions increase phosphorus availability, the matrix of tempeh appears to favor skeletal deposition, whereas pure daidzein preferentially affects pancreatic phosphorus. The divergent tissue patterns—greater femoral phosphorus accumulation following tempeh intake versus preferential pancreatic phosphorus increases with isolated daidzein—are plausibly explained by differences in food matrix, isoflavone speciation, and host–microbial interactions. Fermentation transforms soy into a complex matrix that contains bioactive peptides, converted aglycones, and reduced

phytate levels [8]; these changes enhance overall mineral bioaccessibility and may promote intestinal uptake of phosphate in a form that is preferentially deposited in bone. In addition, tempeh supplies macronutrients and peptide factors that may favor skeletal anabolism and mineral incorporation, thereby biasing newly absorbed phosphate toward the femur. By contrast, the pure daidzein provided here in aglycone form is rapidly absorbed and exerts direct phytoestrogenic and metabolic signaling effects that may modulate pancreatic cellular processes [37]—potentially altering local phosphate transport or retention. Finally, probiotic co-administration may further modify these trajectories through microbial phytase activity and altered metabolite profiles [38], thereby shifting both the amount and the kinetics of phosphate available for organ uptake. Taken together, these mechanisms provide a coherent explanation for why a whole-food, fermented matrix tends to favor skeletal phosphate deposition, whereas an isolated, rapidly absorbed isoflavone may preferentially influence metabolically active organs such as the pancreas; targeted mechanistic studies are warranted to test these hypotheses.

Rats receiving *L. acidophilus* exhibited significantly higher dietary phosphorus content and intake. This effect may be attributed to microbial activity facilitating the release of phosphorus from phytates and other mineral-bound complexes within the diet, thereby enhancing intestinal absorption. Recent advances in probiotic research emphasize the importance of targeted delivery and encapsulation techniques in enhancing probiotic survival and intestinal efficacy, thereby improving host nutrient absorption and metabolic regulation [39]. Mechanistically, probiotics can modulate gut microbiota, release enzymes such as phytases, and enhance intestinal absorption of phosphorus and calcium [40,41]. When combined with isoflavone-containing diets, probiotics may also influence bile acid metabolism [42], improve mineral solubility, and support mineral deposition [43]. These interactions underscore the potential of dietary bioactives and microbial modulation to influence systemic phosphorus homeostasis in estrogen-deficient models.

Although these interventions yielded promising outcomes individually, no evident synergistic effects were observed when daidzein, tempeh, and probiotics were combined. This lack of synergy may be attributed to overlapping mechanisms of action, saturation of phosphorus transport and storage pathways, or limited bioavailability of isoflavones and microbial metabolites under the combined treatment conditions.

Second, OVX rats exhibited a significant association with body mass compared to sham-operated controls (Table 3), consistent with the well-established effects of estrogen deficiency on adiposity and metabolic regulation [44]. Mechanistically, estrogen loss promotes hyperphagia-independent weight gain through reduced energy expenditure [45], altered lipid metabolism [46], and enhanced

adipocyte differentiation, particularly in visceral depots [47]. Additionally, estrogen deficiency shifts the balance of anabolic and catabolic signaling in skeletal muscle and liver, favoring lipid accumulation and altered glucose utilization, which collectively contribute to increased body mass [48].

Despite this weight gain, no significant differences were observed in daily dietary phosphorus intake between the OVX and sham groups (Table 2). This suggests that the observed increase in body mass was not driven by differences in phosphorus consumption. Mechanistically, phosphate homeostasis is tightly regulated by endocrine and renal mechanisms, including FGF23, PTH, and vitamin D-mediated intestinal absorption and renal reabsorption [49]. These regulatory pathways maintain stable systemic and tissue phosphorus concentrations even under conditions of increased bone resorption associated with estrogen deficiency [50].

Alendronate treatment was included as a pharmacological reference to provide context for evaluating the efficacy of diet-based strategies on phosphorus metabolism, given its well-established role in inhibiting osteoclast-mediated bone resorption and clinical relevance in postmenopausal osteoporosis management [51]. In the present study, however, no significant differences in phosphorus concentrations across tissues, body mass, or other measured parameters were observed between alendronate-treated, ovariectomized, and sham-operated groups. This finding contrasts with reports describing alendronate-induced improvements in bone mineral content [52]. Several factors may account for the observed outcome. One important consideration is the applied dose of alendronate (3 mg/kg/week), which may not have been sufficient to elicit measurable alterations in phosphorus distribution. A previous report has demonstrated variability in treatment responses depending on both dosage and frequency of administration [52]. For example, the report has described dosing regimens ranging from 0.1 mg/kg administered repeatedly over an extended period to 7 mg/kg given less frequently, both of which were shown to enhance systemic alendronate delivery and improve osseointegration. However, despite these findings, the optimal dose and frequency required to achieve favorable systemic and tissue-specific effects remain unresolved [52]. In addition, the postmenopausal rat model may exhibit different responsiveness compared to human physiology [53]. Moreover, phosphorus homeostasis alone may not fully capture the pharmacological effects of alendronate treatment, since these compounds primarily act by suppressing osteoclast-mediated bone resorption and indirectly supporting bone formation, rather than exerting direct regulatory control over systemic phosphorus levels [54]. In contrast, dietary interventions with daidzein, tempeh, and probiotics resulted in measurable effects on phosphorus distribution, suggesting that bioactive compounds can modulate mineral metabolism through gut-associated

mechanisms distinct from the antiresorptive pathway targeted by alendronate.

Another noteworthy finding in this study was the positive correlation observed between pancreatic and femoral phosphorus concentrations, suggesting a potential systemic coordination between bone mineralization and pancreatic phosphorus metabolism. This association is particularly intriguing, as links between skeletal mineral dynamics and pancreatic function have rarely been reported in the literature. This relationship may be mediated by endocrine factors such as FGF23, which regulates phosphate homeostasis through its actions on renal excretion and vitamin D metabolism [55]. Given that insulin and IGF-1 are known to suppress FGF23 production via the PI3K/Akt/FOXO1 pathway, pancreatic function could indirectly influence skeletal phosphate regulation. The interplay among insulin, IGF-1, and FGF23 therefore provides a plausible mechanistic link connecting pancreatic phosphorus metabolism with bone mineral dynamics [55]. While the underlying mechanisms remain to be determined, this association generates hypotheses regarding possible regulatory interactions between bone mineralization and pancreatic function. Endocrine factors such as FGF23, which regulate phosphate homeostasis through renal excretion and vitamin D metabolism, as well as insulin and IGF-1, may be involved in this axis. However, these mechanisms were not directly evaluated in the present study, and further investigations are warranted. This observation highlights a novel, underexplored relationship that could inform future studies aimed at understanding systemic mineral regulation and the impact of dietary bioactive on bone and metabolic health.

Recent evidence has highlighted the complex endocrine network regulating phosphate balance, primarily mediated through the FGF23–PTH–vitamin D axis. FGF23, secreted mainly by osteocytes, acts on the kidneys to promote phosphate excretion and suppress 1,25(OH)<sub>2</sub>D synthesis, thereby maintaining systemic phosphate homeostasis [56]. Crosstalk between phosphate sensing, iron metabolism, and FGF23 regulation has also been described, revealing novel pathways that link mineral metabolism to systemic energy status [57]. Moreover, the dynamic interaction between FGF23, PTH, and vitamin D forms a tightly regulated bone–kidney–parathyroid feedback loop essential for mineral balance and skeletal integrity [58]. These insights provide a mechanistic foundation for interpreting the phosphorus-related changes observed in our study and underscore the importance of endocrine regulators in bone–phosphate metabolism.

Moreover, the translational relevance of these findings lies in their potential implications for postmenopausal women, in whom disturbances in mineral metabolism contribute to the risk of osteoporosis and related comorbidities. Dietary sources rich in isoflavones, such as tempeh, together with probiotics, may offer a sustainable nutritional strategy to support phosphorus homeostasis and bone

integrity through gut-mediated and endocrine-modulating pathways. At the same time, it is essential to recognize the importance of maintaining an appropriate calcium-to-phosphorus ratio in dietary interventions aimed at supporting bone health. Together, these findings highlight the promise of food-based approaches as complementary to conventional therapies, while also emphasizing the necessity of carefully balancing benefits with potential systemic risks.

A major strength of the present study lies in its integrative design, which compared the effects of an isolated phytoestrogen, a fermented soy-based food, and a probiotic strain, thereby enabling the distinction between compound-specific, matrix-dependent, and microbiota-mediated effects on phosphorus metabolism. The inclusion of multiple tissues, rather than limiting the analysis to bone, provided a broader perspective on systemic phosphorus handling, and the correlation analysis offered novel insights into inter-organ relationships, such as the pancreas–bone axis.

Nevertheless, several limitations should be acknowledged. The dose-response relationships for daidzein, tempeh, and *L. acidophilus* were not systematically evaluated; therefore, the optimal concentrations required to maximize phosphorus modulation without affecting overall mineral balance remain undetermined. The alendronate group served as a pharmacological reference rather than confirmatory control, as exposure verification was not performed. Therefore, its outcomes should be interpreted descriptively, recognizing potential biological variability and differences in pharmacodynamic response. Furthermore, the present study focused on phosphorus distribution at the tissue level and did not include systemic markers such as FGF23, PTH, or vitamin D metabolites, which could clarify endocrine mechanisms underlying phosphorus regulation. Future studies should integrate these biomarkers to establish causal relationships between dietary interventions and systemic phosphate homeostasis. As dietary intake was measured per cage rather than per individual, statistical analyses were based on individual animal outcomes. This introduces a potential risk of pseudo-replication, which may slightly inflate Type I error rates. Consequently, all correlation analyses should be interpreted as exploratory. Moreover, although dietary phosphorus and Ca:P ratios were analyzed and found to be comparable among groups, minor differences cannot be fully excluded and may have contributed to observed tissue phosphorus variability. This factor should be considered when interpreting between-group effects. Addressing these aspects in future work, ideally through longer-term interventions and the inclusion of human trials, will be crucial to fully establish the translational relevance of these findings.

## 5. Conclusions

Dietary interventions with daidzein, tempeh, and *L. acidophilus* significantly increased phosphorus concen-

trations in the femur, kidney, and pancreas of a postmenopausal rat model. These outcomes extend previous evidence of calcium redistribution under similar interventions, underscoring the interdependence of calcium and phosphorus metabolism in the regulation of mineral balance. The parallel increases in tissue phosphorus and femoral calcium suggest that dietary bioactives promote coordinated deposition across skeletal and metabolic organs. Although no consistent synergistic effects among daidzein, tempeh, and probiotics were identified, the positive correlation between pancreatic and femoral phosphorus points to systemic mechanisms that link bone mineralization with extra-skeletal phosphorus handling. Collectively, these findings provide novel evidence that targeted dietary bioactives may support phosphorus homeostasis and bone mineralization, offering translational potential for nutritional strategies aimed at improving mineral metabolism in postmenopausal women.

### Availability of Data and Materials

Raw per-animal data on phosphorus intake, tissue phosphorus concentrations, and body weight, as well as the analysis code, are available in the REPOd data repository (<https://doi.org/10.18150/QHJUSA>).

### Author Contributions

IAH and JS conceptualized and designed the research study. IAH conducted the investigation, curated the data, performed the formal analysis, acquired funding, administered the project, provided resources, and prepared the visualizations. IAH, KS, PK, and JS developed the methodology. JS supervised the study. IAH and JS validated the results. IAH wrote the original draft of the manuscript. IAH and JS reviewed and edited the manuscript. All authors contributed to editorial changes in the manuscript. All authors read and approved the final manuscript. All authors have participated sufficiently in the work and agreed to be accountable for all aspects of the work.

### Ethics Approval and Consent to Participate

The animal study protocol was approved by the Local Ethical Committee (Lokalna Komisja Etyczna) in Poznań, Poland (No. registration: 21/2021, Issued date: 21 May 2021). Experiments followed EU Directive 2010/63, NIH guidelines for laboratory animal care, and the ARRIVE reporting principles. The completed checklist is provided in a supplementary file. Animal handling adhered to institutional standards at the Poznań University of Life Sciences, ensuring full compliance with national and international regulations.

### Acknowledgment

We would like to express our gratitude to Professor Jerzy Nawracała for providing the soybean used in this study.

### Funding

This study was funded by the Polish National Science Centre/Narodowe Centrum Nauki (Grant no.: 2021/41/N/NZ9/00838; Grant holder: Iskandar Azmy Harahap).

### Conflict of Interest

The authors declare no conflict of interest.

### Supplementary Material

Supplementary material associated with this article can be found, in the online version, at <https://doi.org/10.31083/IJVN46717>.

### References

- [1] Lu L, Tian L. Postmenopausal osteoporosis coexisting with sarcopenia: the role and mechanisms of estrogen. *The Journal of Endocrinology*. 2023; 259: e230116. <https://doi.org/10.1530/JOE-23-0116>.
- [2] Liu J, Kim EK, Ni A, Kim YR, Zheng F, Lee BS, *et al.* Multiscale characterization of ovariectomized rat femur. *Journal of Biomechanics*. 2021; 122: 110462. <https://doi.org/10.1016/j.jbiomech.2021.110462>.
- [3] Wu Y, Guo X, Jiang A, Bai J, Nie X. Estrogen regulates duodenal calcium absorption and improves postmenopausal osteoporosis by the effect of ER $\beta$  on PMCA1b. *Scientific Reports*. 2025; 15: 16053. <https://doi.org/10.1038/s41598-025-00605-2>.
- [4] Tourkova IL, Nelson DJ, Schlesinger PH, Blair HC. Energy-Dependent Phosphate and Acid Transport for Bone Formation and Resorption. *Journal of Cellular Biochemistry*. 2025; 126: e70039. <https://doi.org/10.1002/jcb.70039>.
- [5] Portales-Castillo I, Simic P. PTH, FGF-23, Klotho and Vitamin D as regulators of calcium and phosphorus: Genetics, epigenetics and beyond. *Frontiers in Endocrinology*. 2022; 13: 992666. <https://doi.org/10.3389/fendo.2022.992666>.
- [6] Harahap IA, Schmidt M, Pruszyńska-Oszmałek E, Sassek M, Suliburska J. Impact of *Lactobacillus acidophilus* and Its Combination with Isoflavone Products on Calcium Status, Calcium Transporters, and Bone Metabolism Biomarkers in a Post-Menopausal Osteoporotic Rat Model. *Nutrients*. 2024; 16: 2524. <https://doi.org/10.3390/nu16152524>.
- [7] Harahap IA, Kuligowski M, Cieslak A, Kołodziejki PA, Suliburska J. Effect of Tempeh and Daidzein on Calcium Status, Calcium Transporters, and Bone Metabolism Biomarkers in Ovariectomized Rats. *Nutrients*. 2024; 16: 651. <https://doi.org/10.3390/nu16050651>.
- [8] Harahap IA, Suliburska J, Karaca AC, Capanoglu E, Esatbeyoglu T. Fermented soy products: A review of bioactives for health from fermentation to functionality. *Comprehensive Reviews in Food Science and Food Safety*. 2025; 24: e70080. <https://doi.org/10.1111/1541-4337.70080>.
- [9] Hsiao YH, Ho CT, Pan MH. Bioavailability and health benefits of major isoflavone aglycones and their metabolites. *Journal of Functional Foods*. 2020; 74: 104164. <https://doi.org/10.1016/j.jff.2020.104164>.
- [10] Jin X, Sun J, Yu B, Wang Y, Sun WJ, Yang J, *et al.* Daidzein stimulates osteogenesis facilitating proliferation, differentiation, and antiapoptosis in human osteoblast-like MG-63 cells via estrogen receptor-dependent MEK/ERK and PI3K/Akt activation. *Nutrition Research (New York, NY)*. 2017; 42: 20–30. <https://doi.org/10.1016/j.nutres.2017.04.009>.
- [11] Pawlowski JW, Martin BR, McCabe GP, McCabe L, Jackson



- GS, Peacock M, *et al.* Impact of equol-producing capacity and soy-isoflavone profiles of supplements on bone calcium retention in postmenopausal women: a randomized crossover trial. *The American Journal of Clinical Nutrition*. 2015; 102: 695–703. <https://doi.org/10.3945/ajcn.114.093906>.
- [12] Huynh U, Qiao M, King J, Trinh B, Valdez J, Haq M, *et al.* Differential Effects of Transition Metals on Growth and Metal Uptake for Two Distinct *Lactobacillus* Species. *Microbiology Spectrum*. 2022; 10: e0100621. <https://doi.org/10.1128/spectrum.01006-21>.
- [13] Theilmann MC, Goh YJ, Nielsen KF, Klaenhammer TR, Barangou R, Abou Hachem M. *Lactobacillus acidophilus* Metabolizes Dietary Plant Glucosides and Externalizes Their Bioactive Phytochemicals. *mBio*. 2017; 8: e01421–17. <https://doi.org/10.1128/mBio.01421-17>.
- [14] Figueres L, Beck-Cormier S, Beck L, Marks J. The Complexities of Organ Crosstalk in Phosphate Homeostasis: Time to Put Phosphate Sensing Back in the Limelight. *International Journal of Molecular Sciences*. 2021; 22: 5701. <https://doi.org/10.3390/ijms22115701>.
- [15] Fang J, Yang L, Zhang R, Zhu X, Wang P. Are there differences between Sprague-Dawley and Wistar rats in long-term effects of ovariectomy as a model for postmenopausal osteoporosis? *International Journal of Clinical and Experimental Pathology*. 2015; 8: 1491–1502.
- [16] Harahap IA, Kuligowski M, Schmidt M, Suliburska J. The impact of soybean products and probiotics on calcium bioaccessibility from organic and inorganic calcium salts in an in vitro digestion model. *Food Chemistry Advances*. 2023; 2: 100269. <https://doi.org/10.1016/j.focha.2023.100269>.
- [17] Harahap IA, Olejnik A, Kowalska K, Suliburska J. Effects of Daidzein, Tempeh, and a Probiotic Digested in an Artificial Gastrointestinal Tract on Calcium Deposition in Human Osteoblast-like Saos-2 Cells. *International Journal of Molecular Sciences*. 2024; 25: 1008. <https://doi.org/10.3390/ijms25021008>.
- [18] Kotani M, Kim KH, Ishizaki N, Funaba M, Matsui T. Magnesium and calcium deficiencies additively increase zinc concentrations and metallothionein expression in the rat liver. *The British Journal of Nutrition*. 2013; 109: 425–432. <https://doi.org/10.1017/S0007114512001195>.
- [19] Fu LJ, Tang TT, Hao YQ, Dai KR. Long-term effects of alendronate on fracture healing and bone remodeling of femoral shaft in ovariectomized rats. *Acta Pharmacologica Sinica*. 2013; 34: 387–392. <https://doi.org/10.1038/aps.2012.170>.
- [20] Villares A, Rostagno MA, García-Lafuente A, Guillamón E, Martínez JA. Content and profile of isoflavones in soy-based foods as a function of the production process. *Food and Bioprocess Technology*. 2011; 4: 27–38. <https://doi.org/10.1007/s11947-009-0311-y>.
- [21] Ahn H, Park YK. Soy isoflavone supplementation improves longitudinal bone growth and bone quality in growing female rats. *Nutrition (Burbank, Los Angeles County, CA)*. 2017; 37: 68–73. <https://doi.org/10.1016/j.nut.2016.12.008>.
- [22] Srivastava K, Tyagi AM, Khan K, Dixit M, Lahiri S, Kumar A, *et al.* Isoformononetin, a methoxydaidzein present in medicinal plants, reverses bone loss in osteopenic rats and exerts bone anabolic action by preventing osteoblast apoptosis. *Phytomedicine: International Journal of Phytotherapy and Phytopharmacology*. 2013; 20: 470–480. <https://doi.org/10.1016/j.phymed.2012.12.021>.
- [23] Dar HY, Shukla P, Mishra PK, Anupam R, Mondal RK, Tomar GB, *et al.* *Lactobacillus acidophilus* inhibits bone loss and increases bone heterogeneity in osteoporotic mice via modulating Treg-Th17 cell balance. *Bone Reports*. 2018; 8: 46–56. <https://doi.org/10.1016/j.bonr.2018.02.001>.
- [24] Szulińska M, Łoniewski I, Skrypnik K, Sobieska M, Korybalska K, Suliburska J, *et al.* Multispecies Probiotic Supplementation Favorably Affects Vascular Function and Reduces Arterial Stiffness in Obese Postmenopausal Women-A 12-Week Placebo-Controlled and Randomized Clinical Study. *Nutrients*. 2018; 10: 1672. <https://doi.org/10.3390/nu10111672>.
- [25] Zubek S, Rola K, Szewczyk A, Majewska ML, Turnau K. Enhanced concentrations of elements and secondary metabolites in *Viola tricolor* L. induced by arbuscular mycorrhizal fungi. *Plant and Soil*. 2015; 390: 129–142. <https://doi.org/10.1007/s11104-015-2388-6>.
- [26] Blaise BJ, Correia G, Tin A, Young JH, Vergnaud AC, Lewis M, *et al.* Power Analysis and Sample Size Determination in Metabolic Phenotyping. *Analytical Chemistry*. 2016; 88: 5179–5188. <https://doi.org/10.1021/acs.analchem.6b00188>.
- [27] Allgoewer A, Mayer B. Sample size estimation for pilot animal experiments by using a Markov Chain Monte Carlo approach. *Alternatives to Laboratory Animals: ATLA*. 2017; 45: 83–90. <https://doi.org/10.1177/026119291704500201>.
- [28] Bonjour JP. Calcium and phosphate: a duet of ions playing for bone health. *Journal of the American College of Nutrition*. 2011; 30: 438S–448S. <https://doi.org/10.1080/07315724.2011.10719988>.
- [29] Takasugi S, Shioyama M, Kitade M, Nagata M, Yamaji T. Involvement of estrogen in phosphorus-induced nephrocalcinosis through fibroblast growth factor 23. *Scientific Reports*. 2020; 10: 4864. <https://doi.org/10.1038/s41598-020-61858-7>.
- [30] Bacchetta J, Bardet C, Prié D. Physiology of FGF23 and overview of genetic diseases associated with renal phosphate wasting. *Metabolism: Clinical and Experimental*. 2020; 103: 153865. <https://doi.org/10.1016/j.metabol.2019.01.006>.
- [31] Gupte AA, Pownall HJ, Hamilton DJ. Estrogen: an emerging regulator of insulin action and mitochondrial function. *Journal of Diabetes Research*. 2015; 2015: 916585. <https://doi.org/10.1155/2015/916585>.
- [32] Harahap IA, Kuligowski M, Schmidt M, Kołodziejski PA, Suliburska J. Effects of isoflavone and probiotic intake on calcium transport and bone metabolism biomarkers in female rats. *Food Science & Nutrition*. 2023; 11: 6324–6335. <https://doi.org/10.1002/fsn3.3571>.
- [33] Laddha AP, Kulkarni YA. Pharmacokinetics, pharmacodynamics, toxicity, and formulations of daidzein: An important isoflavone. *Phytotherapy Research: PTR*. 2023; 37: 2578–2604. <https://doi.org/10.1002/ptr.7852>.
- [34] Ahmad S, Ahsan F, Ansari JA, Mahmood T, Shamim A, Bano S, *et al.* A review on daidzein as food supplement: Exploring its phytopharmacological and preclinical status. *EFood*. 2024; 5: e70008.
- [35] Mukherjee A, Breselge S, Dimidi E, Marco ML, Cotter PD. Fermented foods and gastrointestinal health: underlying mechanisms. *Nature Reviews. Gastroenterology & Hepatology*. 2024; 21: 248–266. <https://doi.org/10.1038/s41575-023-00869-x>.
- [36] Shiferaw Terefe N, Augustin MA. Fermentation for tailoring the technological and health related functionality of food products. *Critical Reviews in Food Science and Nutrition*. 2020; 60: 2887–2913. <https://doi.org/10.1080/10408398.2019.1666250>.
- [37] Gundogdu G, Dodurga Y, Cetin M, Secme M, Cicek B. The cytotoxic and genotoxic effects of daidzein on MIA PaCa-2 human pancreatic carcinoma cells and HT-29 human colon cancer cells. *Drug and Chemical Toxicology*. 2020; 43: 581–587. <https://doi.org/10.1080/01480545.2018.1527849>.
- [38] Tang AL, Wilcox G, Walker KZ, Shah NP, Ashton JF, Stojanovska L. Phytase activity from *Lactobacillus* spp. in calcium-fortified soymilk. *Journal of Food Science*. 2010; 75: M373–M376. <https://doi.org/10.1111/j.1750-3841.2010.01663.x>.
- [39] Zhong H, Jiang J, Hussain M, Zhang H, Chen L, Guan R. The Encapsulation Strategies for Targeted Delivery of Probi-



- otics in Preventing and Treating Colorectal Cancer: A Review. *Advanced Science* (Weinheim, Baden-Württemberg, Germany). 2025; 12: e2500304. <https://doi.org/10.1002/adv.202500304>.
- [40] Ptak A, Bedford MR, Świątkiewicz S, Żyła K, Józefiak D. Phytase modulates ileal microbiota and enhances growth performance of the broiler chickens. *PloS One*. 2015; 10: e0119770. <https://doi.org/10.1371/journal.pone.0119770>.
- [41] Wang J, Wu S, Zhang Y, Yang J, Hu Z. Gut microbiota and calcium balance. *Frontiers in Microbiology*. 2022; 13: 1033933. <https://doi.org/10.3389/fmicb.2022.1033933>.
- [42] Sivamaruthi BS, Fern LA, Rashidah Pg Hj Ismail DSN, Chaiyasut C. The influence of probiotics on bile acids in diseases and aging. *Biomedicine & Pharmacotherapy*. 2020; 128: 110310. <https://doi.org/10.1016/j.biopha.2020.110310>.
- [43] Bergillos-Meca T, Cabrera-Vique C, Artacho R, Moreno-Montoro M, Navarro-Alarcón M, Olalla M, *et al*. Does *Lactobacillus plantarum* or ultrafiltration process improve Ca, Mg, Zn and P bioavailability from fermented goats' milk? *Food Chemistry*. 2015; 187: 314–321. <https://doi.org/10.1016/j.foodchem.2015.04.051>.
- [44] Steiner BM, Berry DC. The Regulation of Adipose Tissue Health by Estrogens. *Frontiers in Endocrinology*. 2022; 13: 889923. <https://doi.org/10.3389/fendo.2022.889923>.
- [45] Mamounis KJ, Yang JA, Yasrebi A, Roepke TA. Estrogen response element-independent signaling partially restores post-ovariectomy body weight gain but is not sufficient for 17 $\beta$ -estradiol's control of energy homeostasis. *Steroids*. 2014; 81: 88–98. <https://doi.org/10.1016/j.steroids.2013.10.018>.
- [46] Ko SH, Jung Y. Energy Metabolism Changes and Dysregulated Lipid Metabolism in Postmenopausal Women. *Nutrients*. 2021; 13: 4556. <https://doi.org/10.3390/nu13124556>.
- [47] Zhang Z, Liu J, Veldhuis-Vlug AG, Su Y, Foppen E, van der Eerden BCJ, *et al*. Effects of Chronic Estrogen Administration in the Ventromedial Nucleus of the Hypothalamus (VMH) on Fat and Bone Metabolism in Ovariectomized Rats. *Endocrinology*. 2016; 157: 4930–4942. <https://doi.org/10.1210/en.2016-1481>.
- [48] Sánchez-García M, León-Wu K, de Miguel-Ibáñez R, López-Juárez N, Ramírez-Rentería C, Espinosa-Cárdenas E, *et al*. Metabolic Changes in Patients with Premature Ovarian Insufficiency: Adipose Tissue Focus-A Narrative Review. *Metabolites*. 2025; 15: 242. <https://doi.org/10.3390/metabo15040242>.
- [49] Bergwitz C, Jüppner H. Phosphate sensing. *Advances in Chronic Kidney Disease*. 2011; 18: 132–144. <https://doi.org/10.1053/j.ackd.2011.01.004>.
- [50] Dastghaib S, Koohpeyma F, Shams M, Saki F, Alizadeh A. New concepts in regulation and function of the FGF23. *Clinical and Experimental Medicine*. 2023; 23: 1055–1066. <https://doi.org/10.1007/s10238-022-00844-x>.
- [51] Liu Y, Chen H, Chen T, Qiu G, Han Y. The emerging role of osteoclasts in the treatment of bone metastases: rationale and recent clinical evidence. *Frontiers in Oncology*. 2024; 14: 1445025. <https://doi.org/10.3389/fonc.2024.1445025>.
- [52] Vohra F, Al-Rifaiy MQ, Almas K, Javed F. Efficacy of systemic bisphosphonate delivery on osseointegration of implants under osteoporotic conditions: lessons from animal studies. *Archives of Oral Biology*. 2014; 59: 912–920. <https://doi.org/10.1016/j.archoralbio.2014.05.016>.
- [53] Wang Y, Wu Z, Li C, Ma C, Chen J, Wang M, *et al*. Effect of bisphosphonate on bone microstructure, mechanical strength in osteoporotic rats by ovariectomy. *BMC Musculoskeletal Disorders*. 2024; 25: 725. <https://doi.org/10.1186/s12891-024-07846-8>.
- [54] Russell RGG, Watts NB, Ebetino FH, Rogers MJ. Mechanisms of action of bisphosphonates: similarities and differences and their potential influence on clinical efficacy. *Osteoporosis International: a Journal Established as Result of Cooperation between the European Foundation for Osteoporosis and the National Osteoporosis Foundation of the USA*. 2008; 19: 733–759. <https://doi.org/10.1007/s00198-007-0540-8>.
- [55] Bär L, Feger M, Fajol A, Klotz LO, Zeng S, Lang F, *et al*. Insulin suppresses the production of fibroblast growth factor 23 (FGF23). *Proceedings of the National Academy of Sciences of the United States of America*. 2018; 115: 5804–5809. <https://doi.org/10.1073/pnas.1800160115>.
- [56] Michigami T. Advances in understanding of phosphate homeostasis and related disorders. *Endocrine Journal*. 2022; 69: 881–896. <https://doi.org/10.1507/endocrj.EJ22-0239>.
- [57] Chande S, Bergwitz C. Role of phosphate sensing in bone and mineral metabolism. *Nature Reviews. Endocrinology*. 2018; 14: 637–655. <https://doi.org/10.1038/s41574-018-0076-3>.
- [58] Blau JE, Collins MT. The PTH-Vitamin D-FGF23 axis. *Reviews in Endocrine & Metabolic Disorders*. 2015; 16: 165–174. <https://doi.org/10.1007/s11154-015-9318-z>.