The Poznań University of Life Sciences

The Faculty of Food Science and Nutrition

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Badanie wpływu izoflawonów i probiotyków na biodostępność i gospodarkę wapnia – badania *in vitro* i *in vivo*

The study on the impact of isoflavones and probiotics on calcium bioaccessibility and calcium status – *in vitro* and *in vivo* studies

Doctoral dissertation in the field of agricultural science

in the discipline of food science and nutrition

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Dedication

To my esteemed PhD supervisor, **Professor Joanna Suliburska**, for your invaluable mentorship, wisdom, and belief in my potential. Your guidance has shaped both this work and my development as a researcher.

To my beloved wife, **Dwi Putri Apriyanti**, for her unwavering support, patience, and encouragement throughout this journey. Your love and strength have been my constant source of inspiration.

To my wonderful sons, **Khawarizmi and Kamil**, for bringing endless joy and motivation into my life. May this work inspire you to pursue your dreams with passion and determination.

To my dear **parents**, whose guidance and prayers have been the foundation of all my achievements. Your belief in me has been my greatest blessing.

This work is dedicated to all of you with deep gratitude and love.

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Doctoral dissertation publication list

A. List of articles in the dissertation

No.	Description of Published Articles
P1	**Harahap, I. A.** & Suliburska, J. (2021). *Probiotics and Isoflavones as a Promising Therapeutic for Calcium Status and Bone Health: A Narrative Review*. Foods, 10(11), 2685–2685. DOI https://doi.org/10.3390/foods10112685 Impact Factor 4.7 $MNiSW_{2021}$ Point 70
P2	**Harahap, I. A.**, Olejnik, A., Kowalska, K., & Suliburska, J. (2024). *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, 25(2), 1008–1008. DOI https://doi.org/10.3390/ijms25021008 Impact Factor 4.9 $MNiSW_{2024}$ Point 140
P3	**Harahap, I. A.**, Kuligowski, M., Cieslak, A., Kołodziejski, P. A., & Suliburska, J. (2024). *Effect of Tempeh and Daidzein on Calcium Status, Calcium Transporters, and Bone Metabolism Biomarkers in Ovariectomized Rats*. Nutrients, 16(5), 651–651. DOI https://doi.org/10.3390/nu16050651 Impact Factor 4.8 $MNiSW_{2024}$ Point 140
P4	**Harahap, I. A.**, Schmidt, M., Pruszyńska-Oszmałek, E., Sassek, M., & Suliburska, J. (2024). *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, 16(15), 2524–2524. DOI https://doi.org/10.3390/nu16152524 Impact Factor 4.8 $MNiSW_{2024}$ Point 140
P5	**Harahap, I. A.,** Moszak, M., Czlapka-Matyasik, M., Skrypnik, K., Bogdański, P., & Suliburska, J. (2024). *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*. Frontiers in Nutrition, 11, 1401920. DOI https://doi.org/10.3389/fnut.2024.1401920 Impact Factor 4.0 $MNiSW_{2024}$ Point 70

B. Total Impact Factors and $MNiSW_{2021\text{-}2024}$ Points

Articles in the dissertation	Impact Factors	23.2
	MNiSW Points	560

List of abbreviations

BALP : Bone Alkaline Phosphatase

BMD : Bone mineral density

CTX : C-telopeptide of Type I Collagen

DPD : Deoxypyridinoline

DXA : Dual-energy X-ray Absorptiometry

HRT : Hormone replacement therapy

NCX1 : Sodium-calcium exchanger

OC : Osteocalcin

OPG : Osteoprotegerin

OVX : Ovariectomized

PINP : Procollagen Type I N-Terminal Propeptide

PMCA1 : Plasma membrane Ca^{2+}-ATPase

PYD : Pyridinoline

RANKL : Receptor activator of nuclear factor-κB-ligand

SERMs : Selective estrogen receptor modulators

TRPV5 : Transient receptor potential vanilloid type 5

TRPV6 : Transient receptor potential vanilloid type 6

Streszczenie

Wstęp. Niedobór wapnia jest istotnym problemem zdrowotnym dla kobiet po menopauzie, przyczyniając się do osteoporozy, która charakteryzuje się zmniejszoną gęstością kości i zwiększonym ryzykiem złamań. Stosowane leki, takie jak bisfosfoniany, mogą powodować skutki uboczne, co podkreśla potrzebę poszukiwania bezpieczniejszych i długoterminowych alternatyw.

Cel. Niniejsza dysertacja przedstawia badania potencjału izoflawonów sojowych i probiotyków w zwiększaniu biodostępności wapnia i poprawie zdrowia kości, szczególnie w kontekście osteoporozy po menopauzie. Celem badań było określenie wpływu izoflawonów i probiotyków na biodostępność wapnia i metabolizm kości.

Materiał i metody. Przeprowadzono badania *in vitro* i *in vivo*. Komórki osteoblastopodobne Saos-2 użyto do oceny wpływu daidzeiny, tempehu i *Lactobacillus acidophilus* na zawartość wapnia w komórkach i różnicowanie komórek kości. Model eksperymentalny ze szczurami po owariektomii (OVX) zastosowano do oceny wpływu spożycia tempehu, daidzeiny oraz ich kombinacji z *L. acidophilus* na gospodarkę wapnia, ekspresję transporterów wapnia i biomarkery metabolizmu kości. Badanie kliniczne z udziałem kobiet po menopauzie oceniało wpływ codziennej suplementacji *L. acidophilus* na gospodarkę wapnia, biomarkery metabolizmu kości i gęstość mineralną kości (BMD).

Wyniki. Wyniki wykazały, że w badaniu *in vitro* daidzeina, tempeh i *L. acidophilus* nie zwiększały znacząco zawartości wapnia w komórkach Saos-2, ale wykazały potencjał w promowaniu różnicowania komórek kości. W modelu szczurów OVX codzienne spożywanie tempehu i daidzeiny poprawiło gospodarkę wapnia, zwiększyło ekspresję transporterów wapnia i pozytywnie wpłynęło na biomarkery metabolizmu kości. Te efekty były porównywalne z obserwowanymi po stosowaniu bisfosfonianów. Ponadto, kombinacja *L. acidophilus* i produktów zawierających izoflawony, w tym tempehu i daidzeiny, wykazała korzystny wpływ na poziomy wapnia w kościach udowych i biomarkery metabolizmu kości. Ta kombinacja wpływała również na parametry hematologiczne i profile lipidowe, chociaż prowadziła do podwyższonych poziomów glukozy we krwi. W badaniu klinicznym z udziałem kobiet po menopauzie codzienna suplementacja *L. acidophilus* nie wpłynęła znacząco na wartości BMD, ale stabilizowała obrót kostny. Zaobserwowano jednak, że suplementacja probiotykami zaburzała stężenie wapnia i glukozy w krwi. Wyniki tej dysertacji sugerują, że izoflawony sojowe i probiotyki stanowią obiecujące strategie żywieniowe dla poprawy zdrowia kości. Tempeh, daidzeina i *L. acidophilus* mają potencjał żywieniowy w poprawie gosodarki wapnia i metabolizmu kości, szczególnie w warunkach po menopauzie. Jednakże obserwowane skutki uboczne, takie jak podwyższony poziom glukozy we krwi, podkreślają konieczność dalszych badań klinicznych.

Summary

Introduction. Calcium deficiency is a significant concern for postmenopausal women, contributing to osteoporosis, characterized by reduced bone density and increased fracture risk. Current treatments, such as bisphosphonates, can cause adverse effects, underscoring the need for safer and long-term alternatives.

Objective. This dissertation explored the potential of soy isoflavones and probiotics to enhance calcium bioavailability and improve bone health, particularly in the context of postmenopausal osteoporosis. The aim of this research was to investigate the effects of isoflavones and probiotics on calcium bioavailability and bone metabolism.

Materials and methods. The research was conducted through a series of integrated *in vitro* and *in vivo* studies. Human osteoblast-like Saos-2 cells were used to assess the impact of daidzein, tempeh, and *Lactobacillus acidophilus* on calcium deposition and osteogenic differentiation. Ovariectomized (OVX) rat models were employed to evaluate the effects of daily supplementation with tempeh, daidzein, and their combination with *L. acidophilus* on calcium status, calcium transporter expression, and bone metabolism biomarkers. Additionally, a clinical study involving postmenopausal women examined the impact of daily supplementation with *L. acidophilus* on calcium status, bone metabolism biomarkers, and bone mineral density (BMD) profiles.

Results. The results revealed that, in the *in vitro* study, daidzein, tempeh, and *L. acidophilus* did not significantly enhance calcium deposition in Saos-2 cells. However, these compounds showed potential in promoting osteogenic differentiation. In the OVX rat models, daily intake of tempeh and daidzein improved calcium status, increased the expression of calcium transporters, and positively influenced bone metabolism biomarkers. These effects were comparable to those observed with bisphosphonate drugs. In addition, the combined intake of *L. acidophilus* and isoflavone products, including tempeh and daidzein, showed beneficial effects on femoral bone calcium levels and bone metabolism biomarkers. This combination also impacted haematological parameters and lipid profiles, although it led to elevated blood glucose levels. In the clinical study involving postmenopausal women, daily supplementation with *L. acidophilus* did not significantly alter BMD profiles but appeared beneficial in stabilizing bone turnover. However, probiotic supplementation disrupted calcium and glucose levels in blood.

Conclusion. The findings of this dissertation suggest that soy isoflavones and probiotics offer promising dietary strategies for enhancing bone health. Tempeh, daidzein, and *L. acidophilus* have the potential to improve calcium status and bone metabolism, particularly in postmenopausal conditions. However, the observed side effects, such as elevated blood glucose level, underscore the necessity for further research and careful consideration in clinical applications.

Słowa kluczowe **:** Osteoporoza, Probiotyki, Izoflawony, Metabolizm wapnia, Zdrowie kości

Keywords **:** Osteoporosis, Probiotics, Isoflavones, Calcium Metabolism, Bone Health

1. Research activity of the doctoral candidate

My research career has been marked by a dedication to advancing scientific knowledge and contributing to the field of food science and nutrition. I began this journey by earning a master's degree (M.Sc.) from the Department of Food Science and Technology, at Gadjah Mada University, one of Indonesia's top universities and ranked among the top 300 world universities according to the QS World University Rankings. My master's thesis, titled "Recovery of Indigenous Probiotic *Lactobacillus plantarum* Mut-7 in Healthy Indonesian Adults after Consumption of Fermented Milk Containing These Bacteria," was a clinical trial study that aimed to discover indigenous probiotics in Indonesia, a country known for its abundant natural resources and status as a biodiversity hotspot with immense potential for food and nutraceutical products.

Upon completing my master's degree, I joined the Indonesian Institute of Sciences (LIPI) from the national competition as a civil servant and secured a permanent position as a researcher. During my first year, I was honoured with the award for the Best Young Candidate Researcher by the Chairman of LIPI. With the recent merger of LIPI and other research institutions in Indonesia into the National Research and Innovation Agency (BRIN) under a presidential decree, I now belong to one of the largest research institutions in Southeast Asia, encompassing over 14,000 researchers across Indonesia.

During my tenure as a junior researcher at this research institution, I have secured numerous prestigious research grants, both domestically and internationally. These grants include the INSINAS grant by the Indonesian Ministry of Higher Education, Research, and Technology, the LPDP-RISPRO grant by the Indonesia Endowment Fund part of the Ministry of Finance, and the VLIR-UOS Scholarship by the Flemish Interuniversity Council in Belgium. These grants have afforded me the invaluable opportunity to delve deeper into my research interests.

To further develop my research career, in 2020, I travelled approximately ten thousand kilometres from my home country and commenced my doctoral studies at the Department of Human Nutrition and Dietetics, Poznań University of Life Sciences in Poland under the supervision of Professor Joanna Suliburska. My dissertation title was "The Study on The Impact of Isoflavones and Probiotic on Calcium Bioaccessibility and Calcium Status – *In Vitro* and *In Vivo* Studies."

In my first year of study, I was honoured with several prestigious research grants. I was the first-ranked recipient of the Young Scientist (Młoda Kadra) program from the Faculty of Food Science and Nutrition, Poznań University of Life Sciences. Additionally, I was among the top five recipients of the Preludium grant funded by the Polish National Science Center

(NCN). Furthermore, I received a doctoral support grant from the Nutricia Foundation, being the only PhD student to secure this grant in Poland.

Afterwards, in my second year, as part of the evaluation process for the continuation of my doctoral studies, I successfully defended my PhD progress during the mid-term evaluation. I received an exceptional score of 100 points, making me the doctoral student with the highest points in the Doctoral School of Poznań University of Life Sciences. Additionally, in the same year, I was awarded the ININ 2.0 research grant, an incubator innovation program funded by the Polish Minister of Education and Science. This grant has allowed me to develop and patent a novel idea to formulate a supplement derived from probiotic and isoflavone-based products specifically designed for menopausal women. This supplement aims to improve bone health, addressing a critical need in the demographic most affected by osteoporosis.

The total funding from these grants amounted to nearly 500,000 PLN, significantly supporting my doctoral research endeavours. These milestones in my PhD journey have solidified my contributions to the field of human nutritional science, particularly in understanding the role of probiotics and isoflavones in bone health and mineral metabolism. Much of this success is due to the invaluable guidance and dedication of Professor Joanna Suliburska. Her mentorship has been instrumental in shaping my research trajectory, providing me with critical insights, rigorous academic training, and unwavering support throughout my studies. Her expertise and commitment have not only enhanced the quality of my research but also inspired me to strive for excellence in my academic and professional pursuits.

In addition to these achievements, I have also secured valuable international internship experiences. I participated in an Erasmus+ PhD internship for three months at the University of Porto, Portugal, and was awarded a research grant by the German Academic Exchange Service (DAAD) to conduct research for seven months at Leibniz Universität Hannover, Germany. These experiences have broadened my research perspective and enhanced my skills in a global context.

Furthermore, to disseminate my research findings, I have actively participated in 15 conferences both in Poland and internationally. Recently, I was recognized as one of the Early Research Finalists at the International Conference of Food Digestion (ICFD) in Portugal. Moreover, I received a Travel Grant Award and was invited to speak at the International Scientific Association for Probiotics and Prebiotics (ISAPP) in Ireland, highlighting the international recognition of my work.

To date, I have contributed to the publication of nearly 30 peer-reviewed articles, with almost 20 of them affiliated with Poznań University of Life Sciences under the guidance of Professor Joanna Suliburska. These publications have collectively contributed to my academic profile, resulting in a Google Scholar h-index of 9 and accumulating over 200 citations. These

publications reflect my commitment to advancing knowledge in the fields of food science and nutrition. Through these various accomplishments and experiences, I continue to contribute to the scientific community and strive to make meaningful advancements in nutrition and health research.

Looking ahead, my short-term career goal is to pursue a postdoctoral position at a leading international research institution. This will enable me to further refine my research skills, gain additional expertise, and build upon the solid foundation I have established during my doctoral studies. I am particularly interested in exploring advanced research in nutrition, mineral, and their impact on human health, with a focus on metabolic health. Engaging in cutting-edge projects and collaborating with renowned scientists will be crucial for my professional development and will provide valuable insights and experience that I can bring back to my home country.

In the long term, I aspire to secure a research-focused position. My goal is to establish a dedicated research group that explores the intersection of nutrition, biodiversity resources, and metabolic disease prevention. Previous Nobel Prizes have showcased discoveries of natural resources that inspire me. For instance, Tu Youyou's discovery of artemisinin, a drug derived from the sweet wormwood plant (*Artemisia annua*), has become a cornerstone in the treatment of malaria. Similarly, Christiaan Eijkman's work on vitamin B, inspired by his research on Java Island, Indonesia, led to significant advancements in understanding nutritional deficiencies and their impact on health. These ground-breaking achievements highlight the immense potential of natural resources in advancing medical science and improving health outcomes.

Inspired by these discoveries, I recognize the untapped potential of Indonesia's rich biodiversity, which remains largely unexplored. Indonesia, being a biodiversity hotspot, holds immense promise for the discovery of novel dietary interventions and therapeutic compounds. By leveraging this natural wealth, I aim to develop sustainable solutions for the prevention and treatment of metabolic diseases. For instance, my current dissertation focuses on studying tempeh, an Indonesian fermented soybean product consumed for centuries. This traditional food continues to attract scientific interest due to its remarkable nutritional and health benefits. By developing novel dietary interventions rooted in Indonesia's biodiversity, I aim to improve health outcomes, particularly for vulnerable populations.

In conclusion, I would like to reaffirm that my doctoral study at Poznań University of Life Sciences is a fundamental stepping stone in my education and future research career. I also realize that there is a long and bumpy road ahead that I need to travel before the goals I set can be achieved. But once I make up my mind, I have the determination and patience to accomplish my dreams no matter how hard they may become. I strongly believe that I will overcome any obstacles through my brainpower, willpower, and God's blessings.

2. Discussion of the publication cycle

The publication cycle of this dissertation encompasses a series of studies aimed at understanding the impact of isoflavones and probiotics on calcium bioavailability and bone health, particularly in the context of postmenopausal osteoporosis. The literature study **[P1]** provides a comprehensive overview of existing research on the role of probiotics and isoflavones in enhancing calcium absorption and supporting bone health. This review highlights the potential mechanisms through which these dietary components may exert their beneficial effects, including modulation of gut microbiota, enhancement of intestinal calcium absorption, and direct effects from cells, animal models, and human clinical trial studies. Furthermore, this dissertation provides a comprehensive discussion of the experimental findings from each study **[P2 to P5]** and employs a variety of methodological approaches, including *in vitro* cell culture models, *in vivo* animal studies, and human clinical trials.

P2: Effects of Daidzein, Tempeh, and Probiotic Digestion on Calcium Deposition in Human Osteoblast-like Saos-2 Cells

The first study aimed to investigate the effects of daidzein, tempeh, and *Lactobacillus acidophilus* digestion on calcium deposition in human osteoblast-like Saos-2 cells using an artificial gastrointestinal model. Despite initial expectations, the results showed no significant improvement in calcium deposition at the cellular level. However, there were indications that these dietary components might enhance osteogenic differentiation, suggesting a potential influence on bone cell activity beyond calcium deposition.

P3: Effect of Tempeh and Daidzein on Calcium Status, Calcium Transporters, and Bone Metabolism Biomarkers in Ovariectomized Rats

The second study evaluated the effects of daily supplementation with tempeh and daidzein on calcium status, calcium transporters expression, and bone metabolism biomarkers in an ovariectomized rat model, which simulates postmenopausal osteoporosis. The results demonstrated that isoflavone products, such as tempeh and daidzein, improved calcium status, enhanced the expression of calcium transporters, and positively influenced bone metabolism biomarkers. These effects were comparable to those of bisphosphonate drugs, commonly used to treat osteoporosis.

P4: Impact of Lactobacillus acidophilus and Isoflavone Combination on Calcium Status, Calcium Transporters, and Bone Metabolism Biomarkers in a Post-menopausal Osteoporotic Rat Model

The third study explored the combined effects of *L. acidophilus* and isoflavone products (tempeh and daidzein) on calcium status, calcium transporters, and bone metabolism biomarkers in an ovariectomized rat model, which simulates postmenopausal osteoporosis. The findings revealed that the combination of probiotics and isoflavones enhanced femoral bone calcium levels and improved bone metabolism biomarkers, while reducing serum calcium levels. Additionally, this combination positively influenced hematological parameters and lipid profiles, though it also led to elevated blood glucose levels.

P5: Effects of Daily Probiotic Supplementation with Lactobacillus acidophilus on Calcium Status, Bone Metabolism Biomarkers, and Bone Mineral Density in Postmenopausal Women

The fourth study involved a clinical trial with postmenopausal women, examining the effects of daily supplementation with *L. acidophilus* on calcium levels, bone metabolism biomarkers, and bone mineral density (BMD) profiles. The results indicated that while the probiotic supplementation did not significantly alter BMD profiles, it helped stabilize bone turnover. However, the results of this human clinical study demonstrated that this intervention also disturbed calcium and glucose levels.

Collectively, these studies provide a holistic view of the potential benefits and limitations of using isoflavones and probiotics to enhance calcium bioavailability and promote bone health. While the findings highlight promising avenues for dietary interventions, these results also underscore the necessity for further research to fully understand the long-term effects and optimize these strategies for practical application.

Overall, the publication cycle reflects a comprehensive and integrative approach to addressing the primary objective of the dissertation. By combining *in vitro*, *in vivo*, and human clinical studies, this research offers valuable insights into the roles of isoflavones and probiotics in calcium metabolism and bone health.

2.1. Introduction

2.1.1. Background

Calcium is indispensable for maintaining bone health and overall skeletal integrity. As a primary mineral constituent of bone, calcium contributes significantly to the hardness and strength of the skeletal system. Bones serve as the major reservoir for calcium in the body, storing over 99% of the body's calcium and providing a structural framework that supports movement, protects internal organs, and contains bone marrow, which is essential for producing blood cells. The remaining 1% of calcium circulates in the blood and is crucial for various

physiological functions, including muscle contraction, nerve signal transmission, vascular contraction and dilation, and hormonal secretion (Trailokya et al., 2017).

During childhood and adolescence, calcium is critical for bone growth and development, facilitating the attainment of peak bone mass. In adults, calcium plays a continuous role in bone remodeling, a dynamic process where old bone is resorbed, and new bone is formed. This remodeling is vital for repairing micro-damages and maintaining bone strength. As individuals age, maintaining adequate calcium intake becomes increasingly important to counterbalance the gradual loss of bone mass, which can lead to conditions such as osteopenia and osteoporosis. These conditions are characterized by reduced bone density and an increased risk of fractures, significantly impacting the quality of life and mobility (Brunetti et al., 2014; Mesías et al., 2011).

Ensuring adequate calcium bioavailability and absorption poses significant challenges, especially for populations at high risk for osteoporosis, such as postmenopausal women. Calcium bioavailability refers to the proportion of dietary calcium that is absorbed and utilized by the body, a process influenced by several factors including age, hormonal status, dietary composition, and overall health. As women age, particularly during and after menopause, there is a notable decline in estrogen levels, a hormone crucial for calcium absorption and bone metabolism. This hormonal change leads to decreased calcium absorption efficiency and increased bone resorption, heightening the risk of osteoporosis (Quesada-Gomez et al., 2013).

Calcium absorption in humans and mammals primarily occurs in the small intestine, particularly in the duodenum and jejunum, through two main pathways: active transcellular transport and passive paracellular diffusion. The active transcellular transport pathway is predominant when dietary calcium intake is low or moderate. This pathway involves the entry of calcium into intestinal epithelial cells via transient receptor potential vanilloid type 6 (TRPV6) channels, intracellular transport facilitated by the calcium-binding protein calbindin-D, and active extrusion into the bloodstream through plasma membrane Ca^{2+}-ATPase (PMCA1) and sodium-calcium exchanger (NCX1) transporters. In contrast, when dietary calcium intake is high, passive paracellular diffusion becomes the primary mechanism. This pathway allows calcium to move along its concentration gradient through the tight junctions between enterocytes, without the need for energy (Areco et al., 2015).

Postmenopausal women face a unique set of challenges in maintaining adequate calcium levels. The reduction in estrogen not only affects calcium absorption in the intestines but also accelerates bone loss, as estrogen normally helps to regulate bone turnover by inhibiting osteoclast activity. Consequently, the decline in estrogen results in an imbalance

between bone resorption and formation, leading to a net loss of bone density (Park et al., 2020). This makes postmenopausal women particularly susceptible to fractures and osteoporosis, a condition characterized by weak and brittle bones.

Existing recommendations for the treatment and prevention of postmenopausal osteoporosis typically include estrogen replacement therapy and pharmacological interventions. Estrogen treatment, also known as hormone replacement therapy (HRT), aims to compensate for the decline in estrogen levels, thereby improving calcium absorption and reducing bone resorption. While effective in mitigating bone loss and reducing fracture risk, HRT is associated with significant adverse effects (Gambacciani & Levancini, 2014). Furthermore, pharmacological treatments for osteoporosis, such as bisphosphonates, selective estrogen receptor modulators (SERMs), and monoclonal antibodies like denosumab, offer alternative approaches to enhance bone density and reduce fracture risk. Bisphosphonates, for example, work by inhibiting osteoclast-mediated bone resorption, thereby preserving bone mass. However, long-term use of these medications is not without complications. Patients may experience gastrointestinal issues, atypical femoral fractures, and osteonecrosis of the jaw, raising concerns about their safety and adherence over extended periods (Langdahl, 2021).

Given the limitations and adverse effects associated with conventional treatments, there is a pressing need for alternative strategies that are both effective and safe for long-term use. Natural compounds and dietary interventions that can enhance calcium bioavailability without adverse effects are of particular interest. Isoflavones, naturally occurring compounds found in soy products, and probiotics like *Lactobacillus acidophilus*, have emerged as promising candidates (Harahap & Sulibursk a, 2021). Isoflavones have estrogen-like effects that can help modulate bone metabolism, while probiotics can improve gut health and enhance calcium absorption. These compounds offer a potential synergistic approach to improving calcium bioavailability and supporting bone health, presenting a viable alternative for populations at risk of osteoporosis.

2.1.2. Research assumptions

Our literature study reported that therapeutic nutrients such as probiotics and isoflavones may be useful in controlling calcium absorption and bone metabolism **[P1]** (Harahap & Sulibursk a, 2021). Probiotics can regulate calcium uptake in paracellular and transcellular pathways. Isoflavones facilitate calcium homeostasis by mobilizing calcium from skeletal muscles. *Lactobacillus* and *Bifidobacteria* promote the enrichment and diversity of gut microbiota, leading to enhanced immune function and improved bone health. Moreover, isoflavones and their metabolites have been shown to enhance the bone mineral density by

stimulating osteoblast activity, inhibiting bone resorption, and regulating bone remodeling processes (Harahap & Sulibursk a, 2021). In addition, probiotics may mitigate bone loss following menopause by enhancing the expression of tight junction proteins in the gut, reducing antigen transport to the intestines, and activating immune cells in the intestines (Amin et al., 2020). Osteoprotective characteristics of *L. acidophilus* have been shown to benefit bone health. Ovariectomized (OVX) mice that received *L. acidophilus* showed improved bone microarchitecture, mineral density, and heterogeneity (Dar et al., 2018).

Estrogen deficiency is a leading cause of bone loss and osteoporosis in postmenopausal women (Tai et al., 2012). The three major chemical types of phytoestrogens that have been identified are isoflavones, lignans, and coumestans. The primary isoflavones in the aglycone form are genistein, daidzein, and glycitein. These isoflavones can be found in soybeans and are considered potential alternatives to hormone therapy (Nikolić et al., 2017). Daidzein, a soybean isoflavone, is metabolized to equol by the gut microflora in the gastrointestinal tract (Rafii, 2015). Equol itself may be involved in the regulation of androgens in the adrenal cortex in women (Liu et al., 2020). Besides, equol inhibits bone loss, apparently without affecting the reproductive organs, in OVX mice (Nishide et al., 2013). An association has also been found between the status of equol production and gut microbiota (Iino et al., 2019). Daidzein is present in a variety of soy products, including fermented soy, especially tempeh, in which the level of isoflavones is significantly higher than in unfermented soy (Kuligowski et al., 2017).

Our previous investigations in digestion simulation studies and healthy female rat models have yielded promising findings regarding the impact of isoflavones and probiotics on calcium bioaccessibility, bone health, and overall mineral status. For instance, studies have shown that soybean products and probiotics can enhance calcium bioaccessibility from both organic and inorganic calcium salts (Harahap, Kuligowski, Schmidt, & Sulibursk a, 2023b), highlighting their potential to improve mineral absorption and utilization. Additionally, the combination of isoflavones and *L. acidophilus* has been observed to positively influence iron status and morphological parameters (Harahap, Kuligowski, Schmidt, & Sulibursk a, 2023a), further emphasizing the role of these compounds in supporting overall mineral balance and health.

Moreover, the effects of isoflavones and probiotics on bone calcium and bone cell activity have demonstrated significant benefits in terms of bone mineral density and bone strength in healthy female rat models (Harahap, Kuligowski, Schmidt, Kurzawa, et al., 2023b). These findings were complemented by studies showing that isoflavones and probiotics can enhance calcium transport in the small intestine and improve bone metabolism parameters,

suggesting a direct beneficial impact on bone health and calcium absorption processes (Harahap, Kuligowski, Schmidt, Kołodziejski, et al., 2023a). The influence of these compounds on magnesium status also indicates their broad-spectrum efficacy in modulating essential mineral levels in the body (Harahap, Kuligowski, Schmidt, Kurzawa, et al., 2023c).

However, a notable research gap exists regarding the effects of isoflavone products and probiotics on these parameters in a postmenopausal osteoporotic context. While our previous study provided valuable insights into the baseline physiology of bone health, there remains a need to assess how dietary interventions, specifically the combination of isoflavone products and probiotics, impact bone health in the context of menopausal osteoporosis.

Therefore, by an understanding of this background, this dissertation aims to delve deeper into the cellular mechanisms and *in vivo* effects of isoflavones and probiotics, focusing on calcium deposition and bone metabolism in human osteoblast-like Saos-2 cells, OVX rats, and postmenopausal women. By exploring these mechanisms across different models, we seek to elucidate the potential therapeutic benefits of these compounds for enhancing bone health and preventing osteoporosis, particularly in vulnerable populations such as postmenopausal women.

These serial studies include investigations, *first* **[P2]**, conducted an *in vitro* experiment employing Caco-2 and Saos-2 cells to simulate human digestion and osteoblast activity of bone mineralization. *Second* **[P3 and P4]**, the *in vivo* OVX model, particularly in elucidating the intricate mechanisms underlying menopause-related health conditions and exploring potential therapeutic interventions of *L. acidophilus* and its combination with isoflavone products. In this model, female rats underwent surgical removal of their ovaries to induce a state of hormonal deficiency akin to menopause in humans. *Third* **[P5]**, the inclusion of human clinical trials involving postmenopausal women in Poland adds a crucial translational dimension to this research, bridging the gap between preclinical findings and clinical interventions. **Figure 1** illustrates the sequential progression of studies, beginning with *in vitro* experiments, followed by animal studies, and culminating in a human clinical trial. Each phase builds upon the findings of the previous stage, providing a comprehensive understanding of the impact of isoflavones and probiotics on calcium bioavailability and bone health across different model experiments.

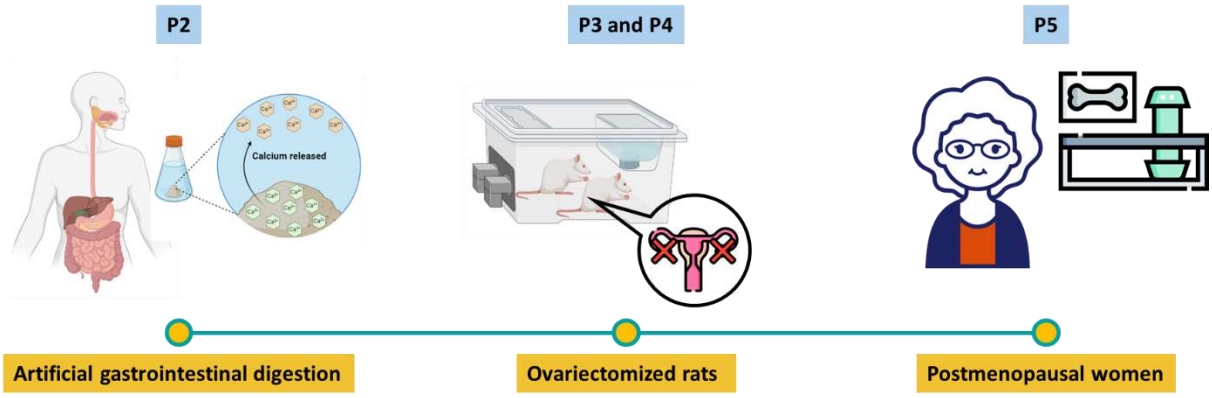

Figure 1. Sequential research scheme of studies investigating the impact of isoflavones and probiotics on calcium bioavailability and bone health.

2.1.3. Research hypotheses

1. Tempeh, daidzein, and *L. acidophilus* enhance calcium bioaccessibility and calcium status in organisms.
2. Isoflavones and probiotics improve bone health in postmenopausal conditions.
3. Isoflavones and probiotics, when taken simultaneously, have beneficial synergistic effects on calcium status and bone health.

2.1.4. Research objectives

General Objective

The primary objective of this dissertation is to investigate the impact of isoflavones and probiotics on enhancing calcium bioavailability and promoting bone health, particularly in the context of postmenopausal osteoporosis, integrating both *in vitro* and *in vivo* studies to provide comprehensive insights.

Specific Objectives

The specific objectives of this dissertation are as follows:

1. To investigate the impact of daidzein, tempeh, and probiotic digestion on calcium deposition in human osteoblast-like Saos-2 cells;
2. To evaluate the effects of tempeh and daidzein supplementation on calcium status, calcium transporters expression, and bone metabolism in an ovariectomized rat model;
3. To determine the effects of *L. acidophilus* and its combination with isoflavone products on calcium status, calcium transporters, and bone metabolism biomarkers in an ovariectomized rat model;
4. To investigate the effects of consuming *L. acidophilus* probiotics on calcium levels, biomarkers of bone metabolism, and BMD profiles in postmenopausal women.

2.2. Justification for combining publications into one cycle for the doctoral thesis

The decision to combine multiple publications into a single cycle for this doctoral thesis is grounded in several reasons that underscore the scientific, methodological, and thematic coherence of the research. This approach not only enhances the robustness and comprehensiveness of the dissertation but also aligns with the principal research objectives and academic standards. The key justifications for this integrative strategy are as follows:

1. Unified research objective

The primary objective of this dissertation is to investigate the impact of isoflavones and probiotics on enhancing calcium bioavailability and promoting bone health, particularly in the context of postmenopausal osteoporosis. Each study within the publication cycle contributes to this overarching goal, addressing different facets and stages of the research question. By combining these publications, the thesis presents a holistic view of the research problem, offering a thorough understanding that would be less cohesive if the studies were presented independently.

2. Methodological synergy

The studies in this dissertation employ a variety of methodological approaches, including *in vitro* cell culture models, *in vivo* animal studies, and human clinical trials. This methodological diversity is important for providing a comprehensive analysis of the research question. By integrating these methodologies into one cycle, the dissertation demonstrates a well-rounded and multi-dimensional investigation, highlighting the progression from basic science to applied research. This synergy enhances the credibility and depth of the findings.

3. Thematic consistency

Each publication within the cycle explores different aspects of the same thematic area, namely the association between dietary components (isoflavones and probiotics), calcium bioavailability, and bone health. The thematic consistency across the studies ensures that the dissertation maintains a clear and focused narrative. This coherence allows for a more compelling argument regarding the potential therapeutic implications of the findings and ensures that the reader can easily follow the logical progression of the research.

4. Comprehensive data interpretation

Combining the publications into one cycle facilitates a comprehensive interpretation of the data. It allows for cross-referencing findings from different studies, providing a richer context and deeper insights. For instance, the *in vitro* results can be compared with *in vivo*

outcomes, and animal model findings can be compared with human clinical trial data. This integrative approach leads to a more understanding of the research problem and helps in identifying patterns and drawing more robust conclusions.

To sum up, the integration of multiple publications into a single cycle for this doctoral thesis is justified by the unified research objective, methodological synergy, thematic consistency, and comprehensive data interpretation. This approach not only strengthens the scientific validity and impact of the research but also ensures that the dissertation provides a thorough, coherent, and compelling investigation into the effects of isoflavones and probiotics on calcium bioavailability and bone health in the context of postmenopausal osteoporosis.

2.3. Materials and methods

2.3.1. Materials

2.3.1.1. General materials

Soybeans of the Augusta variety were obtained from the Department of Genetics and Plant Breeding at Poznań University of Life Sciences, Poland. *Rhizopus oligosporus* NRRL 2710 came from the Agricultural Research Service Culture Collection in Illinois, USA. The media and reagents, including Potato Dextrose Agar (PDA), skim milk, and maltodextrin, were sourced from Merck in Darmstadt, Germany. *Lactobacillus acidophilus* DSM20079 was provided by the Leibniz-Institut Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ; German Collection of Microorganisms and Cell Cultures). De Man, Rogosa, and Sharpe (MRS) broth were procured from Oxoid in Hampshire, UK. Enzymes such as pepsin from porcine gastric mucosa and pancreatin from porcine pancreas, along with hydrochloric acid (HCl), sodium bicarbonate ($NaHCO_3$), and lanthanum chloride ($LaCl_3$), were acquired from Sigma-Aldrich in Steinheim, Germany. Additionally, calcium citrate tetrahydrate was purchased from Warchem Sp. z o.o. in Warsaw, Poland. The detailed descriptions of the materials used for cell assessment are included in the method sections, with all other chemicals meeting analytical grade standards.

2.3.1.2. Materials used in cell experiment

The materials utilized in this study included the human intestinal epithelial Caco-2 cell line (HTB-37™) sourced from the American Type Culture Collection (ATCC), Manassas, VA, USA. Dulbecco's Modified Eagle's Medium (DMEM) and non-essential amino acids (100X NEAA) were obtained from Sigma-Aldrich. Fetal bovine serum (FBS) was provided by Gibco BRL, Grand Island, NY, USA, while gentamicin was also sourced from Sigma-Aldrich. Polycarbonate membranes with a pore size of 0.4 μm (3.14 cm2) were used, specifically

NuncTM polycarbonate cell culture inserts. The Millicell Electrical Resistance System (ERS-2, Millipore) was employed for measuring transepithelial electrical resistance (TEER). For the cytotoxicity analysis, calcium citrate, probiotic, and daidzein were used. The MTT assay reagent, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide, was sourced from Sigma-Aldrich. Acidic isopropanol was used for formazan crystal extraction, and absorbance was measured with a Tecan Infinite M200 microplate reader from Tecan Group Ltd., Männedorf, Switzerland.

Furthermore, the materials utilized in this study included Saos-2 cells (HTB-85^{TM}), a human osteosarcoma cell line, cultured in ATCC-formulated McCoy's medium supplemented with 15% fetal bovine serum (FBS) and gentamicin. For the induction of osteogenesis, β-glycerophosphate, dexamethasone, and L-ascorbic acid were used. The alizarin red staining involved a 40 mM Alizarin Red Solution and 10% cetylpyridinium chloride for quantification, both sourced from Sigma-Aldrich, Merck Group. For intracellular calcium measurement, a Radioimmunoprecipitation assay (RIPA) lysis buffer and a Colorimetric calcium assay kit from Sigma-Aldrich were employed. The alkaline phosphatase activity was determined using an Alkaline phosphatase assay kit, with p-nitrophenyl phosphate as the substrate, and the total protein content was quantified using the Pierce® BCA Protein Assay Kit from Thermo Scientific Inc., USA. Gene expression analysis involved TRI reagent, cDNA Transcriptor First-Strand kit from Roche Diagnostics GmbH, and SYBR® Select Master Mix from Life Technologies. Primers for cDNA amplification and GAPDH normalization were synthesized according to specified sequences. Absorbance measurements were performed using a Tecan Infinite M200 microplate reader.

2.3.1.3. Materials used in animal study

The research utilized 72 female Wistar rats, aged 3 months, sourced from the Nencki Institute of Experimental Biology at the Polish Academy of Sciences in Warsaw, Poland. The rats were provided with a standard diet of AIN 93M, obtained from Zoolab in Sędziszów, Poland. Pure daidzein from Gentaur Molecular Products BVBA in Kampenhout, Belgium. Additionally, alendronate sodium trihydrate, purchased from TCI Europe N.V. in Zwijndrecht, Belgium, was incorporated.

Moreover, enzyme-linked immunosorbent assay (ELISA) kits were obtained from Qayee Bio-Technology Co., Ltd. (Shanghai, China) for the quantification of serum levels of bone metabolism markers, including Pyridinoline (PYD), Deoxypyridinoline (DPD), C-telopeptide of Type I Collagen (CTX), Bone Alkaline Phosphatase (BALP), Osteocalcin (OC),

and Procollagen Type I N-Terminal Propeptide (PINP). Absorption spectrophotometry was performed using LEDetect96 (Labexim, Lengau, Austria).

For the analysis of transient receptor potential vanilloid type 5 (TRPV5) and TRPV6 calcium transporters, EXTRAzol (DNA Gdansk, Poland), High-Capacity cDNA Reverse Transcription Kit (Life Technologies, Grand Island, NY, USA), gene-specific primers and 5 X HOT FIREPol® Eva-Green® qPCR Mix Plus (ROX), Gapdh (forward primer: TGACTTCAACAGCGACACCCA, reverse primer: CACCCTGTTGCTGTAGCCAAA), TRPV5 (forward primer: CGAGGATTCCAGATGC, reverse primer: GACCATAGCCATTAGCC), and TRPV6 (forward primer: GCACCTTCGAGCTGTTCC, reverse primer: CAGTGAGTGTCGCCCATC) were procured.

2.3.1.4. Materials used in human study

The probiotic supplement administered was an oral daily dose of 1×10^9 colony-forming units of *L. acidophilus* UALa-01™, with excipients comprising microcrystalline cellulose, silica, magnesium stearate, and a gelatin natural capsule, all sourced from Swanson Europe (Gdansk, Poland). The dietary assessment was performed using the 6.0 Diet Program from the National Food and Nutrition Institute, Warsaw, Poland. Body composition was assessed using the InBody 270 system (Cerritos, CA, US). Dual-energy X-ray Absorptiometry (DXA) scans were conducted with a GE Lunar Prodigy® machine from General Electric Healthcare (Madison, WI, US) at the Department of Human Nutrition and Dietetics, Poznań University of Life Sciences – Poland.

In addition, commercial ELISA kits procured from Qayee Bio-Technology Co., Ltd., Shanghai, China, were utilized in conjunction with absorption spectrophotometry (LEDetect96, Labexim, Lengau, Austria) to quantify serum levels of markers associated with bone metabolism. Specifically, C-telopeptide of Type I Collagen (CTX) and Tartrate-Resistant Acid Phosphatase 5b (TRAP-5b), indicative of bone resorption, were assessed, along with Bone-Specific Alkaline Phosphatase (BSAP) and Procollagen Type I N-Terminal Propeptide (PINP), biomarkers reflective of bone formation.

2.3.2. Methods

2.3.2.1. Measurement of calcium concentration

Calcium concentrations in diets, hair, and fecal samples were determined by ashing 2 g of each diet in a muffle furnace at 450°C until complete mineralization. The ashes were then dissolved in 1 N nitric acid (Suprapure, Merck). In the meantime, for digested samples, calcium levels in tissues, digestion was performed in 65% (w/w) spectra pure HNO3 (Merck, Kenilworth, NJ, USA) using a Microwave Digestion system (Speedwave Xpert, Berghof,

Eningen, Germany). Calcium concentrations in diets, serum, fecal, and bone samples were determined by flame atomic absorption spectrometry (AAS-3, Carl Zeiss, Jena, Germany) after dilution with Lanthanum (III) chloride (Merck KGaA, Darmstadt, Germany) and deionized water. The analysis utilized a specific wavelength of 422.7 nanometers. Method accuracy and dependability were assessed using Bovine liver 1577C (Sigma-Aldrich), a certified reference material, which demonstrated a calcium quantification accuracy rate of 92%.

2.3.2.2. Measurement of blood morphology and bone histopathology

Whole-blood morphology and bone histopathology assessments were conducted at Alab Laboratories, Poznań, Poland. Tissue samples were prepared by initial fixation, followed by 14-hour decalcification in EDTA solution and over 24-hour immersion in 70% ethanol. Prepared sections were placed in labeled histology cassettes and processed using standard paraffin techniques. Haematoxylin-eosin staining was used to visualize cellular and tissue structures. Pathological evaluations were carried out by experienced veterinarian-pathologists using Zeiss Axiolab 5 microscopes in Halle, Germany, at 5×, 10×, and 40× magnifications. Histopathological changes were graded, and trabecular bone morphometry was analyzed using established protocols. Representative areas were captured with a 3DHISTECH PANNORAMIC 250 Flash III microscope, and digital slides were generated using a Grundium Ocus®20 microscope slide scanner.

2.3.2.3. Measurement of calcium transporters

Quantitative real-time polymerase chain reaction (qRT-PCR) was employed to measure calcium transporters. Total RNA was extracted from the duodenum and jejunum tissues using EXTRAzol and mechanically homogenized with TissueLyser II (Qiagen, USA). The High-Capacity cDNA Reverse Transcription Kit (Life Technologies, Grand Island, NY, USA) was used to reverse-transcribe 1 μg of RNA into cDNA. RT-PCR was performed on QuantStudio 12K Flex™ using gene-specific primers and 5 X HOT FIREPol® Eva-Green® qPCR Mix Plus (ROX). DNA melting points were determined at a transition rate of 0.1 C/s to ensure specificity. Relative gene expression was calculated with the delta–delta CT method using Gapdh as a reference. TRPV5 and TRPV6 mRNA levels were quantified relative to Gapdh mRNA levels.

2.3.2.4. Measurement of nutritional values of daily diet

Participants' daily dietary intake was evaluated using a standardized 3-day food recall method. Utilizing the 6.0 Diet Program from the National Food and Nutrition Institute, Warsaw, Poland, participants recorded their food consumption for three days before and after the

intervention. Data collected were entered into the 6.0 Diet Program for analysis, enabling the calculation of nutrient intake levels such as energy, macronutrients, and micronutrients.

2.3.2.5. Measurement of body composition

Anthropometric measurements and body composition analyses were conducted at baseline and post-trial. Measurements were taken in a metabolic laboratory with participants in light clothing and after an overnight fast. Body mass, BMI, waist circumference, hip circumference, and waist-hip ratio were measured using standardized techniques. Body composition was analyzed using the InBody 270 system, which employs multifrequency 8-point tetrapolar touch electrodes to measure parameters such as body adiposity index, minerals, and lean body mass. Measurements were taken barefoot, with electrodes placed on the hands and feet, and results were generated within seconds using proprietary algorithms. Quality control measures were implemented to ensure accuracy and reliability, with all study personnel trained in the proper use of the InBody 270.

2.3.2.6. Measurement of DXA Bone Mineral Density

Participants underwent DXA scans before and after the intervention at the Department of Human Nutrition and Dietetics, Poznań University of Life Sciences, Poland. A GE Lunar Prodigy® machine was used for the scans, administered by the same researcher. Participants were instructed to remove all metal components from their clothing and accessories to ensure accurate measurements. The BMD of the lumbar spine (L1–L4), left femur, and total body were assessed using DXA software (enCORE by General Electric Healthcare, Madison, WI, US). Daily calibration and quality control of the DXA equipment were conducted to maintain stability and reliability, enhancing the precision of BMD assessments.

2.3.2.7. Statistical analysis

In the cell and rat studies, statistical analysis was conducted to ascertain the significance of observed differences. The normal distribution of variables was assessed using the Shapiro-Wilk method. Statistical significance regarding identified differences was evaluated through analysis of variance (ANOVA), followed by Tukey's post hoc test for multiple comparisons among the groups. Specifically, in an animal study, a power analysis determined that achieving an 80% power level required a sample size of 8 rodents in each group to detect statistical significance at the 0.05 level. Pearson's correlation analysis was utilized to assess relationships among serum calcium levels, bone metabolism biomarkers, and calcium transporters. These statistical analyses were performed using SPSS version 22 for Windows, and the results were presented with mean values accompanied by their corresponding standard deviations.

In human studies, statistical analysis was employed to determine the significance of observed differences. Comparisons within the same group before and after the intervention (dependent groups) were analyzed using the Wilcoxon matched pairs test. For comparisons between the placebo and probiotic groups (independent groups), the Mann-Whitney U test was employed. Spearman's correlation analysis was used to investigate relationships among serum calcium levels, bone biomarkers, and BMD profiles. IBM SPSS Statistics version 22 for Windows was utilized for statistical analysis and figure generation. Mean and median values, along with their corresponding standard deviations and interquartile ranges, were presented to provide a comprehensive understanding of the data distribution and variability within the dataset.

2.4. Research findings

2.4.1. Effect on calcium deposition in human osteoblast-like Saos-2 cells

In the first step of the study, the experiment assessed the effects of daidzein, tempeh, and *L. acidophilus* on calcium uptake and deposition in human osteoblast-like Saos-2 cells **[P2]**. In the initial phase, the calcium bioaccessibility from these dietary components and their combinations using an artificial gastrointestinal digestion model was assessed. Following digestion, the resulting products were tested for simulated intestinal absorption using the Caco-2 cell model, representing intestinal epithelial cells. The fractions that permeated the intestinal barrier were then analyzed in cultures of osteoblast-like Saos-2 cells. This approach enabled us to investigate the interactions and effects of the studied dietary compounds on calcium bioavailability and bone-related cellular processes in our experimental models as illustrated in **Figure 2**.

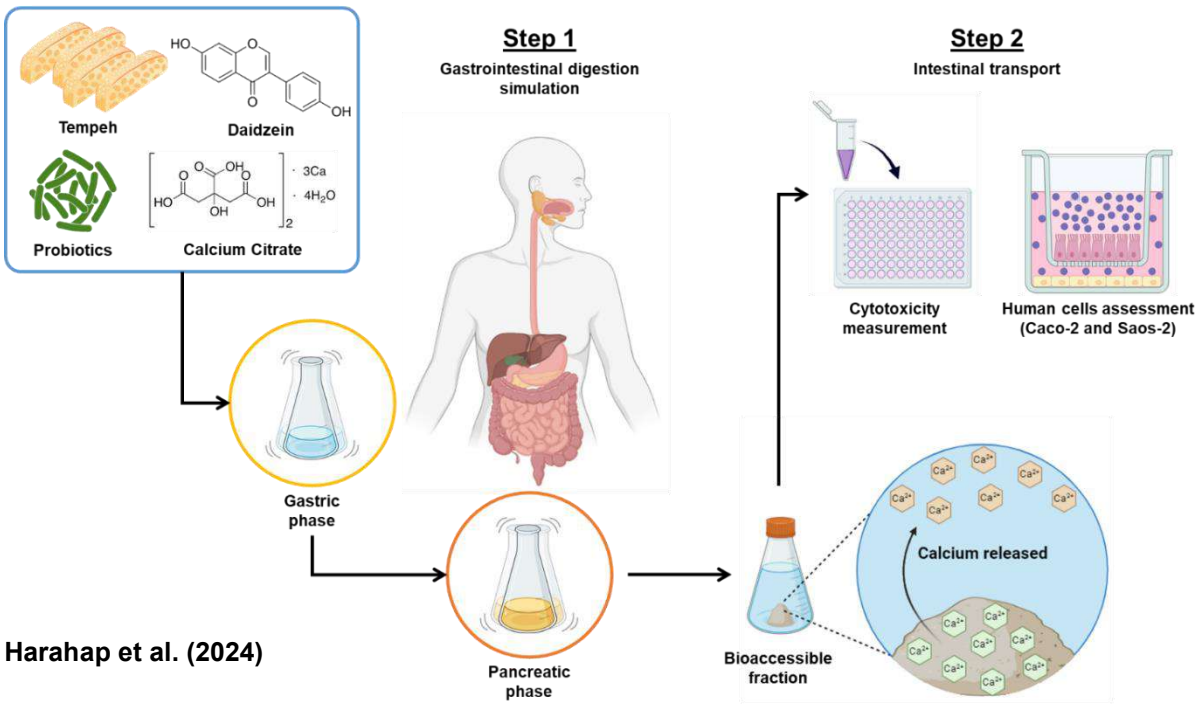

Figure 2. Design experiment of *in vitro* artificial digestion with human cells.

The digestion simulations revealed significant differences in calcium release and bioaccessibility among the tested combinations. The combination of pure daidzein, calcium citrate, and *L. acidophilus* (D1:1:1) demonstrated the highest levels of calcium release and bioaccessibility. Specifically, this combination yielded a calcium release of 17.38 mg/100 g and a bioaccessibility percentage of 15.28%. In contrast, the combination involving tempeh, calcium citrate, and *L. acidophilus* (T1:1:1) resulted in much lower values, with a calcium release of 6.12 mg/100 g and a bioaccessibility of only 1.19%.

Although the digestion simulations indicated that the combination of D1:1:1 significantly enhanced calcium release and bioaccessibility compared to the combination of T1:1:1, this did not translate into increased calcium deposition within the Saos-2 cells. The higher calcium bioaccessibility of the D1:1:1 combination underscores its potential for improving calcium availability in the gastrointestinal tract, but its limited impact on cellular calcium uptake suggests additional regulatory mechanisms at play within osteoblast-like cells.

One possible explanation for the lack of enhanced calcium deposition could be related to the specific cellular mechanisms governing calcium uptake and storage in osteoblasts. Calcium citrate alone was found to increase intracellular calcium accumulation, highlighting its direct role in promoting calcium uptake. However, the D1:1:1 combination did not significantly affect intracellular calcium levels, suggesting that while it facilitates calcium release and absorption, it may not directly interact with cellular pathways responsible for calcium bioavailability. This discrepancy indicates that the bioavailability of calcium in the digestive

system does not necessarily correlate with its deposition in bone cells, pointing to a complex interplay between nutrient absorption and cellular metabolism.

Interestingly, the study also revealed that tempeh (T) and the tempeh combination (T1:1:1) reduced intracellular calcium deposits in Saos-2 cells. This result suggests that certain compounds present in tempeh might inhibit calcium uptake or promote calcium efflux, thereby reducing intracellular calcium levels. The phenomenon of reduced calcium bioavailability and intracellular content in Saos-2 cells treated with tempeh can be attributed to the presence of antinutritional compounds such as phytic acid, oxalate, fiber, tannins, and saponin, which act as chelating agents and inhibit calcium absorption. Isoflavones, including daidzein, exhibit chelation activity towards metal ions, further decreasing calcium bioaccessibility. The study samples underwent enzymatic digestion, potentially increasing the presence of isoflavone aglycones, which are more rapidly absorbed and may enhance chelation. Despite fermentation, which reduces antinutritional factors like phytic acid, the inhibitory effects on calcium deposition in Saos-2 cells persisted. This highlights the need for further research into the chelating activity of isoflavonoids and their impact on calcium uptake, particularly in the context of fermented soy products like tempeh.

Despite the lack of enhanced calcium deposition, the findings indicate that tempeh, daidzein, and *L. acidophilus* may support osteogenic differentiation through alternative pathways. Osteogenic differentiation involves a series of processes that lead to the formation of bone matrix and mineralization. Compounds such as isoflavones and probiotics could influence these processes by modulating the expression of genes related to osteoblast differentiation and activity. For example, isoflavones like daidzein are known to interact with estrogen receptors, potentially enhancing the differentiation and function of osteoblasts even if they do not directly increase calcium deposition.

Moreover, the lack of synergistic effects between isoflavones and probiotics on calcium deposition and osteogenic differentiation emphasizes the need for a deeper understanding of how these compounds interact at the cellular level. While both isoflavones and probiotics have individually shown potential benefits for bone health, their combined effects may not be additive or synergistic. This underscores the importance of investigating the individual and combined impacts of these dietary components to develop effective dietary strategies for enhancing bone health, particularly in populations at risk of osteoporosis, such as postmenopausal women.

In conclusion, this first study provides valuable insights into the differential effects of daidzein, tempeh, and *L. acidophilus* on calcium bioaccessibility and deposition in osteoblast-

like cells. Our findings indicate that tempeh, daidzein, and *L. acidophilus* did not enhance cellular calcium deposition in Saos-2 cells. However, these dietary components may promote osteogenic differentiation in Saos-2 cells. No synergistic effects on calcium deposition or osteogenic differentiation were observed when isoflavones and probiotics were combined. These results enhance our comprehension of the interactions between dietary compounds, calcium bioaccessibility, and bone cells. Further *in vivo* models and clinical trials are needed to validate and apply these cell culture findings to more biologically relevant conditions.

2.4.2. Effect on calcium status, calcium transporters, and bone metabolism biomarkers in a post-menopausal osteoporotic rat model

The second serial study was divided into two parts: the first part examined the impact of pure daidzein and tempeh on calcium status, calcium transporters, and bone metabolism biomarkers in OVX rats **[P3]**; the second part evaluated the effects of probiotics *L. acidophilus* and its combination with isoflavone products, such as daidzein and tempeh **[P4]**, on the same parameters in the same animal model. This model was chosen to simulate the postmenopausal condition in women, where decreased estrogen levels lead to increased bone resorption and decreased bone density.

Additionally, a three-week period of calcium deficiency treatment was implemented post-surgery to induce a state of calcium deficiency and exacerbate the osteoporosis condition in the rats. This intervention was designed to simulate a more severe osteoporotic condition, allowing for a more rigorous assessment of the potential therapeutic effects of pure daidzein and tempeh on calcium status and bone metabolism during this stage. Following this, the study incorporated probiotic *L. acidophilus*, pure daidzein, and tempeh into the diets of the OVX rats over a six-week period to explore their effects on calcium status and bone metabolism comprehensively. The research scheme for this study is illustrated in **Figure 3**.

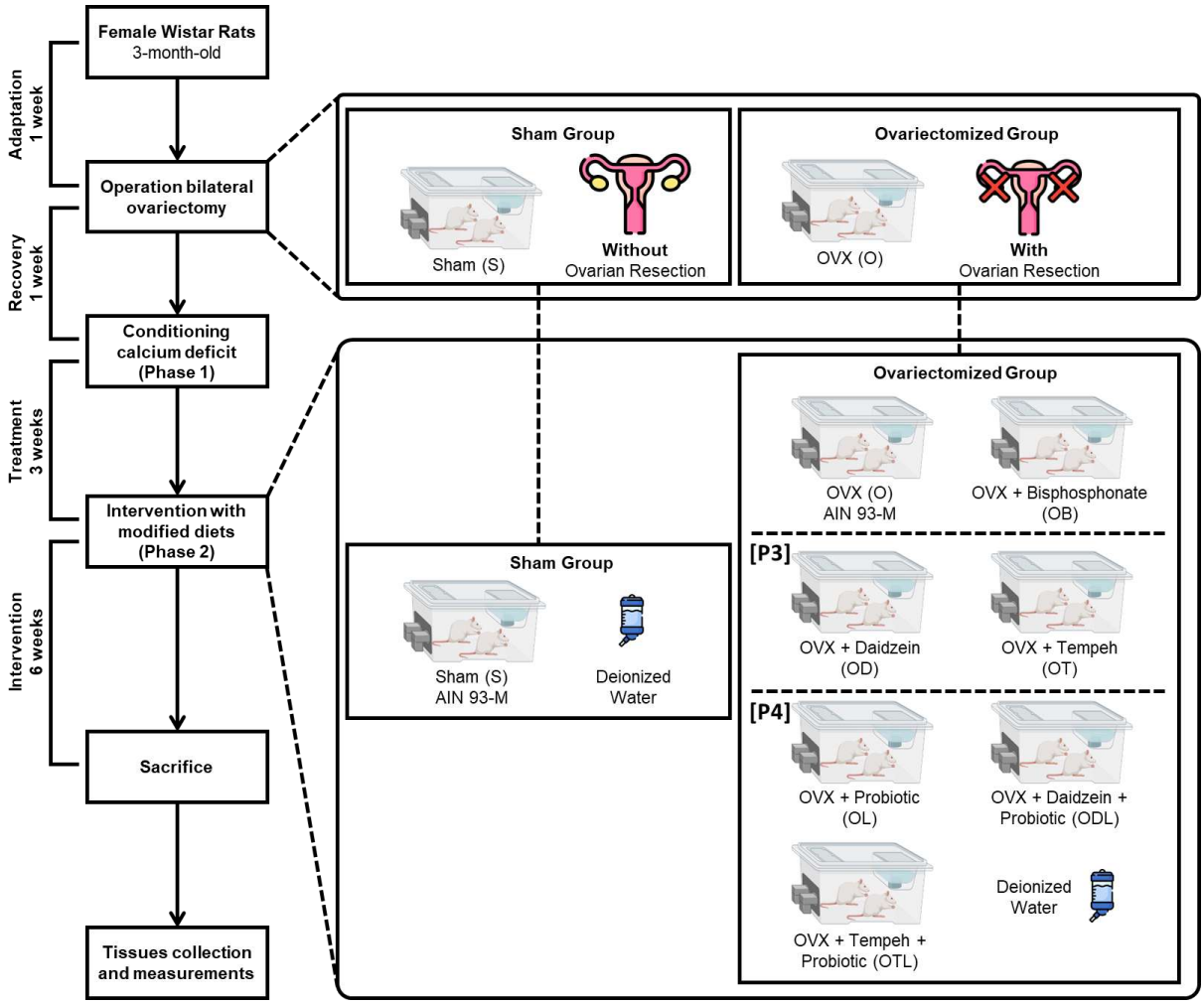

Figure 3. Research scheme of ovariectomized rats study.

Table 1 provides a comprehensive overview of the matrix findings derived from a study conducted on OVX rats. The comparison between the isoflavone products **[P3]** and probiotic and its combination with isoflavone products **[P4]** interventions in OVX rats revealed intriguing findings regarding calcium status, calcium transporters, and bone metabolism biomarkers.

Table 1. Matrix findings of ovariectomized rats study.

Parameters	Findings	
	Isoflavone products [P3]	**Probiotic and its combination with isoflavones products [P4]**
Calcium status	• OB group showed a significant increase in calcium content in femur bones compared to the O group. • A significant reduction in serum calcium content was observed in the OD group compared to the S group and the OT group compared to the O group. • While significant differences were not observed, both the OD and OT groups showed elevated calcium content in feces compared to the O group.	• OL, ODL, and OTL groups showed a significant decrease in serum calcium levels compared to the O group. • OB, OL, ODL, and OTL groups displayed significant increases in calcium levels in the femoral bone compared to the O group. • Although no significant differences were noted, the OL, ODL, and OTL groups demonstrated increased calcium content in feces compared to the O group.
Calcium transporters	• No statistically significant differences in TRPV5 and TRPV6 mRNA expression levels in duodenum and jejunum were observed in the OB, OD, and OT groups relative to the O group.	• A significant decrease in the mRNA expression of TRPV5 was observed in the duodenum for all groups (OB, OL, ODL, and OTL) compared to the O group. • A significant reduction in the mRNA expression of TRPV5 was noted in the jejunum of the OTL group compared to the S group.
Bone metabolism biomarkers	• Within the OVX rat groups, the OT group showed a significant elevation in PYD, CTX, BALP, and PINP levels compared to both the S and O groups.	• OL, ODL, and OTL groups exhibited a significant increase in PYD and CTX levels compared to the S group. • OL group showed a significant increase in PYD levels compared with the O group. • OL, ODL, and OTL groups demonstrated a significant increase in OC and PINP levels compared to the S and O groups. • The OTL group showed a significant increase in DPD levels compared to the S and O groups. • The OB group presented a significant increase in BALP levels compared to the O group.

OVX: ovariectomized rats; S: sham rats fed AIN 93M; O: OVX rats fed AIN 93M; OB: OVX rats fed AIN 93M with a bisphosphonate; OD: OVX rats fed AIN 93M with daidzein; OT: OVX rats fed AIN 93M with tempeh; OL: OVX rats fed AIN 93M with probiotic; ODL: OVX rats fed AIN 93M with daidzein and probiotic; OTL: OVX rats fed AIN 93M with tempeh and probiotic.

In terms of calcium status, both interventions led to significant alterations in serum calcium levels, with the isoflavone products **[P3]** group showing a reduction in serum calcium content in the OD and OT groups compared to the S and O groups, respectively, while the

probiotic and its combination with isoflavones products **[P4]** group displayed a decrease in serum calcium levels in the OL, ODL, and OTL groups compared to the O group. Conversely, both interventions resulted in increased calcium levels in femoral bones, as observed in the OB, OD, OT, OL, ODL, and OTL groups compared to the O group.

In addition to these findings **[P4]**, a significant decrease in TRPV5 mRNA expression levels in the duodenum was noted across all groups (OB, OL, ODL, and OTL) compared to the O group. Moreover, the OTL group exhibited a significant reduction in TRPV5 mRNA expression in the jejunum compared to the S group.

Furthermore, both interventions demonstrated significant effects on bone metabolism biomarkers, with the OT group in the isoflavone products **[P3]** group and the OL, ODL, and OTL groups in the probiotic and its combination with isoflavones products **[P4]** group showing elevated levels of bone turnover markers such as PYD, CTX, OC, PINP, and DPD compared to the respective control (S and O) groups. **Figure 4** illustrates histopathological alterations observed in femoral bone following a 6-week intervention.

Comparing these findings to the current bisphosphonate intervention (OB group), both isoflavone products **[P3]** and probiotic and its combination with isoflavone products **[P4]** interventions demonstrated similar effects on calcium status and bone metabolism biomarkers. While our study does not yield any spectacular results, it suggests the potential of these dietary interventions as alternatives for osteoporosis management. Overall, the findings from both studies **[P3 and P4]** shed light on the interrelation between dietary components, calcium metabolism, and bone health in menopausal conditions.

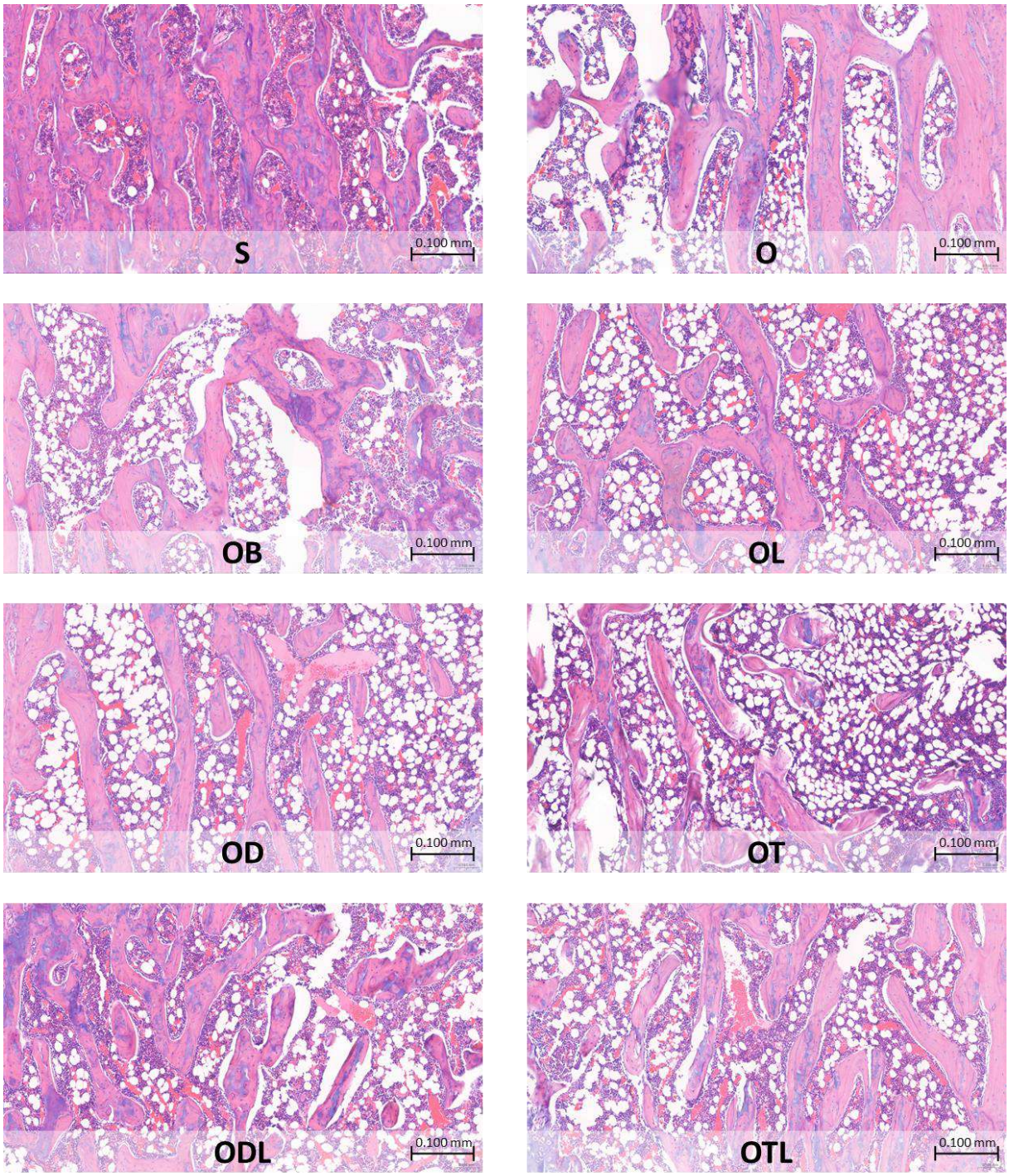

Figure 4. Histopathological changes in femoral bone following a 6-week intervention. Images were captured using a 10× objective lens with a scale of 0.100 mm.

2.4.3. Effect on calcium status, bone metabolism biomarkers, and bone mineral density in postmenopausal women

The final serial study in this doctoral research was designed to investigate the effects of probiotic supplementation with *L. acidophilus* on calcium status, bone metabolism biomarkers, and BMD profiles in postmenopausal women **[P5]**. This decision was underpinned by several important considerations. While previous studies with OVX rats did not yield statistically significant effects on calcium transport and bone metabolism biomarkers, they revealed promising trends, including a significant increase in calcium content in the femur of the OVX rats following *L. acidophilus* supplementation **[P3 and P4]**. These findings underscored the necessity of further exploration in human trials, particularly in a demographic at high risk for osteoporosis postmenopausal women.

The study aimed to fill notable gaps in understanding how probiotics influence the gut-bone axis and bone health, an area that remains underexplored despite extensive research on probiotics' effectiveness in other health conditions. By focusing on 12 weeks of daily oral consumption of *L. acidophilus* UALa-01™, the study sought to provide comprehensive insights into its impact on calcium status, bone metabolism biomarkers, and BMD profiles. This research not only aimed to advance the understanding of probiotic supplementation as a potential intervention for managing osteoporosis but also aspired to illuminate its possible role in reducing osteoporotic fracture risk in postmenopausal women. **Figure 2** illustrates the experimental design of the probiotic intervention study.

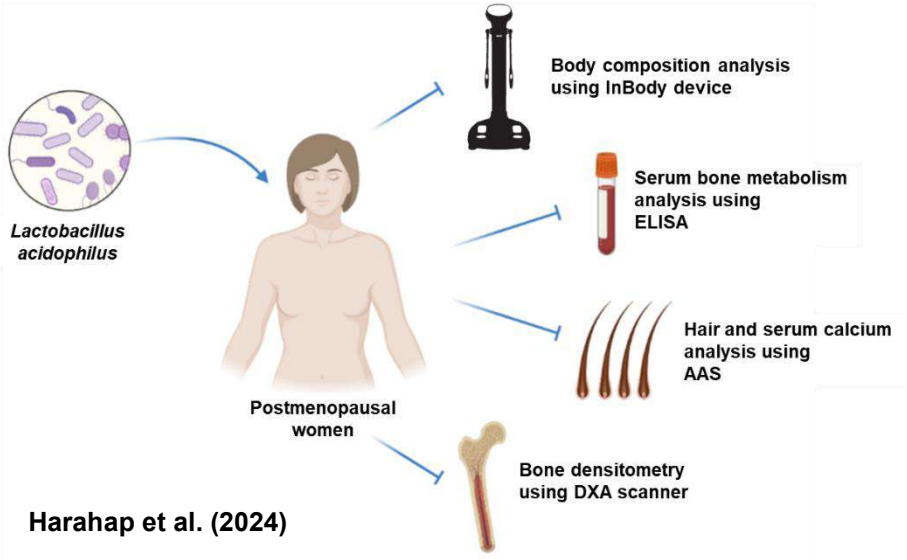

Figure 5. Experimental design of probiotic intervention.

The results demonstrated that initial comparisons between the placebo and probiotic groups revealed significantly lower serum calcium concentrations in the placebo group at

baseline, although no differences were noted in hair calcium levels or bone metabolism biomarkers. This baseline discrepancy underscores the importance of accounting for initial nutritional and metabolic differences when evaluating the effects of dietary interventions.

In addition to the results of this study, significant differences were found between the placebo and probiotic groups in terms of calcium concentrations in serum, while no significant differences were found in hair after a 12-week intervention. Interestingly, the placebo group experienced a significant increase in serum calcium levels from baseline to endline, whereas the probiotic group showed a significant decrease in serum calcium levels. This phenomenon was similarly noted in a previous OVX rat study **[P4]**, where probiotic supplementation also led to reduced serum calcium levels.

Furthermore, in terms of bone metabolism biomarkers, the placebo group exhibited a significant decrease in CTX levels and a significant increase in TRAP-5b levels after the 12-week period. These changes suggest alterations in bone resorption and formation dynamics within the placebo group, potentially indicating a response to the intervention period. Conversely, the probiotic group did not show significant changes in these biomarkers, suggesting that *L. acidophilus* supplementation might stabilize bone turnover rates, preventing fluctuations commonly observed in postmenopausal women. The stability of these biomarkers in the probiotic group indicates a potential benefit of *L. acidophilus* in maintaining balanced bone metabolism, though it did not lead to marked improvements.

The DXA assessment provided further insights into the effects of probiotic supplementation on BMD. Despite no significant changes in BMD levels observed in both groups from baseline to endline, it is noteworthy that the probiotic group exhibited a slightly larger difference in BMD across various skeletal sites compared to the placebo group. This marginal improvement, although not statistically significant, suggests a potential trend towards better bone density maintenance with probiotic supplementation. Specifically, the probiotic group showed minor variations in BMD levels at the lumbar spine, left femur, and total body, which, while small, could indicate a positive direction in bone health that might become more pronounced with a longer intervention period or a larger sample size.

Overall, these findings highlight the complexity of bone health management in postmenopausal women. While *L. acidophilus* supplementation did not result in significant improvements in calcium status or BMD, the observed trends and the stabilization of bone metabolism biomarkers point towards a potentially beneficial role of probiotics in maintaining bone health. The decrease in serum calcium levels within the probiotic group warrants further investigation to understand the underlying mechanisms and ensure that probiotic

supplementation is optimized for enhancing bone health outcomes. Future research should explore longer intervention periods, higher doses, or combinations with other nutrients to fully elucidate the therapeutic potential of probiotics in managing osteoporosis and improving bone health in postmenopausal women.

2.4.4. Mechanism of action underlying the obtained findings

This section delves into the mechanisms of action underlying the findings obtained from cell studies, OVX animal models, and human clinical trials. The observed effects of isoflavones and probiotics on calcium metabolism and bone health are explored, highlighting their interactions within the body. By examining these mechanisms, we aim to provide a comprehensive understanding of how these dietary components influence calcium absorption, transport, and deposition in bones.

2.4.4.1. Fermentation increases calcium concentration in tempeh

Rhizopus oligosporus, a dimorphic fungus, undergoes a transformation from filamentous mycelium to producing pseudohyphae and yeast-like cells when exposed to Griseofulvin, a mitotic inhibitor, and Fluconazole, a cytochrome P450 1,4-α-demethylase inhibitor. This transformation involves the activation of chloride channels by calcium ions (Ca^{2+}), which helps restore proper cell volume and maintain resting membrane potential. Consequently, the presence of Ca^{2+} ions promotes mycelial aggregation during fungal growth. This process is significant in the context of tempeh production, where fermentation not only enhances the nutrient profile but also increases the calcium content of tempeh compared to raw soybeans. The higher calcium content in tempeh can be attributed to the role of Ca^{2+} ions during the fermentation process, which supports better calcium absorption and bioavailability (Harahap, Kuligowski, Schmidt, & Suliburska, 2023b). While the exact source of calcium for tempeh fermentation is not explicitly stated, it is commonly sourced from tap water, which contains dissolved minerals, including calcium ions. Therefore, the higher calcium content in tempeh can be attributed to the role of Ca^{2+} ions present in the water used during the process.

2.4.4.2. Probiotics and isoflavones decrease serum calcium levels but increase calcium levels in bone

This study highlights the potential pathways through which soy isoflavones and probiotics modulate calcium homeostasis in the context of menopausal osteoporosis. Our investigation provides compelling evidence suggesting that isoflavones, particularly the abundance of daidzein found in fermented soy foods, contribute to restoring calcium balance during menopause. These isoflavones, through their estrogenic activity, may act as partial substitutes for declining endogenous estrogen levels, thereby regulating bone turnover and

calcium homeostasis. The enhancement in intestinal calcium absorption associated with isoflavone consumption could play a pivotal role in countering the diminished calcium absorption commonly encountered during menopause. These multifaceted effects collectively underscore the potential of isoflavones to positively influence calcium metabolism (Harahap et al., 2024; Harahap, Kuligowski, Schmidt, Kołodziejski, et al., 2023a; Harahap, Kuligowski, Schmidt, Kurzawa, et al., 2023b).

Estrogen pathways play an essential role in bone metabolism, which is significant in the pathophysiology of osteoporosis and justifies the use of isoflavones for its prevention and therapy (Khosla et al., 2012). Isoflavones exert their effects by interacting with estrogen receptors and modulating estrogenic pathways. They act as selective estrogen receptor modulators, exhibiting both estrogenic and antiestrogenic properties depending on the tissue and cellular context (Setchell, 2001; Vitale et al., 2013). In bone metabolism, isoflavones can replace endogenous estrogens in metabolic pathways crucial for bone health. One such pathway is the RANKL/RANK/OPG system, which regulates osteoclast differentiation and bone resorption (Hooshiar et al., 2022). Additionally, isoflavones enhance the production of osteoprotegerin (OPG), a decoy receptor that binds to the receptor activator of nuclear factor-κB-ligand (RANKL) and prevents its interaction with RANK, inhibiting osteoclast formation and activity (Chen et al., 2002).

Probiotic supplementation also led to reduced serum calcium levels. Several factors could contribute to this counterintuitive outcome. One reasonable explanation lies in the interactions between probiotics and the gut microbiota. Probiotics modulate the composition and activity of gut microbiota, which can influence nutrient absorption processes, including calcium uptake (Zhou et al., 2023). Alterations in microbial populations within the gut could impact the overall efficiency of calcium absorption. Probiotics might also indirectly influence calcium homeostasis by modifying the intestinal pH, which plays a crucial role in the solubility and absorption of calcium. This hypothesis aligns with the observed increase in femoral calcium content noted in both the current human study and previous OVX rat studies.

Moreover, probiotics might upregulate the expression of calcium-binding proteins and transporters in the intestinal epithelium and other tissues, such as TRPV5 and TRPV6, enhancing calcium absorption efficiency at the cellular level. This enhanced absorption might result in more calcium being directed towards bone deposition and less remaining in the bloodstream, contributing to the decreased serum calcium levels observed. Additionally, the influence of probiotics on systemic inflammatory markers and endocrine factors should not be overlooked. Probiotics can modulate the immune response and inflammatory status, which are

known to affect bone metabolism and calcium homeostasis. A reduction in systemic inflammation could enhance bone formation and calcium incorporation into the bone matrix, again explaining the reduced serum calcium levels.

To sum up, the decrease in serum calcium levels following probiotic and isoflavones supplementation observed in both the studies **[P3 and P4]** on the OVX rat model can be attributed to a multifaceted interaction of gut microbiota modulation, changes in intestinal pH, upregulation of calcium transport mechanisms, and systemic inflammatory responses. These findings highlight the role of probiotics and isoflavones in nutrient metabolism and underscore the need for further research to unravel the precise mechanisms and optimize probiotic use for bone health.

2.4.4.3. Probiotics and its combination with isoflavone products influence blood biochemistry and glucose levels

The daily consumption of probiotic *L. acidophilus* and its combination with tempeh in ovariectomized rats led to significant changes in various blood biochemistry parameters. Notably, there was an increase in hemoglobin, hematocrit, and glucose levels, while cholesterol and triglycerides were significantly reduced. These changes suggest a multifaceted impact of the combined intake of probiotics and isoflavones on blood chemistry. One significant observation was the increase in leukocytes and lymphocytes in the ovariectomized rats. This could be attributed to the dysregulation of the immune system resulting from estrogen deficiency due to ovariectomy. Estrogen is known to modulate immune function, including the production and activity of various immune cells (Chakraborty et al., 2023). The removal of estrogen may thus lead to an imbalance in immune cell populations, triggering an increase in leukocytes and lymphocytes as a response to hormonal changes associated with menopause.

Consistent with a previous human clinical trial where a combination of probiotics including *Lactobacillus casei*, *Lactobacillus rhamnosus*, *Lactobacillus acidophilus*, and *Bifidobacterium bifidum*, along with fructooligosaccharides, led to increased hemoglobin and hematocrit levels (dos Santos et al., 2017), our study demonstrated similar outcomes in ovariectomized rats. However, our prior study on healthy female rats did not show significant changes in hemoglobin and hematocrit levels with *L. acidophilus* and isoflavones (Harahap, Kuligowski, Schmidt, & Suliburska, 2023a). The increase observed in the study **[P5]** could be due to several mechanisms. *L. acidophilus* has mechanisms for transporting Fe^{2+} ions and possesses an intracellular ferroxidase enzyme similar to those in bifidobacteria. The lactate produced by *L. acidophilus* facilitates the oxidation of Fe^{2+} ions, and the resulting Fe(III) ions remain bound to the lactate within the cell (Kot et al., 1995), enhancing iron bioavailability

which is crucial for hemoglobin synthesis (Oda et al., 1994). Additionally, the fermentation process in tempeh production generates peptides that may promote erythropoiesis and red blood cell production (Bunn, 2007), further contributing to the increased hemoglobin and hematocrit levels observed.

The study also highlighted favorable effects on lipid metabolism, with reductions in cholesterol and triglyceride levels following the daily intake of probiotic *L. acidophilus* and tempeh. These findings align with previous research showing that dietary interventions, such as consuming tempeh and probiotics during soy tempeh fermentation, can improve lipid profiles in diabetic rat models (Huang et al., 2018; Su et al., 2023). The improvements in lipid metabolism may be attributed to the bioactive compounds generated during fermentation and the probiotic activity enhancing lipid metabolism and reducing cholesterol synthesis.

However, an adverse effect was noted with an increase in blood glucose levels in the groups consuming probiotics alone and their combination with isoflavones **[P4]**. This finding was corroborated by our human study on menopausal women, which also showed increased glucose levels **[P5]**. Despite previous studies indicating that tempeh consumption can improve blood glucose levels in diabetic models, possibly due to short-chain fatty acids like propionate enhancing glucose uptake and regulating glucose levels, the observed increase in our study suggests a complex interaction between diet, gut microbiota, and metabolic health. The reduction in propionate producers due to alterations in gut microbiota composition may lead to elevated glucose levels, highlighting the intricate balance required for managing metabolic health through dietary interventions (Singh et al., 2023).

The increase in glucose levels affected by probiotic may be due to several factors. Probiotics can influence glucose metabolism through interactions with the gut microbiota, which affects carbohydrate fermentation and the production of short-chain fatty acids involved in glucose homeostasis (Falcinelli et al., 2018). Additionally, the decrease in serum calcium levels observed in the probiotic group might indirectly impact glucose metabolism. Calcium plays a role in insulin secretion and sensitivity, and changes in calcium levels can affect glucose uptake and utilization by pancreatic β-cells (Gilon et al., 2014). Moreover, the slight increase in the HOMA-IR index in the probiotic group suggests changes in insulin resistance, contributing to higher glucose levels. These findings highlight a complex interplay between probiotic supplementation, calcium metabolism, and glucose homeostasis, warranting further research to understand the underlying mechanisms. Factors such as the type of probiotic administered, the duration of the intervention, and the diversity of probiotic strains used could help clarify the observed discrepancies in research findings (Li et al., 2023).

In summary, while the study revealed beneficial effects on lipid metabolism with the daily intake of probiotic *L. acidophilus* and tempeh, the adverse impact on glucose levels suggests a multifaceted relationship between gut microbiota and metabolic processes. The enhancement of isoflavone absorption by lactic acid bacteria underscores the potential for these dietary interventions to influence overall metabolic health. However, the increase in glucose levels indicates a need for further research to optimize these interventions for better health outcomes, particularly in managing glucose and lipid metabolism in postmenopausal women.

2.5. Conclusions

1. The effects of daidzein, tempeh, and *L. acidophilus* on calcium deposition in human osteoblast-like Saos-2 cells did not show significant enhancement in calcium deposition. However, there were indications that these dietary components might promote osteogenic differentiation.
2. The effects of daily intake of tempeh and daidzein improved calcium status, enhanced the expression of calcium transporters, and positively influenced bone metabolism biomarkers. These effects were comparable to those seen with bisphosphonate drugs.
3. The effects of daily intake with a combination of *L. acidophilus* and isoflavone products, including tempeh and daidzein, demonstrated beneficial effects on femoral bone calcium levels and bone metabolism biomarkers. The intervention also might influence hematological parameters and lipid profiles, although it led to elevated blood glucose levels.
4. The effects of daily supplementation with *L. acidophilus* did not significantly alter bone mineral density profiles but may help stabilize bone turnover in postmenopausal women. However, the probiotic supplementation disturbed calcium and glucose levels.

2.5.1. Overall conclusion

In conclusion, the findings from these studies collectively suggest that isoflavone products, such as tempeh and daidzein, along with probiotics like *L. acidophilus*, exhibit potential benefits for bone health. Specifically, these dietary components were shown to positively influence calcium status and bone metabolism biomarkers. While there are promising indications, particularly in animal models, the findings also highlight the complexity of these interactions and the need for further research to fully understand their implications and optimize their use in preventing or treating osteoporosis. Further research is necessary to fully elucidate their mechanisms of action, optimize their use, and ensure their safety and efficacy in diverse populations.

2.5.2. Practical conclusion

Intake of good sources of soy isoflavones and probiotics through diet or dietary supplements may be beneficial in supporting the prevention and treatment of osteoporosis in postmenopausal women. However, the use of *Lactobacillus acidophilus* may lead to an increase in blood glucose levels. This side effect of probiotics should be the focus of long-term clinical trials.

2.6. Novelty of the research

The research presented in this dissertation introduces several novel contributions to the field of nutritional science and osteoporosis treatment, particularly in the context of postmenopausal osteoporosis. The comprehensive approach, integrating both *in vitro* and *in vivo* studies, provides new insights into the roles of isoflavones and probiotics in enhancing calcium bioavailability and promoting bone health.

1. Integration of isoflavones and probiotics

   This research is among the first to systematically explore the combined effects of daidzein and tempeh with probiotics like *L. acidophilus* on calcium metabolism and bone health. By examining these dietary components in various models, the studies provide a holistic understanding of their potential synergistic effects. The use of an artificial gastrointestinal model to study the digestion and subsequent impact on calcium deposition in osteoblast-like cells is a particularly novel aspect, shedding light on the bioactive potential of these compounds post-digestion.

2. Comprehensive *in vitro* and *in vivo* analysis

   The dissertation uniquely combines *in vitro* cell culture studies with *in vivo* animal models and human clinical trials, creating a comprehensive framework for assessing the effects of dietary interventions on bone health. The multi-faceted approach allows for a detailed investigation into the mechanisms of action at the cellular level, their physiological impacts in animal models, and their potential translational benefits in human subjects.

3. Potential therapeutic alternatives

   The research provides evidence suggesting that isoflavone-rich foods like tempeh and daidzein, in combination with probiotics, could serve as viable alternatives or complements to conventional osteoporosis treatments. This is particularly significant given the side effects and limitations associated with drugs currently used in osteoporosis management. The studies in ovariectomized rats, which simulate

postmenopausal osteoporosis, highlight the potential of these dietary interventions to improve calcium status and bone metabolism biomarkers effectively.

4. Impact on postmenopausal women

   The clinical trial involving postmenopausal women adds a critical translational component to the research, offering practical insights into how these dietary interventions could be implemented in real-world scenarios. The findings that daily supplementation with *L. acidophilus* helps stabilize bone turnover, despite not significantly altering bone mineral density profiles, provide a basis for further exploration and potential dietary recommendations for postmenopausal women.

5. Innovation in probiotic and isoflavone products

   Although this dissertation has not delved deeply into the innovation of probiotic and isoflavone products, the findings provide a substantial foundation for future exploration in this area. The research also explores the innovative potential of developing new dietary supplements and formulas based on probiotics and isoflavones, aimed specifically at improving bone health in postmenopausal women. The ININ 2.0 research grant, which facilitated the patenting of such a formula, underscores the practical applications and commercial potential of the research findings.

Overall, the novelty of this research lies in its integrative approach, the exploration of synergistic effects between isoflavones and probiotics, and the translational potential of these findings in the context of postmenopausal osteoporosis. The comprehensive nature of the studies, spanning from cellular models to human trials, provides a robust foundation for future research and development of dietary interventions aimed at enhancing bone health.

2.7. Limitations and future prospective study

This research demonstrated the originality and strength of using probiotics and isoflavones as interventions to analyze calcium bioavailability, calcium status, and bone metabolism in various experimental designs. However, there are certain limitations that should be acknowledged, and future prospective studies should address these aspects to further enhance the scientific understanding in this area.

2.7.1. Limitations

1. Analysis only selected parameters

This dissertation focused on a selected set of parameters to evaluate the effects of isoflavones and probiotics on calcium bioavailability and bone health. However, several important aspects were not analyzed, which may limit the comprehensiveness of the findings.

Vitamin D and K analysis

The studies did not include an analysis of vitamin D and vitamin K levels, which play crucial roles in calcium metabolism and bone health. Vitamin D is important for calcium absorption in the intestines, while vitamin K is important for the carboxylation of osteocalcin, a protein involved in bone mineralization. The omission of these vitamins means that potential interactions between these dietary components and the interventions studied were not explored, possibly limiting the understanding of the full impact of the dietary interventions on bone health.

Gut microbiota and its metabolites

The research did not comprehensively analyze changes in gut microbiota composition and the production of microbial metabolites, such as short-chain fatty acids, in response to probiotic and isoflavone consumption. Given the known influence of gut microbiota on nutrient absorption and bone health, this is a significant limitation. A detailed analysis of gut microbiota and their metabolites would provide deeper insights into how these dietary interventions affect bone health through the modulation of gut microbiota.

Bone microarchitecture

The study did not assess bone microarchitecture, which is crucial for understanding bone quality and strength beyond what BMD can reveal. Parameters such as trabecular bone structure, cortical thickness, and overall bone geometry significantly impact bone resilience and fracture risk. Future studies should include advanced imaging techniques, like micro-CT, to evaluate these aspects.

2. Study duration and long-term effects

The duration of the studies, particularly the human clinical trial, was relatively short (12 weeks). This timeframe may not be sufficient to observe the long-term effects of probiotic and isoflavone supplementation on bone health. Long-term studies are necessary to determine the sustained impact of these interventions and to monitor any potential adverse effects that may arise over extended periods.

3. Group size and diversity

The group sizes used in the human study were limited, which may affect the generalizability of the findings. Additionally, the human study population lacked diversity in terms of age, ethnicity, and health status, which could influence the outcomes. Larger, more diverse study populations would enhance the robustness and applicability of the results to broader populations.

2.7.2. Future prospective study

Future research should aim to address the limitations identified in this dissertation to provide a more comprehensive understanding of the role of isoflavones and probiotics in bone health. Expanding the range of parameters assessed, including bone microarchitecture, mechanical strength, and additional biomarkers of bone turnover, will offer a more detailed data of how these dietary interventions impact bone health. Additionally, incorporating an analysis of vitamin D and K levels will help elucidate the interactions between these crucial vitamins and the studied interventions, providing a fuller understanding of their combined effects on calcium metabolism and bone health.

Long-term studies with larger and more diverse populations are important to determine the sustained impact and safety of probiotic and isoflavone supplementation. These studies should also include a detailed examination of gut microbiota composition and the production of microbial metabolites, given their significant role in nutrient absorption and bone health. Additionally, the effects would be more precisely defined by examining the potential interactions between the interventions and other dietary factors. Addressing these aspects in future research will enhance the understanding and application of these dietary interventions in the prevention and management of osteoporosis, especially among postmenopausal women.

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List of tables

List of figures

List of supporting articles involved in the dissertation

No.	Description of Published Articles
1.	Katarzyna Skrypnik, Schmidt, M., Agnieszka Olejnik-Schmidt, **Harahap, I. A.**, & Suliburska, J. (2024). *Influence of supplementation with iron and probiotic bacteria Lactobacillus plantarum and Lactobacillus curvatus on selected parameters of inflammatory state in rats on a high-fat iron-deficient diet.* Journal of the Science of Food and Agriculture, 104(7), 4411–4424. DOI https://doi.org/10.1002/jsfa.13329 Impact Factor 4.1 MNiSW$_{2024}$ Point 100
2.	**Harahap, I. A.**, Kuligowski, M., Schmidt, M., Kurzawa, P., & Suliburska, J. (2023). *Influence of Isoflavones and Probiotics on Magnesium Status in Healthy Female Rats*. Foods, 12(21), 3908–3908. DOI https://doi.org/10.3390/foods12213908 Impact Factor 5.2 MNiSW$_{2023}$ Point 100
3.	**Harahap, I. A.**, Kuligowski, M., Schmidt, M., Kołodziejski, P. A., & Suliburska, J. (2023). *Effects of isoflavone and probiotic intake on calcium transport and bone metabolism biomarkers in female rats*. Food Science & Nutrition, 11(10), 6324–6335. DOI https://doi.org/10.1002/fsn3.3571 Impact Factor 3.9 MNiSW$_{2023}$ Point 100
4.	**Harahap, I. A.**, Kuligowski, M., Schmidt, M., Kurzawa, P., Pruszyńska-Oszmałek, E., Maciej Sassek, & Suliburska, J. (2023). *Isoflavones and probiotics effect on bone calcium and bone cells in rats*. Heliyon, 9(6), e16801–e16801. DOI https://doi.org/10.1016/j.heliyon.2023.e16801 Impact Factor 4.0 MNiSW$_{2023}$ Point 40
5.	**Harahap, I. A.**, Kuligowski, M., Schmidt, M., & Suliburska, J. (2023). *The impact of soy products, isoflavones, and Lactobacillus acidophilus on iron status and morphological parameters in healthy female rats*. Journal of Trace Elements in Medicine and Biology, 78, 127183–127183. DOI https://doi.org/10.1016/j.jtemb.2023.127183 Impact Factor 3.5 MNiSW$_{2023}$ Point 100
6.	**Harahap, I. A.**, Kuligowski, M., Schmidt, M., & Suliburska, J. (2023). *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, 2, 100269–100269. DOI https://doi.org/10.1016/j.focha.2023.100269 Impact Factor 0.0 MNiSW$_{2023}$ Point 0
7.	**Harahap, I. A.**, & Suliburska, J. (2023). *Can probiotics decrease the risk of postmenopausal osteoporosis in women?*. PharmaNutrition, 24, 100336–100336. DOI https://doi.org/10.1016/j.phanu.2023.100336 Impact Factor 3.2 MNiSW$_{2023}$ Point 40

8.	**Harahap, I. A.**, Kuligowski, M., Schmidt, M., Brzozowska, A., & Suliburska, J. (2022). *Impact of isoflavones and Lactobacillus acidophilus on the fecal microbiology status in healthy female rats*. Acta Scientiarum Polonorum. Technologia Alimentaria, 21(2), 223–231. DOI https://doi.org/10.17306/J.AFS.2022.1059 Impact Factor 1.5 MNiSW$_{2022}$ Point 40
9.	**Harahap, I. A.**, Landrier, J. F., & Suliburska, J. (2022). *Interrelationship between Vitamin D and Calcium in Obesity and Its Comorbid Conditions*. Nutrients, 14(15), 3187–3187. DOI https://doi.org/10.3390/nu14153187 Impact Factor 5.9 MNiSW$_{2022}$ Point 140
10.	**Harahap, I. A.**, Sobral, M. M. C., Casal, S., Pinho, S., Faria, M. A., Suliburska, J., & Ferreira, I. M. P. L. V. O. (2022). *Fat Oxidation of Fatty Fish vs. Meat Meal Diets Under in vitro Standardized Semi-Dynamic Gastric Digestion*. Frontiers in Nutrition, 9. DOI https://doi.org/10.3389/fnut.2022.901006 Impact Factor 5.0 MNiSW$_{2022}$ Point 70
11.	**Harahap, I. A.**, & Suliburska, J. (2022). *An overview of dietary isoflavones on bone health: The association between calcium bioavailability and gut microbiota modulation*. Materials Today: Proceedings, 63, S368–S372. DOI https://doi.org/10.1016/j.matpr.2022.03.549 Impact Factor 0 MNiSW$_{2022}$ Point 0
12.	Suliburska, J., **Harahap, I. A.**, Wawrzyniak, N., & Gramza-Michałowska, A. (2021). *The calcium deficit diet does not affect body composition, glucose, and lipid status in ovariectomized rats*. Acta Scientiarum Polonorum. Technologia Alimentaria, 20(4), 459–464. DOI https://doi.org/10.17306/J.AFS.2021.1004 Impact Factor 1.5 MNiSW$_{2021}$ Point 40
13.	Suliburska, J., **Harahap, I. A.**, Katarzyna Skrypnik, & Paweł Bogdański. (2021). *The Impact of Multispecies Probiotics on Calcium and Magnesium Status in Healthy Male Rats*. Nutrients, 13(10), 3513–3513. DOI https://doi.org/10.3390/nu13103513 Impact Factor 5.9 MNiSW$_{2021}$ Point 140

Total Impact Factors and MNiSW$_{2021\text{-}2024}$ Points

Articles involved in the dissertation	Impact Factors	43.7
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Articles in the dissertation

foods

MDPI

Review

Probiotics and Isoflavones as a Promising Therapeutic for Calcium Status and Bone Health: A Narrative Review

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Abstract: Probiotics have potential clinical effects for treating and preventing osteoporosis. Meanwhile, isoflavones have attracted much attention due to their ability to prevent postmenopausal symptoms. Research has established that probiotics and isoflavones can regulate hormones, immune cells, and the gastrointestinal system, acting as links in the gut–bone axis. However, combining the effects of probiotics and isoflavones on calcium status and bone health is a more novel and a still-evolving research area. *Lactobacillus* and *Bifidobacterium* are the foremost strains that influence bone health to a significant extent. Among the isoflavones, daidzein, genistein, and the metabolites of genistein (such as equol) stimulate bone formation. It can be concluded that probiotics and isoflavones promote bone health by regulating calcium uptake, gut microbiota, and various metabolic pathways that are associated with osteoblast activity and bone formation. Nevertheless, further experiments of probiotics and isoflavones are still necessary to confirm the association between calcium bioavailability and bone health.

Keywords: probiotics; isoflavones; calcium status; bone health; osteoporosis

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1. Introduction

Osteoporosis is progressively becoming a very grave issue worldwide. This condition affects numerous people of all races and genders, and its occurrence will expand with the population, most frequently in Caucasians, women, and older people [1]. The gradual loss of bone with ageing is a normal condition. However, it may be accelerated by internal and external variables such as menopause, severe health conditions, and life factors such as an inadequate diet, insufficient exercise, smoking, or intemperate alcohol consumption [2]. On the one hand, the interactions between dietary calcium intake and bone health seem to differ; no consistent relationships have been demonstrated between dietary calcium and bone strength parameters. Besides, there may not be a significant association between dietary calcium intake and bone mineral density (BMD) in those with sufficiently (or high) vitamin D levels [3]. A positive interaction effect on the skeleton has also been seen between dietary protein and dietary calcium in older subjects who met their requirements but not those with lower calcium intakes [4]. On the other hand, there is acknowledged evidence of the interaction between calcium and vitamin D [5].

At the same time, vegetarians and vegans may be at greater risk of lower BMD and fractures than omnivores because of deficiency in calcium, vitamin D, vitamin B12, protein, and *n*-3 (ω-3) fatty acids in their diets. All of these nutrients play essential roles in maintaining bone health [6]. Furthermore, the association between calcium absorption and lactose intake [7] has not been clearly defined, nor between calcium absorption and caffeine intake [8]. However, fiber intake (soluble corn fiber and soluble fiber dextrin) has been found to increase calcium absorption [9].

A few current recommendations for treating and preventing osteoporosis among postmenopausal women are estrogen therapy and pharmacological agents. Estrogen treatment

is also valuable in maintaining or improving bone mineral density [10] but presents an increased risk of reproductive system cancers [11]. However, pharmacological agents such as bisphosphonates, calcitonin, and denosumab (a receptor activator of nuclear factor-κB ligand / RANKL inhibitor) are not prescribed for long-term use, frequently require regular infusion appointments, and carry their own risk of adverse drug responses [12]. Furthermore, a meta-analysis has shown that a daily intake of calcium and vitamin D could decrease the risk of a total fracture by 15% and hip fracture by 30% [13]. It thus appears that clinicians' current treatment and management of osteoporosis by employing dietary supplements alone may not be sufficient to prevent menopausal bone loss entirely.

Considering the duration of the treatment that would be needed to maintain bone health before and after menopause, it would be of great clinical value to create viable treatments with negligible side effects that are appropriate for longer-term use. Recent studies have found that probiotics and isoflavones have a significant impact on calcium absorption and bone health. With recent direct and indirect evidence in mind, this review focuses on the combination of probiotics and isoflavones and their effect on the host's calcium status and bone health. There have been no high-quality reviews on this topic since 2010, to the best of our knowledge. We thus considered it necessary to describe the current state of knowledge on this very significant topic.

1.1. Probiotics

Probiotics are microorganisms that deliver health benefits to the host when consumed in the appropriate amounts [14]. The health benefits they deliver include potential clinical effects that are useful in treating and preventing osteoporosis [15]. Furthermore, probiotics could be used to decrease postmenopausal bone loss by increasing gut epithelial stability, increasing the expression of tight junction proteins, or reducing antigen transfer and lowering the activation of intestinal immune cells [16]. *Lactobacillus acidophilus* appears to be a promising strain with beneficial effects in ovariectomy [17] and osteoarthritis [18]. This strain is capable of colonizing the human colon, has antimicrobial effects, and can be used to treat intestinal infections [19]. Additionally, *L. acidophilus* has therapeutic potential as an osteoprotective agent in enhancing bone health. *L. acidophilus* in ovariectomized mice was able to improve both the trabecular and cortical bone microarchitecture, which also increased the mineral density and heterogeneity of bones. This effect of *L. acidophilus* administration is due to its immunomodulatory effect on the host immune system. *L. acidophilus* skews the Treg-Th17 cell balance by inhibiting osteoclastogenic Th17 cells and promoting antiosteoclastogenic Treg cells in ovariectomized mice. The administration of *L. acidophilus* also suppresses the expression of osteoclastogenic factors (interleukin 6 / IL-6, interleukin 17 / IL-17, tumor necrosis factor-alfa / TNF-α, and RANKL) and increases the expression of antiosteoclastogenic factors (such as interleukin 10 / IL-10, interferon gamma / IFN-γ) [17]. Moreover, *Lactobacillus casei* and *L. acidophilus* showed both the highest serum calcium level and the highest bone marrow concentration. This result indicated that the elevation of alkaline phosphatase (ALP), calcium (Ca), and phosphorus (P) is directly related to bone loss [20]. Therefore, these studies have shown a significant effect on various biochemical parameters that are triggered in postmenopausal osteoporosis and consequently prevent bone loss.

1.2. Isoflavones

Estrogen deficiency is a leading cause of bone loss and osteoporosis in postmenopausal women [21]. The three major chemical types of phytoestrogen that have been identified are isoflavones, lignans, and coumestans. The primary isoflavones in aglycone form are genistein, daidzein, and glycitein. These isoflavones can be found in soybeans and are considered potential alternatives to hormone therapy [22].

Daidzein, a soybean isoflavone, is metabolized to equol by the gut microflora in the gastrointestinal tract [23]. Nevertheless, daidzein reduces serum testosterone (T) and androstenedione (AD) levels in steroid metabolism between equol producing and non-

equol producing women [24]. Unlike in women, both soy-based food and isoflavones have no consequence of free testosterone levels in men [25]. Equol itself may be involved in the regulation of androgens of the adrenal cortex in women [24]. Besides, equol inhibits bone loss, apparently without affecting the reproductive organs, in ovariectomized mice [26]. An association has also been found between the status of equol production and gut microbiota [27].

1.3. Calcium Status and Bone Health

Dietary supplements have become clinicians' and patients' preferred treatment for preventing and managing bone disease. The entry point of Ca into the body in humans and mammals is the intestine. There are two pathways for Ca absorption: through the paracellular pathway and the transcellular pathway [28]. First, a non-saturable process depends on the electrical gradient between the lumen and intestinal mucosa, allowing calcium ions' passive diffusion across the intestinal epithelium [29]. Second, a transcellular saturable multistep process is initiated by transient receptor potential vanilloid type 6 (TRPV6). Once calcium has entered the cell through the TRPV6 channel, calcium-buffering proteins bind to the calcium and transport it. Finally, calcium is transferred from the cell to the blood vessels through plasma membrane ATPase 1b (PMCA1b) that is located in the basolateral membrane [30]. Hormones, nutrients, and other factors regulate both of the pathways. The major regulating hormone is calcitriol [1,25(OH)2D3], which works via vitamin D receptor signaling [31].

1.4. Gut Microbiota and Bone Health

The microbiota benefits the host's health by producing essential nutrients, digesting food components, and enhancing the maturation's immune system. However, dysbiosis, an unhealthy imbalance in the microbiota community composition, is linked to various metabolic, inflammatory, and immunologic diseases [32]. The imbalance of the gut microbiome can cause the imbalance of osteogenesis and osteoclast reaction [33]. When dysbiosis occurs, the gut microbiome loses its protective capabilities, and the gut barrier is impaired. The host fails to effectively control the dissemination of the gut microbiome components into the tissues [34]. The gut microbiota is critical for developing the immune system since both the microbiota and the immune system can regulate bone health [35]. Recent studies have shown that the gut microbiota significantly influences bone health (Table 1).

Table 1. Dysbiosis microbial signatures in bone health issues.

	Study Design	Specific Dysbiosis Microbial Signatures	Ref
Human	48 primary osteoporosis 48 healthy	*Bacteroidetes* phylum, *Bacterioidia* class, *Bacteroidetes* order, *Ruminococcaceae* family, *Prevotellaceae* family, *Dialister* genus, and *Faecalibacterium* genus have been revealed as the key microbes related to primary osteoporosis	[33]
Human	6 Normal control (NC) (5F,1M) 6 Osteopenia (ON) (5F,1M) Osteoporosis (OP) (5F,1M)	Higher *Firmicutes* and lower *Bacteroidetes* were in the osteoporosis group than in the normal group. *Gemmatimonadetes* and *Chloroflexi* were different between bone health issues groups and control group	[36]
Human	108 postmenopausal women	*Klebsiella*, *Morganella*, *Escherichia/Shigella*, *Enterobacter*, *Citrobacter*, *Pseudomonas*, *Succinivibrio*, and *Desulfovibrio* were significantly higher in the postmenopausal osteopenia group	[37]
Animal	40 female Sprague Dawley	*Firmicutes/Bacteroidetes* Ratio, *Clostridium*, *Robinsoniella*, *Coprococcus*, and *Dialister* increased significantly after ovariectomy. *Ruminococcus flavefaciens* was the greatest abundance.	[38]
Animal	6 Male C57BL mice	A strong positive correlation was demonstrated between members of the *Actinobacteria phylum* (including the *Bifidobacteriaceae* family) and bone volume fraction ratio	[39]

Hormones, immune cells, and the gastrointestinal system can regulate the balance of bone resorption by osteoclasts and bone formation by osteoblasts. Specifically, the gastrointestinal system contributes to absorbing bone mineralization [40], and it produces endocrine factors that signal to bone cells, such as incretins [41] and serotonin [42]. Probiotics that modify the microbiota composition or function and promote intestinal health can also benefit bone health [43]. The gut microbiome is essential for the efficient maturation of the immune system and cytoprotection against exogenous insults. The gut microbiota produces metabolites that account for anatomically distant biological effects. Indole derivatives were among the first bacterial metabolites to be described as affecting intestinal immunity. In addition, insulin-like growth factor 1 (IGF-1), produced predominantly in the liver in response to food intake and regulated by microbes and microbial products, was the first metabolite that was identified as a link in the gut–bone axis [44]. The crosstalk between the growth hormone/insulin-like growth factor-1 (GH/IGF-1) axis and the gut microbiota correlates with marked changes in microbial abundance (both phylum and genus shifts), richness, evenness, maturity, and levels of metabolites such as short-chain fatty acids, branched-chain amino acids, ammonia, and neurotransmitters [45].

2. Methods

The databases we searched included Medline via PubMed and Scopus. The search focused on citations after the year 2010 to capture the most recent evidence. To restrict the search results, we used the search terms (probiotic *) AND (isoflavone * OR daidzein OR equol) AND (calcium) AND (intake OR supplement * OR consum *) AND (bone OR osteo *) AND (cell * OR vitro *) AND (animal * OR rat * OR mice * OR mouse *) AND (human OR subject * OR volunteer * AND participant * OR women OR female). The inclusion and exclusion criteria were carefully chosen and are laid out in Table 2. The method encompassed in vitro, animal, and human studies in health and disease. Both observational and intervention studies were included. Studies with data duplication, lacking a description of their method, not in English, or published before 2010 were excluded, as were case reports. Therefore, this article was based on actual literature from the last ten years. Studies with other dietary supplements that were supplied simultaneously with probiotics or minerals were excluded. Figure 1 shows the search flowchart for the review.

Table 2. Study inclusion and exclusion criteria.

Parameter	Inclusion Criteria	Exclusion Criteria
Materials	In vitro, animal, and human studies, both in health and disease.	Not to be defined.
Intervention	Probiotic supply. Isoflavones supply.	Studies with other dietary supplements supplied simultaneously with probiotic, synbiotic, or isoflavones.
Comparator	Comparison of bacterial strains and isoflavones. Placebo or no comparator.	Not to be defined.
Outcomes	There are justifications for the use of probiotics and isoflavones, and they interact with the health of the host.	Not to be defined.
Study design	Intervention studies. Review articles only if they contain data on important issues not available elsewhere.	Case reports Studies duplicating data, lacking a description of method, not in English, or published before 2010.

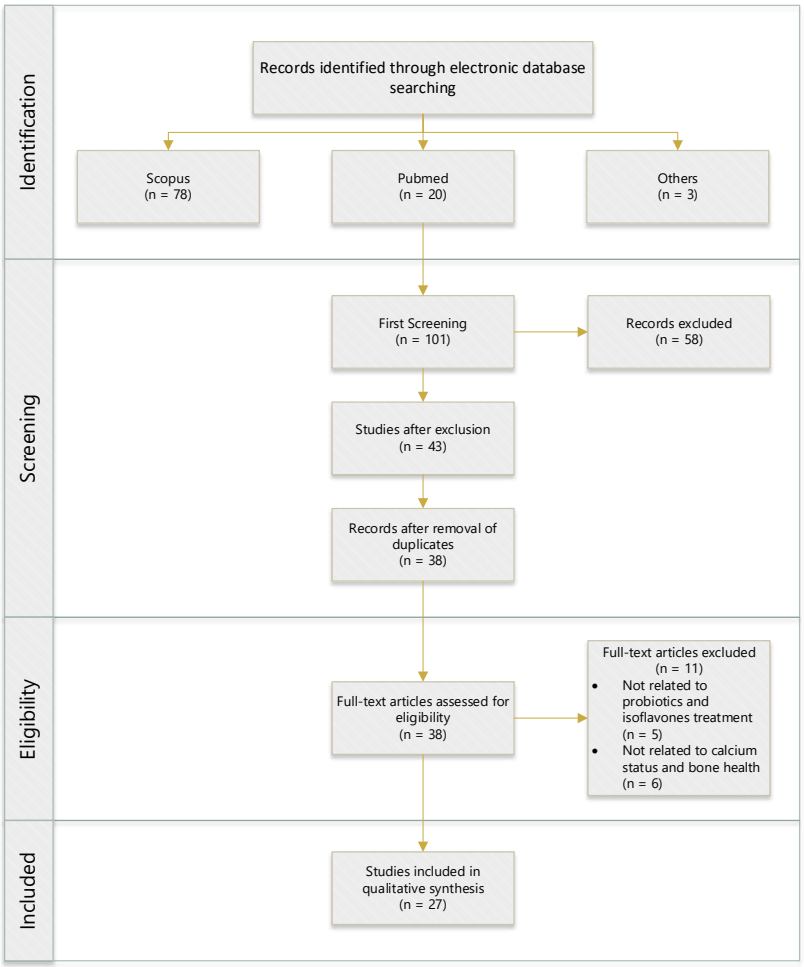

Figure 1. Flow diagram of the literature search and process of selection. (n = numbers).

Data Synthesis

We synthesized the data narratively by summarizing the study design and mechanism findings in tables. The overall findings are explored in the Results (Section 3) and Discussions (Section 4).

3. Results

3.1. In Vitro Studies

Research on the effects of probiotics and their effects on the calcium status and bone health is limited compared to isoflavone in in vitro studies (Table 3). In vitro data are also helpful in determining the calcium status, bone health regulation, and other metabolites, elucidating their potential mechanisms. Probiotics and isoflavones may affect gastrointestinal health indicators, justifying animal and human studies.

Raveschot and colleagues [46] studied the probiotic properties of 174 *Lactobacillus* strains that were isolated from Mongolian dairy products and their impact on intestinal calcium uptake and absorption. A total of five *Lactobacillus* strains displayed good probiotic characteristics and modulated calcium absorption by intestinal cells—namely *L. casei* 9b, *L. kefiranofaciens* 15b, *L. plantarum* 46a, *L. helveticus* 49d, and *L. delbrueckii* 50b. *L. casei* 9b, *L. kefiranofaciens* 15b and *L. helveticus* 49d increased the total calcium transport by Caco-2 cells, most probably by improving calcium solubility. *L. delbrueckii* 50b impacted the paracellular pathway of calcium absorption by upregulation of the cld-2 gene. *L. plantarum* 46a improved the intestinal calcium uptake and absorption through the transcellular pathway involving VDR and TRPV6.

Table 3. Summary of in vitro studies.

Probiotic	Study Design	Mechanism Findings	Ref
L. casei, L. kefiranofaciens, L. plantarum, L. fermentum, L. helveticus and L. delbrueckii	Six *Lactobacillus* strains from different species were selected, and their effect on intestinal calcium uptake and transport was investigated using Caco-2.	The *L. plantarum* strain modulates the transcellular pathway by regulating the expression of vitamin D receptor and calcium transporter. In contrast, the *L. delbrueckii* strain acts on the paracellular pathway by modulating claudin-2 expression.	[46]
Isoflavones	**Study Design**	**Mechanism Findings**	**Ref**
Genistein	Isoflavone aglycone was tested proliferation activity to osteoblast cell as in vitro	Genistein obtained as fermentation process of soybean by *Lactobacillus bulgaricus* is active to osteoblast cell proliferation.	[47]
Daidzein	The study examined the role of daidzein in the proliferation of OCT1 cells by the assay of XTT.	Daidzein enhanced the phosphorylated protein level of Smad1/5/8 and protein expression of Osterix (Osx, a direct target gene of BMP signaling) and increased BMP signaling activity reporter (12xSBE-OC-Luc).	[48]
Daidzein	The effects of daidzein compared with 17β-estradiol on proliferation, differentiation, and cisplatin-induced apoptosis in human osteoblast-like MG-63 cells containing 2 ER isoforms.	Daidzein promoted cell viability, enhanced ALP activity, collagen type 1 levels, and protected against cisplatin-induced apoptosis in human osteoblast-like MG-63 cells.	[49]
Daidzein	The study investigated the effects of daidzein, raloxifene, and E2 on expression of the osteoblast-produced bone regulatory factors OPG, RANKL, and IL-6 in human osteoblastic MG-63 cells.	Daidzein promoted the classic estrogen response element (ERE) pathway through increasing ERα, ERβ, and steroid hormone receptor coactivator (SRC)-1 expression.	[50]
Genistein and menaquinone-4 (MK-4)	The study evaluated the effects of genistein and MK-4 at obtainable dietary concentrations on the level of mRNAs and their protein products in MC3T3-E1 cells derived from neonatal mouse calvaria.	Genistein and/or MK-4 treatments increased BGLAP, indicating that this promoted an osteoblastic phenotype in the MC3T3-E1 cells.	[51]

In another in vitro study on isoflavones, Fawwaz and colleagues [47] reported that soybean aglycone isoflavones were extracted by *L. bacillus* to test the effects on osteoblast cell proliferation in vitro, using a control, calcitonin, and natrium fluoride (NaF) as a positive control in this assay. The study results showed that NaF and calcitonin have 100% cell viability. The percentage of cell viability was taken to indicate the grade of cell proliferation; the greater the increase in osteoblast cell proliferation, the greater the increase in bone mass.

Daidzein shows promise as a potential antiosteoporosis agent. Hu and colleagues [48] determined the mechanisms underlying daidzein's effects on osteoblast differentiation. The role of daidzein in bone morphogenetic protein (BMP)-2 gene expression was tested in organic cation transporter 1 (OCT1) cells. It was found to upregulate the expression of BMP-2, enhance the phosphorylated protein level of Smad1/5/8 and protein expression of Osterix (Osx, a direct target gene of BMP signaling), increase the activity of BMP signaling reporter (12xSBE-OC-Luc), and stimulate Col I, Runx2, and ALP expression. It could be

concluded that daidzein acts by enabling the BMP-2/Smads pathway to promote osteoblast proliferation and differentiation.

Furthermore, daidzein stimulates osteogenesis through estrogen receptor-dependent signal pathways. An investigation was performed to compare the effects of daidzein and 17β-estradiol on the proliferation, differentiation, and cisplatin-induced apoptosis in human osteoblast-like MG-63 cells containing two estrogen-receptor (OR) isoforms. There were several effects of daidzein, including promoting cell viability, enhancing ALP activity and collagen type 1 content, and protecting against cisplatin-induced apoptosis in human osteoblast-like MG-63 cells [49]. Similarly, in human osteoblastic MG-63 cells, daidzein improved the protein and mRNA expression levels of osteoprotegerin (OPG) and simultaneously decreased the receptor activator of the nuclear factor-κB ligand (RANKL) and interleukin-6 (IL-6). Moreover, daidzein promoted the activation of the classic estrogen response element (ERE) pathway by increasing the expression of ERα, ERβ, and steroid hormone receptor coactivator (SRC)-1 [50]. Besides daidzein, Katsuyama and colleagues [51] reported a study that determined the beneficial effects of genistein or menaquinone-4 (MK-4) on osteoblastic MC3T3-E1 cell functions. They showed that GATA6, NOTCH2, and WNT5A were associated with osteoclast function. At the same time, the alterations in osteoblast function, BGLAP, and CHAD were increased in each treatment group at 48 h.

3.2. Animal Studies

Table 4 summarizes the experimental data that were collected from animal studies. Numerous animal studies have reported an altered calcium status and bone health following nourishment with probiotic and isoflavone ingredients. Parvaneh and colleagues [52] fed 24 10-week-old female mature Sprague-Dawley rats that were randomly grouped into a sham group, an ovariectomized group (OVX), and an OVX group that was supplemented with 1 mL of *B. longum* 10^8–10^9 CFU/mL. *B. longum* was given once daily for 16 weeks, starting from two weeks after the surgery. The effects of *B. longum* on bone mass density (BMD), bone mineral content (BMC), bone remodeling, bone structure, and gene expression in OVX rats were examined. The study concluded that *B. longum* increased the bone formation, decreased bone resorption, and altered the femur's microstructure. The femur BMD was increased due to the upregulation of the Sparc and Bmp-2 genes.

Scholz-Ahrens and colleagues [53] tested whether the combination of a probiotic with a defined microbial strain resulted in improved bone mineralization and whether this effect was associated with gut ecology changes. A total of 80 ovariectomized adult rats were grouped into sham-operated group 1 and the ovariectomized groups 2–5. The rats were fed for 16 weeks on semi-purified diets containing 0.7% calcium and 0.5% phosphorus. Groups 1 and 2 received no supplements, group 3 was supplemented with a potential probiotic (*L. acidophilus* NCC90), group 4 was given prebiotics (oligofructose + acacia gum), and group 5 was given synbiotics (probiotics + prebiotics). The study demonstrated that the bone mineral loss following an ovariectomy was significantly prevented mainly by combining the specific prebiotic (oligofructose + acacia gum) with *L. acidophilus* NCC90.

Yang and colleagues [54] tested the beneficial effects of two novel *Lactobacilli* strain probiotics on bone health in ovariectomized induced osteoporotic mice and investigated its underlying mechanisms. A total of 45 9-week-old mice were grouped into a sham-operation (n = 9) or OVX (n = 36) groups. After four days post-operation, one group was treated with CMC (the control group), one was treated with alendronate at 2.5 mg/kg (the positive control), and the remaining two groups were orally treated with *Lactobacillus plantarum* GKM3 and *Lactobacillus paracasei* GKS6, both at a dose of 20.5 mg/kg. This study measured osteoporotic parameters by measuring the bone volume/tissue volume ratio, trabecular thickness, trabecular number, trabecular separation, and bone mineral density. The results showed that both of the probiotic strains inhibited bone loss, with GKS6 outperforming GKM3. Furthermore, GKS6 and GKM3 encouraged osteoblast differentiation via bone morphogenetic proteins (BMP) and inhibited RANKL-induced osteoclast differentiation through RANKL pathways. These findings demonstrated that *Lactobacilli* strains are poten-

tial candidates for treating and managing osteoporosis, particularly in postmenopausal osteoporosis.

Table 4. Summary of animal studies.

Probiotic	Study Design	Mechanism Findings	Ref
Bifidobacterium longum	The rats were randomly assigned into three groups (sham, OVX, and an OVX group supplemented with 1 mL of *B. longum* 10^8–10^9 CFU/mL). *B. longum* was given once daily for 16 weeks, starting two weeks after surgery.	Femur BMD increased due to the upregulation of Sparc and Bmp-2 genes.	[52]
L. acidophilus NCC90	80 ovariectomized adult rats were allocated to five groups: Group 1: sham-operated; groups 2–5: ovariectomized. Groups 1 and 2 got no supplements. Group 3 was given a potential probiotic (*L. acidophilus* NCC90), group 4 was fed prebiotics (oligofructose + acacia gum), and group 5 was fed synbiotics (probiotics + prebiotics).	Lowering pH has less impact on bone mineralization than the mass of digesta in the gut lumen and the mass of intestinal tissue. The luminal bowel content of the lower gastrointestinal tract is mainly composed of microbes known to release growth factors and to exert trophic effects on the intestine.	[53]
Lactobacillus plantarum GKM3, *Lactobacillus paracasei* GKS6	45 9-week-old mice underwent either a sham-operation (n = 9) or OVX (n = 36). In the four OVX groups, there were four groups (n = 9): the control group was treated with CMC; the positive control group was treated with alendronate at 2.5 mg/kg; the remaining two groups were orally treated with 20.5 mg/kg *L. plantarum* GKM3 and *L. paracasei* GKS6, respectively.	Both GKS6 and GKM3 promoted osteoblast differentiation and inhibited RANKL-induced osteoclast differentiation via bone morphogenetic proteins (BMP) and RANKL pathways, respectively.	[54]
Lactobacillus rhamnosus	Sprague–Dawley model rats with colitis were randomly divided into a control group (n = 25) and an observation group (n = 25). The observation group was treated with probiotics by gastric gavage, while the control group was treated with the same volume of physiological saline. The rats in the observation group underwent an enema with 12.5 g/kg *L. rhamnosus*.	*L. rhamnosus* elevates the level of serum inflammatory cytokines in rats to improve osteoporosis. IL-6 functions primarily in the early stage of osteoclast and stimulates the division and proliferation of osteoclast precursors. TNF-α is a bone absorption promoter, which suppresses bone formation and osteoclast apoptosis.	[55]
Powdered whole grape and probiotics (*Bifidobacterium bifidum*, *B. breve*, *Lactobacillus casei*, *L. plantarum*, and *L. bulgaricus*)	A group (n = 6) of mice was used to provide bones for baseline reference measurements to which age and diet-related changes in 16-month-old mice were compared. The remaining groups (n = 7) were fed one of six diets for six months (to age 16 months): 10% grape powder with sugar corrected to 20%; 20% grape powder; 1% probiotic with sugar corrected to 20%; 10% grape powder + 1% probiotic with sugar corrected to 20%; 20% grape powder + 1% probiotic; and 20% sugar control.	Dietary coenrichment with grape powder and probiotics does not produce a synergistic beneficial bone response in aging mice.	[56]
Hwangryun-haedok-tang (HRT), a Korean traditional herbal medicine fermented using *Lactobacillus curvatus*	Sprague-Dawley female rats (10 weeks old) were randomly divided into a sham-operated group (n = 8) and an OVX group (n = 24). The OVX rats were further assigned to three groups of eight rats each: (1) bilateral OVX administered with saline; (2) bilateral OVX administered 0.3 g/kg of HRT; (3) bilaterally OVX administered 0.3 g/kg of fHRT.	fHRT has inhibitory activity on RANKL-induced osteoclastogenesis by suppressing NFATc1 expression, resulting in an improvement of BMD and bone parameter in OVX rats.	[57]

Table 4. *Cont.*

Isoflavones	Study Design	Mechanism Findings	Ref
Soy isoflavone (ISO) daidzein with Resistant starch (RS)	Eight-week female ddY mice were randomly divided into five groups (n = 7 each): sham-operated; OVX control; OVX fed 0.05% ISO diet; OVX fed 9% RS diet; and OVX fed 0.05% ISO-and 9% RS diet. The supplemented ISO contained the purified ISO conjugates daidzin (55.8%), glycitin (27.3%), genistin (10.3%). and others (1.1%).	ISO and RS suppressed the increase in OVX-induced IL-7R mRNA expression and slightly decreased the expression of CD40L. IL-7R and CD40L play a crucial role in bone resorption stimulated by estrogen deficiency. ISO and combinations altered bone marrow inflammation status, resulting in attenuated bone loss in OVX mice.	[58]
Soy isoflavones (ISOs) and resveratrol (RES)	Eight-week female ddY mice were divided into six groups (n = 6–8 each): normally housed mice, loading mice, hindlimb-unloading (UL) mice fed a control diet, UL mice fed a 0.16% ISO conjugates, UL mice fed a 0.15% RES diet, and UL mice fed a 0.16% ISO and 0.15% RES diet.	ISO and RES prevent the bone resorption caused by hindlimb-unloading through regulating RANKL and OPG mRNA expression in bone marrow cells.	[59]
Cladrin and formononetin	Daily oral administration of each of these compounds at 10.0 mg/kg/day dose to recently weaned female Sprague-Dawley rats for 30 consecutive days increased bone mineral density at various anatomic positions studied.	Cladrin stimulated osteoblast proliferation and differentiation by activating the MEK-Erk pathway, while formononetin exerted its differentiation-promoting action by activating the p38 MAPK pathway.	[60]
Daidzein and equol	Female Sprague-Dawley rats, aged three weeks, were divided into four groups (n = 8 per group), orally administered corn oil, 8 mg/day of daidzein, 4 mg/day of equol, or 8 mg/day of equol in corn oil for four weeks.	Equol stimulates endocortical apposition as well as estradiol during the growth period.	[61]
Genistein and daidzein	30 healthy cyclic female Wistar rats were performed, where ten females were sham-operated, and twenty females were subjected to ovariectomy. The ovariectomized female rats were then randomly divided into two groups: the control group was fed a casein-based diet and the second was fed a high soy isoflavone diet. Both groups were compared to a sham-operated group fed a casein-based diet.	Ovariectomy accelerates bone turnover, which manifests as increased ionized Ca^{2+} and phosphorous levels, while decreased alkaline phosphatase activity denotes osteoblast activity. Elevated alkaline phosphatase activity may indicate active bone formation, as it is a byproduct of osteoblast activity.	[62]
Genistein	After eight weeks, the sham and OVX mice were administered genistein (5 mg/kg body weight in 200 µL polyethylene glycol) via gavage.	Gut microbiota converts daidzein and genistein to equol. Most of the circulating equol is in the form of glucuronidated or sulfated conjugates, which exert estrogen agonist activity.	[63]

Zhong and colleagues [55] tested the relationship between changes in intestinal flora and osteoporosis in rats with inflammatory bowel disease and the improvement effect of probiotics. A total of 100 Sprague-Dawley rats were randomly divided into two groups: a bowel disease group and an osteoporosis group (ovaries on both sides of the abdominal incision of all rats were removed), with 50 rats in each group. The osteoporosis group rats were randomly grouped into the control group (n = 25) and the experimental group (n = 25). The rats in the observation group underwent an enema with 12.5 g/kg *Lactobacillus rhamnosus*. The results showed that the serum values of OPG, PICP, TRACP, and Ca in the experimental group were higher than those in the control group. However, the serum

values of RANKL, bone-specific alkaline phosphatase (BALP), IL-6, TNF-α, and INF-γ in the experimental group were lower than those in the control group. The authors concluded that the probiotics increased the value of serum inflammatory cytokines in rats; the activity of IL-6 and TNF-α may cause this with IL-6 stimulating the osteoclast precursors and TNF-α suppresses bone formation and osteoclast apoptosis. Whereas the occurrence of osteoporosis in rats with inflammatory bowel disease was positively related to the counts of *Lactobacillus* and *Bifidobacteria*. This study concluded that probiotics improved inflammatory bowel disease symptoms in rats with osteoporosis by influencing the level of the corresponding cytokines.

However, a study found that a combination of probiotics and polyphenol did not affect osteoporosis. Blanton [56] tested dietary enrichment with powdered whole grape and probiotics (composed of equal parts *Bifidobacterium bifidum*, *B. breve*, *Lactobacillus casei*, *L. plantarum*, and *L. bulgaricus*) on bone microarchitecture in a mouse model of age-related osteoporosis. Male mice that were ten months-old were grouped (n = 7 each) and fed one of six diets for six months: 10% grape powder with sugar corrected to 20%; 20% grape powder; 1% probiotic with sugar corrected to 20%; 10% grape powder + 1% probiotic with sugar corrected to 20%; 20% grape powder + 1% probiotic; and a 20% sugar control. The results showed that merging probiotics with 10% and 20% of grape diets exerted no effect on the bone microarchitecture measures, unlike independent probiotic and grape dietary enrichment. The authors concluded that there was no increased benefit to the bone by using the combined supplementation instead of the independent supplementation with probiotics or whole grape powder.

Unlike the above approaches, combining fermentation probiotics and the bioactive components of flavonoids showed a positive result in treating postmenopausal osteoporosis. Shim and colleagues [57] evaluated the effect of Hwangryun-haedok-tang (HRT) and its fermented product (fHRT), that was fermented by *Lactobacillus curvatus* KFRI-166, on postmenopausal bone loss using an ovariectomy rat model. The hormone replacement therapy contained 250 g *Coptis japonica Makino*, 250 g *Scutellaria baicalensis Georgi*, 250 g *Phellodendron chinense Schneider*, and 250 g *Gardenia jasminoides fructus*. Female Sprague-Dawley rats that were ten weeks-old were randomly grouped into sham-operated (sham, n = 8) and surgically ovariectomized (OVX, n = 24) groups. One week after surgery, the OVX rats were randomly assigned to an OVX that was administered with saline group 1; an OVX that was administered with 0.3 g/kg HRT group 2; and an OVX that was administered with 0.3 g/kg fHRT group 3. Inhibitory activity on RANKL-induced osteoclastogenesis by suppressing NFATc1 expression was found in the fHRT group, indicating an improvement in the BMD and bone parameters in the OVX rats. It was concluded that the administration of fHRT significantly slowed the decline of the bone mineral density and improved the femur bone parameters more than in the case of HRT and the bone parameter in OVX rats.

In another in vivo study on isoflavones, Tousen and colleagues [58] tested the combined effects of a diet that was supplemented with soy isoflavone (ISO) and resistant starch (RS) on intestinal microbiota, equol production, bone mineral density (BMD), and inflammatory gene expression in the bone marrow of ovariectomized (OVX) mice. Female ddY strain mice that were eight weeks-old were either sham-operated (n = 7) or underwent OVX on the same day. The OVX mice were placed into the following groups: (n = 7 each): OVX control (group 1); OVX fed 0.05% ISO-supplemented diet (group 2); OVX fed 9% RS-supplemented diet (group 3); and OVX fed 0.05% ISO-and 9% RS-supplemented diet (group 4). IL-7R and CD40L played a key role in bone resorption stimulated by estrogen deficiency. In the OVX mice, two weeks of diet supplementation with equol prevented an OVX-induced increase in IL-7R and CD40L. The authors concluded that ISO or its combination with RS supplement improved the bone marrow inflammation status, resulting in decreased bone loss in the OVX mice.

Furthermore, Tousen and colleagues [59] tested the combined effects of soy isoflavones (ISOs) and resveratrol (RES) on bone loss that was induced by hindlimb-unloading in mice. Female mice (ddY strain, 8 weeks) were randomly divided into six body weight-matched

groups: a normally housed group (n = 6), a loading group (n = 6), a hindlimb-unloading group of mice that were fed a control diet (n = 6), aa hindlimb-unloading group of mice that were fed a 0.16% ISO conjugate diet (n = 8), a hindlimb-unloading group of mice that were fed a 0.15% RES diet (n = 8), and a hindlimb-unloading group of mice that were fed a 0.16% ISO conjugate and RES diet (n = 8). The ISO and RES treatment decreased the RANKL/OPG gene expression ratio in bone marrow cells in unloading mice. It also prevented the bone resorption that was caused by hindlimb-unloading by regulating RANKL and OPG mRNA expression in bone marrow cells.

Gautam and colleagues [60] investigated the effects of formononetin and cladrin (two structurally related methoxydaidzeins that are found in soy food and other natural sources) in osteoblast functions in bone formation in vivo. A total of 21 day-old immature female Sprague–Dawley rats were treated with 10.0 mg kg−1 body weight doses of an individual compound or vehicle (gum acacia in distilled water) once daily for 30 consecutive days by oral gavage. Each animal received an intraperitoneal injection of fluorochrome tetracycline (20 mg/kg body weight dose) and calcein (20 mg kg−1 body weight dose) on days 15 and 28 of treatment. This study analyzed the osteoblast proliferation, differentiation, and mineralization of bone marrow osteoprogenitor cells. The findings demonstrated that rats that were treated with cladrin had increased bone formation rates with the cladrin treatment but not in the control. Cladrin had much better plasma bioavailability than formononetin.

Tousen and colleagues [61] examined the effects of orally administered daidzein or equol on bone formation and bone mineral density in growing female rats. Female Sprague-Dawley rats, aged three weeks, were given 0.2mL of corn oil (the control group), 8 mg/day of daidzein, 4 mg/day of equol, or 8 mg/day of equol in a corn oil suspension (n = 8 per group). The results showed that daidzein and equol improved BMD in growing female rats by stimulating bone formation without showing a substantial effect on the weight of the reproductive organs. The bone growth was caused by increasing the mineralizing surface/bone surface ratio, and the bone formation rate in the equol group was approximately twice that of the rates that were observed in the daidzein group rats.

Abdelrazek and colleagues [62] tested the effects of soy isoflavones as hormone replacement therapy (HRT) on immunological and bone health. A total of 30 healthy cyclic female Wistar rats were grouped into a sham-operated group (n = 10) and an ovariectomy group (n = 20). The ovariectomized (OVX) female rats were randomly grouped into a control group that were fed a casein-based diet and the second group that were fed a high soy isoflavone diet (genistein and daidzein). Both of the groups were compared to a sham-operated group. Ovariectomy accelerated bone turnover, as manifested by increased ionized Ca^{2+} and phosphorous levels; decreased alkaline phosphatase activity would denote decrease osteoblast activity. Reduced calcitonin hormone levels also accompanied these changes. Elevated ALP activity may indicate active bone formation, as it is a byproduct of osteoblast activity. Hence, this study concluded that supplementing with soy isoflavones improved bone mineralization via the calcitonin hormone and improved lipid profile, and subsequently, the antioxidant reserve exerted an anti-inflammatory effect.

Lee and colleagues [63] investigated the effects of ovariectomy on the nutrikinetics of genistein metabolites. After eight weeks, female sham-operated and OVX mice (nine weeks old) were administered genistein (5 mg/kg body weight in 200 µL polyethylene glycol) via gavage. They found that the gut microbiota plays a significant role in the metabolism, bioavailability, and bioactivity of dietary compounds. The gut microbiota converts daidzein and genistein to equol.

3.3. Human Studies

Table 5 summarizes the experimental data that was collected from human studies. The results of several studies suggest that probiotics and isoflavones may have favorable effects on the calcium status and bone health for the treatment and prevention of osteoporosis in postmenopausal women. The study of Jafarnejad and colleagues [64] tested the effects of a

multispecies probiotic supplementation on bone biomarkers and bone density in osteopenic postmenopausal women. This randomized, double-blind, placebo-controlled, clinical trial was performed on 50 patients with osteopenia aged 50–72. The participants were randomly grouped into a multispecies probiotic supplement group (GeriLact; n = 25) and a placebo group (n = 25) for six months. The GeriLact supplement contained seven probiotic bacteria species (*Lactobacillus casei, Bifidobacterium longum, Lactobacillus acidophilus, Lactobacillus rhamnosus, Lactobacillus bulgaricus, Bifidobacterium breve, and Streptococcus thermophilus*). The participants received 500 mg Ca plus 200 IU vitamin D daily. Decreases in BALP and collagen type-1 cross-linked C-telopeptide (CTX) levels and in serum parathyroid hormone (PTH) and tumor necrosis factor (TNF)-α were found in the intervention group but not in the placebo group.

Table 5. Summary of human studies.

Probiotics	Study Design	Mechanism Findings	Ref
7 bacteria species (*Lactobacillus casei*, *Bifidobacterium longum*, *Lactobacillus acidophilus*, *Lactobacillus rhamnosus*, *Lactobacillus bulgaricus*, *Bifidobacterium breve*, and *Streptococcus thermophilus)*	This randomized, double-blind placebo-controlled clinical trial was performed on 50 patients with osteopenia aged 50–72. Participants were randomly assigned to take either a multispecies probiotic supplement (GeriLact; n D 25) or placebo (n D 25) for six months.	Various strains of probiotics on bone may produce several short-chain fatty acids, which decrease PTH, followed by an increase in mineral absorption by solubilization. Probiotic administration reduces the expression of several proinflammatory and osteolytic cytokines (TNF-a and IL-1b).	[64]
Lactobacillus paracasei DSM 13434, *Lactobacillus plantarum* DSM 15312, and *Lactobacillus plantarum* DSM 15313	Early postmenopausal women were randomized to receive three *Lactobacillus* strains (1×10^{10} CFU/capsule) or placebo once daily for 12 months.	The bone protective effect of probiotics reduces gut permeability, increases short-chain fatty acids, reduces inflammation in the gut, reduces levels of proinflammatory cytokines in bone, and decreases osteoclastic bone resorption.	[65]
Lactobacillus reuteri ATCCPTA 6475	In this double-blind, placebo-controlled study, women aged 75 to 80 with low BMD were randomized to orally receive 10^{10} colony-forming units of *L. reuteri* 6475 daily or placebo. The predefined primary end-point was a relative change after 12 months in tibia total volumetric BMD (vBMD).	*L. reuteri* 6475 for 12 months reduced loss of tibia total vBMD in older women with low BMD. The underlying mechanism for this has not been elucidated, and further studies are needed to evaluate this strain supplementation's clinical usefulness.	[66]
Bacillus subtilis C-3102	76 healthy postmenopausal Japanese women were treated with a placebo or probiotic *B. subtilis* C-3102 spore-containing tablets for 24 weeks.	C-3102 improves BMD by inhibiting bone resorption and modulating gut microbiota in healthy postmenopausal women.	[67]
Isoflavones	**Study Design**	**Mechanism Findings**	**Ref**
Red clover extract (RCE) rich in isoflavone aglycones and probiotics (lactic acid bacteria)	A 12-month, double-blind, parallel design, placebo-controlled, randomized controlled trial of 78 postmenopausal osteopenic women supplemented with calcium (1200 mg/d), magnesium (550 mg/d), and calcitriol (25 mg/d) given either 60 mg isoflavone aglycones/d and probiotics (RCE) or a masked placebo (CON)	Twice-daily RCE intake over one year attenuated BMD loss caused by estrogen deficiency, improved bone turnover, promoted a favorable estrogen metabolite profile (2-OH:16a-OH), and stimulated equol production in postmenopausal women with osteopenia.	[68]

Table 5. Cont.

Isoflavones	Study Design	Mechanism Findings	Ref
Daidzein, genistein, and glycitein	99 healthy premenopausal women were randomized to isoflavones (136.6 mg aglycone equivalence) and 98 to placebo for five days per week for up to two years. BMD, serum calcium and urinary excretion of daidzein and genistein were measured before and during treatment.	Isoflavone exposure interacted with serum calcium in affecting whole-body BMD, but not hip and spine BMD.	[69]
Genistein, daidzein, and glycitein	A double-blind, randomized controlled trial in healthy postmenopausal women (46–63 yr) were studied. There were two soy isoflavone doses (80 or 120 mg/d) vs. placebo tablets on volumetric bone mineral density and strength (using peripheral quantitative computed tomography)	Soy isoflavone exerted a modest beneficial effect on the percentage change in the midshaft femur vBMD as TLMP increased and a modest beneficial effect on the midshaft femur SSI as bone turnover (reflected by serum BAP) increased.	[70]
Soy isoflavone, calcium, and soy isoflavone combined with calcium	160 women with osteoporosis or osteopenia were enrolled and randomized into four groups, namely control, soy isoflavone, calcium, and soy isoflavone combined with calcium.	Isoflavone combined with calcium increases estradiol level and reduces osteocalcin level, while increasing plasma calcium concentration.	[71]
Daidzein and genistein and green kiwifruit	33 healthy postmenopausal Caucasian women were randomly allocated to two groups: Group A received isoflavones for the first six weeks, followed by isoflavones and kiwifruit for the following six weeks. Group B had the same intervention sequence in reverse. Isoflavone capsules and kiwifruit were taken in the morning with breakfast.	Osteocalcin (OC) is a vitamin K-dependent protein produced by the osteoblasts and is the primary noncollagenous protein in bone. Vitamin K acts as an essential cofactor for the enzymatic carboxylation of OC's glutamyl side chains.	[72]

Jansson and colleagues [65] investigated how the combination of three bacterial strains protects against rapid spine bone loss in healthy early postmenopausal women. A total of 249 participants were randomly assigned in a 1:1 ratio to receive probiotic treatment consisting of three Lactobacillus strains (*Lactobacillus paracasei* DSM 13434, *Lactobacillus plantarum* DSM 15312, and *Lactobacillus plantarum* DSM 15313; 1×10^{10} CFU/capsule) or placebo once daily for 12 months. This study revealed that the loss of lumbar spine bone mineral density decreased in the *Lactobacillus*-treated group. It can be concluded that the probiotic treatment using a mix of three *Lactobacillus* strains protects against lumbar spine bone loss in healthy postmenopausal women.

Nilsson and colleagues [66] examined the effects of daily supplementation with *Lactobacillus reuteri* 6475 in bone loss in older women with low bone mineral density (BMD). A total of 90 subjects were enrolled and randomized to orally receive 10^{10} CFU of *L. reuteri* 6475 daily or placebo. In this randomized, placebo-controlled, double-blind, clinical trial, supplementation with *L. reuteri* 6475 for 12 months resulted in reduced bone loss in older women with low bone density. The experiment found that the daily supplementation with *L. reuteri* 6475 for 12 months reduced tibia total volumetric bone mineral density (vBMD) in older women with low BMD. It demonstrated that supplementation intake of *Lactobacillus* strain reserved bone mineral density in older women.

Takimoto and colleagues [67] examined the effect of the probiotic *Bacillus subtilis* C-3102 on BMD and gut microbiota in healthy postmenopausal Japanese women. A total of 76 participants were assigned an individual trial identification number and randomly allocated into two groups that were treated with placebo or C-3102 spore-containing tablets for 24 weeks. The experiment results showed that total hip BMD was enhanced in the C-3102 group compared with the placebo group. *Bifidobacterium* also increased in the C-3102 group compared with the baseline, and *Fusobacterium* decreased in the C-3102 group compared with the baseline. This study illustrated that probiotics improved BMD and modulated host-gut microbiota.

In another clinical trial with isoflavones, Lambert and colleagues [68] tested the beneficial effects of a bioavailable isoflavone and probiotic lactic acid bacteria treatment against postmenopausal osteopenia. This study was a 12-month parallel-design, placebo-controlled, double-blind, randomized controlled trial. A total of 78 postmenopausal osteopenic women that were supplemented with calcium (1200 mg/d), magnesium (550 mg/d), and calcitriol (25 mg/d) were given either red clover extract (RCE) (60 mg isoflavone aglycones/d and probiotics) or a masked placebo (control). The results demonstrated that estrogen deficiency attenuated BMD loss, improved bone turnover, and promoted a favorable estrogen metabolite profile (2-OH:16a-OH). Potent stimulation of equol was found in these women that were taking RCE twice-daily over one year.

Nayeem and colleagues [69] investigated how soy isoflavones affect bone mineral density (BMD). A total of 99 healthy premenopausal women were randomized to isoflavones (136.6 mg aglycone equivalence) and 98 to placebo for five days per week for up to two years. BMD and serum calcium of daidzein and genistein were measured before and during treatment. The result showed that daidzein had a similar but marginal effect, and genistein significantly decreased whole-body BMD at low normal serum calcium levels but increased whole-body BMD at higher serum calcium levels. This study showed that there was an interaction between the isoflavones and serum calcium on whole-body BMD changes.

Shedd-Wise and colleagues [70] examined the three-year effects of soy isoflavones on BMD and strength in postmenopausal women. A double-blind, randomized controlled trial in 224 eligible women examined the effects of two soy isoflavone doses (80 or 120 mg/d) or placebo tablets on vBMD and strength (using peripheral quantitative computed tomography) in healthy postmenopausal women (age 46–63). The study found that isoflavone supplements' beneficial effects on the percentage change in the midshaft femur vBMD and on the midshaft femur strength-strain index improved for three years.

Zhang and colleagues [71] tested the effect of soy isoflavones that were combined with calcium on bone mineral density in perimenopausal Chinese women. A total of 160 perimenopausal women with osteoporosis or osteopenia were enrolled and randomized into four groups receiving control, soy isoflavone, calcium, or soy isoflavone that was combined with calcium. The isoflavone that was combined with calcium was found to decrease osteocalcin, luteinizing hormone (LH), follicle stimulating hormone (FSH), and malondialdehyde (MDA) levels, while increasing glutathione peroxidase (GSH) activity and serum calcium and vitamin D levels, as compared with the control, the isoflavone, and calcium groups. The authors concluded that isoflavone that was combined with calcium was effective and safe in attenuating BMD loss in perimenopausal women was better than soy isoflavone and calcium alone.

Kruger and colleagues [72] investigated the effects of green kiwifruit combined with isoflavones on equol production, bone turnover, and gut microflora in healthy postmenopausal women. Healthy women one to ten years after menopause were randomly allocated to group A (n = 16) or B (n = 17) for a 16-week crossover trial. A two-week lead-in period initiated the diet and was followed by two six-week interventions, with a two-week washout in between. Group A was fed isoflavones for the first six weeks, followed by isoflavones and kiwifruit for the following six weeks. In comparison, group B had the same intervention sequence in reverse. The participants received 50 mg isoflavones daily from

an oral supplement containing daidzein and genistein. The results showed that kiwifruit supplementation did not alter the gut microbiota profile (*Bifidobacterium*, *Lactobacillales*, *Bacteroides*, *Prevotella*, and *Clostridium*). The absence of an effect on gut microbiota may be due to the interventions being of short duration. Isoflavone supplementation alone significantly increased serum undercarboxylated osteocalcin (ucOC), whereas isoflavone supplementation with kiwifruit had a beneficial effect by significantly decreasing serum ucOC.

4. Discussions

This literature review has examined a broad spectrum of evidence from studies of various designs to determine if probiotics and isoflavones affect calcium status and bone health. The demonstrated beneficial mechanism of probiotics and isoflavones in bone is shown in Figure 2. This figure illustrates the crosstalk mechanism of probiotics and isoflavones in the gut microbiota and calcium absorption.

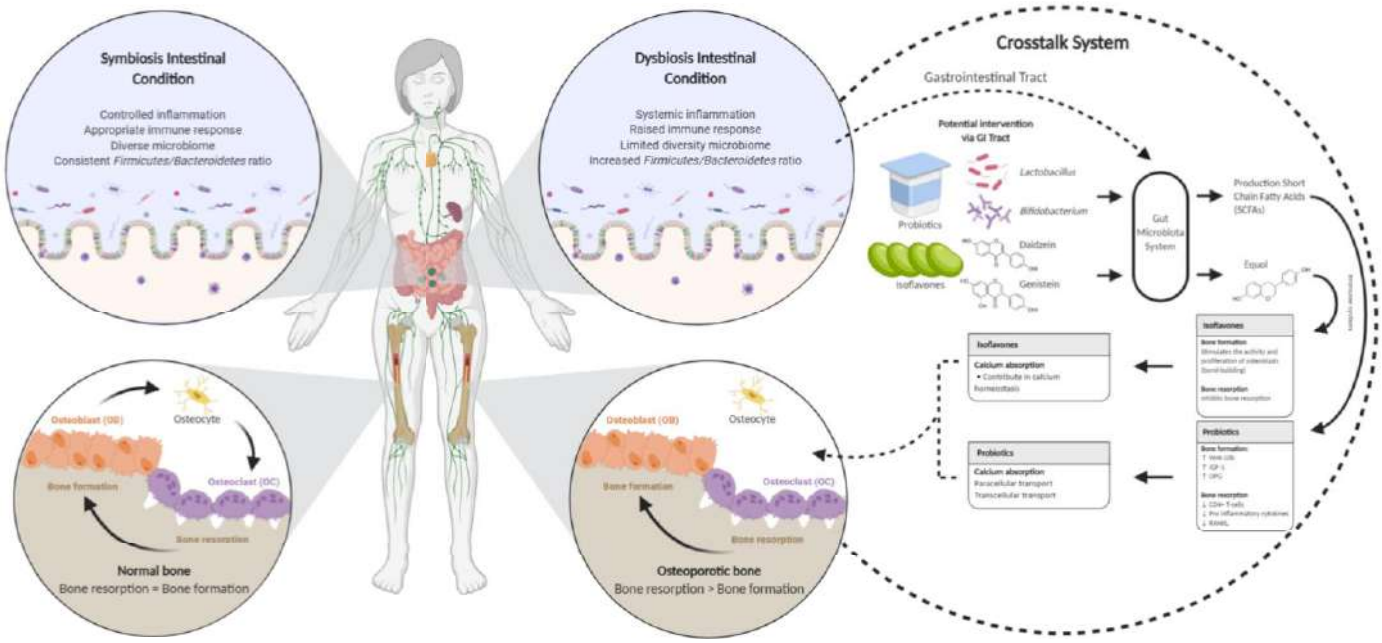

Figure 2. The crosstalk system mechanism of probiotics and isoflavones that benefits bone. An unhealthy balance in the microbiota community composition is linked to various metabolic, inflammatory, and immunologic diseases [32], and vice versa. The imbalance of the gut microbiome can cause the imbalance of osteogenesis and osteoclast reaction [33]. The gastrointestinal (GI) tract system contributes to absorbing bone mineralization [40]. The mechanism of action between probiotics and isoflavones regulates the GI tract system and calcium homeostasis. In the GI tract system, probiotics increase short-chain fatty acids [65] and elevate the immune system [55] to regulate the function of bone formation by increasing the level of Wnt Family Member 10B (Wnt10b), insulin-like growth factor 1 (IGF-1), and osteoprotegerin (OPG) [15] and lead the function of bone resorption by decreasing the level of CD4+ T cells, pro-inflammatory cytokines, and receptor activator of nuclear factor-κB ligand (RANKL) [15,54,55]. Furthermore, gut microbiota converts isoflavones (daidzein and genistein) to equol in the GI tract [63]. Isoflavones stimulate the activity and proliferation of osteoblasts [47] and inhibit bone resorption [26]. Isoflavones prevent bone resorption [58,59]. Isoflavones contribute to calcium homeostasis [70], and probiotic modulates the transcellular and paracellular pathways [46]. ↑: increase; ↓: decrease.

In this review, the evidence on calcium status from in vitro studies suggests that the *L. plantarum* strain regulated the expression of vitamin D receptor and calcium transporter and modulated the transcellular pathway, while *L. delbrueckii* modulated claudin-2 expression on the paracellular pathway [47]. In human studies, micronutrients such as soy isoflavones represent a new class of compounds that can participate in calcium homeostasis by mobilizing calcium from bone into circulation [70]. Calcium absorption mostly occurs

in the small intestine through paracellular and intracellular pathways. These calcium transport systems rely on the steroid hormone 1,25 dihydroxyvitamin D, and its interaction with the nuclear vitamin D receptor (VDR). The primary controlling hormone of intestinal Ca^{2+} transport is calcitriol [1,25(OH)2D3], which increases both pathways' gene and protein expression [26–29].

Table 6 demonstrates the number of probiotics and isoflavones studies in previous experiments in vitro, animal, and human interventions to affect calcium status and bone health. The doses were spread out from 10^6 CFU to 10^{10} CFU for probiotics and from 10 mg/kg to 4000 mg/kg for isoflavones. The results that were gathered in the current review established confirm the favorable impact of dietary consumption of probiotics and isoflavones on calcium absorption and bone health. It also showed that probiotics [73] and isoflavones [74] are safe to consume for humans, based on their safety profile according to general toxicity assessments.

Table 6. Dosage intervention of probiotics and isoflavones in in vitro, animal, and human studies.

Study Design	Intervention	Dosage	Unit	Reference
Probiotics				
In vitro	*L. casei*, *L. kefiranofaciens*, *L. plantarum*, *L. fermentum*, *L. helveticus and* *L. delbrueckii*	10^7	CFU/mL	[46]
Animal	*B. longum*	10^8–10^9	CFU/mL	[52]
	L. acidophilus NCC90	1–5 × 10^6	CFU	[53]
	L. plantarum GKM3 and *L. paracasei* GKS6	2 × 10^{11}	CFU/g	[54]
	L. rhamnosus	12.5	g/kg	[55]
	B. bifidum, *B. breve*, *L. casei*, *L. plantarum*, and *L. bulgaricus*	10^{11}	CFU/g	[56]
	L. curvatus KFRI-166	0.3	g/kg	[57]
Human	*L. casei* 1.3 × 10^{10} CFU, *B. longum* 5 × 10^{10} CFU, *L. acidophilus* 1.5 × 10^{10} CFU, *L. rhamnosus* 3.5 × 10^9 CFU, *L. bulgaricus* 2.5 × 10^8 CFU, *B. breve* 1 × 10^{10} CFU, and *S. thermophilus* 1.5 × 10^8 CFU	500	mg	[64]
	L. paracasei DSM 13434, *L. plantarum* DSM 15312, and *L. plantarum* DSM 15313	1 × 10^{10}	CFU	[65]
	L. reuteri ATCCPTA 6475	1 × 10^{10}	CFU	[66]
	Bacillus subtilis C-3102	3.4 × 10^9	CFU	[67]
Isoflavones				
In vitro	Daidzein	0.001; 0.005; 0.01; 0.03; 0.06	mg	[48]
	Daidzein	0.01; 0.1; 1; 10	µmol/L	[49]
	Daidzein	0.01; 0.1; 1	µM	[50]
	Genistein	1	µM	[51]

Table 6. *Cont.*

Study Design	Intervention	Dosage	Unit	Reference
Animal	Daidzin (55.8%); glycitin (27.3%); genistin (10.3%) and others (1.1%).	530	mg/kg	[58]
	Daidzein (33 mg); genistein (8.5 mg); and glycitein (15 mg)	4000	mg/kg	[59]
	Cladrin and formononetin	10	mg/kg	[60]
	Daidzein	8	mg	[61]
	Genistein; daidzein	1500; 800	mg/kg	[62]
	Genistein	5	mg/kg	[63]
Human	Red clover extract (RCE) rich in isoflavone aglycones and probiotics (lactic acid bacteria)	60	mg	[68]
	Daidzein; genistein; glycitein	30; 30; 8.3	mg	[69]
	Genistein:daidzein:glycitein (1.3:1:0.3)	80 and 120	mg	[70]
	Soy isoflavone; calcium	15; 125	mg	[71]
	Daidzein and genistein	50	mg	[72]

Furthermore, in terms of bone health, the gut microbiota involves millions of bacteria and can be modified by numerous environmental factors, including diet. Numerous studies have established the bacteria in the human gut microbiota, including *Ruminococcaceae*, *Faecalibaculum*, *Lachnospiraceae*, and *Bacteroides*, as well as in bacterial phyla (e.g., *Actinobacteria*) and genera (e.g., *Lactobacillus* and *Bifidobacterium*) [45]. Ma and colleagues [69] reported that the identification of Firmicutes and Bacteroidetes is the gut microbiota's main phylum. An increased *Firmicutes*/*Bacteroidetes* ratio after an ovariectomy can be identified as a useful biomarker for osteoporosis. Probiotics can alter the gut's microbiota alignment and improve the solubility and absorption of minerals, leading to the immune system's modulation. An example of a system that is modulated by probiotics is the process of bone re-modelling [53]. Rodent mechanistic experiments have investigated whether probiotics can reduce gut permeability, while increasing levels of short-chain fatty acids, reducing inflammation in the gut, reducing proinflammatory levels cytokines in bone, and reducing osteoclastic bone resorption [66]. Isoflavones are metabolized to equol by the gut microbiota in the intestinal tract [23]. Subsequently, equol increases bone mineral density by stimulating bone formation [69].

Overall, the evidence from human studies suggests that short-term or long-term probiotic and isoflavone treatment on bone health are minor compared with the effects of osteoporosis treatment with bisphosphonates [64,70] This evidence from in vitro, animal, and human studies generally suggests that probiotics and isoflavones can serve as a promising treatment or adjunct therapy for bone health issues or osteoporosis.

Limitations

This study's strength includes the updated scientific articles that considered probiotics and isoflavones supplementation within the last 10 years. It represents the beneficial effects of probiotics and isoflavones metabolism on calcium levels, bone health, and osteoporosis. We considered it essential to study the effects of probiotics and isoflavones because their dietary exposure is usually life-long. On the other hand, weaknesses of this review include pre-clinical data and observational studies in humans, such as risk factors, pathogenesis, and medications of bone health management, which would require a more comprehensive study. We did not explain the adverse event of probiotics and isoflavones as therapeutic management of bone health, whose effects would be anticipated to show the toxicity levels.

5. Conclusions and Future Trends

Both the in vitro and in vivo studies show that the probiotics that positively affect bone health are *Lactobacillus* and *Bifidobacterium*. The isoflavones that show most bone formation activity are daidzein and genistein, along with their metabolites, such as equol. In the calcium uptake, probiotics regulate calcium either on the transcellular pathway or on the paracellular pathway. Isoflavones participate in calcium homeostasis by mobilizing calcium from bone into circulation. In the gastrointestinal tract, *Lactobacillus* and *Bifidobacteria* improve the imbalance in the microbiota community composition and influence the immune system to regulate bone health. Isoflavones, including their metabolites, increase bone mineral density by stimulating bone formation.

From the authors' viewpoint, future research and development could include several investigation areas that are particularly urgent, such as (1) developing nutritional and medical strategies to determine the prevention activity of probiotics and isoflavones as an epidemiological decision, (2) designing innovative products and diet recommendations of probiotics and isoflavones that correspond to the sensory characteristics, nutritional value, and physicochemical properties of older adults and postmenopausal women, and (3) exploring the natural resources of probiotics and isoflavones is crucial in designing novel dietary consumption to support the sustainable food systems. Finally, future research and industrial investments are vitally important to expand the application of probiotics and isoflavones to benefit food safety and public health.

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Effects of Daidzein, Tempeh, and a Probiotic Digested in an Artificial Gastrointestinal Tract on Calcium Deposition in Human Osteoblast-like Saos-2 Cells

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Abstract: Adequate calcium intake is crucial for the prevention and treatment of bone-related issues. Developing a nutritional source of readily bioavailable calcium is particularly significant for individuals deficient in this essential element and at risk of developing osteoporosis. This research aimed to evaluate the impact of tempeh (T), daidzein (D), and *Lactobacillus acidophilus* (LA) within a simulated intestinal environment consisting of Caco-2 epithelial and Saos-2 cells, focusing on their implications for bone mineralization mechanisms. In the initial phase, calcium bioaccessibility from calcium citrate (CaCt), LA, D, the daidzein combination D–CaCt–LA (D1:1:1), and the tempeh combination T–CaCt–LA (T1:1:1) was assessed through digestion simulation. The calcium content of both untreated and digested samples was determined using atomic absorption spectrometry (AAS). In the subsequent stage, the digested samples were used to induce intestinal absorption in differentiated enterocyte-like Caco-2 cells. The permeable fractions were then evaluated in a culture of osteoblast-like Saos-2 cells. Preliminary cellular experiments employed the MTT assay to assess cytotoxicity. The results indicated that the analyzed products did not influence the deposition of extracellular calcium in Saos-2 cells cultured without mineralization stimulators. The combined formulations of permeable fractions of digested CaCt, LA, D, and T demonstrated the capacity to enhance the proliferation of Saos-2 cells. In Saos-2 cells, D, D1:1:1, and LA showed no discernible impact on intracellular calcium accumulation, whereas T and T1:1:1 reduced the calcium deposits. Additionally, mRNA transcripts and alkaline phosphatase (ALP) activity levels in Saos-2 cells cultured without mineralization induction were unaffected by the analyzed products. An examination of the products revealed no discernible effect on ALP activity or mRNA expression during Saos-2 cell differentiation. Our findings suggest that tempeh, daidzein, and *L. acidophilus* did not positively impact cellular calcium deposition in Saos-2 cells. However, tempeh, daidzein and its combination, and *L. acidophilus* might enhance the process of osteogenic differentiation in Saos-2 cells. Nevertheless, this study did not identify any synergistic impact on calcium deposition and the process of osteogenic differentiation in Saos-2 cells of isoflavones and probiotics.

Keywords: isoflavones; probiotics; tempeh; calcium; Caco-2; Saos-2

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1. Introduction

Insufficient calcium intake significantly contributes to the onset of osteoporosis and an increased vulnerability to fractures [1]. The likelihood of calcium deficiency is notably pronounced in postmenopausal and elderly women, owing to age-related physiological and metabolic changes, along with heightened calcium requirements. These age-associated alterations frequently lead to inadequate calcium intake, compromised absorption, and

altered calcium metabolism, collectively raising the risk of calcium deficiency. The interaction between calcium supply and its bioavailability plays a pivotal role in establishing optimal calcium levels and, consequently, in maintaining bone health [2].

The formation of bone tissue involves two distinct processes: endochondral ossification, characterized by the sequential development of a cartilage template followed by its replacement with bone tissue, and intramembranous ossification, where bone tissue forms directly through the concentration and osteogenic differentiation of mesenchymal stromal cells [3–5]. In intramembranous ossification, osteoblasts derived from mesenchymal stromal cells contribute to bone matrix deposition by generating collagen type I fibrils and regulating the deposition of minerals within the collagenous matrix [3]. Osteoblasts play a crucial role in the mineralization of the collagenous matrix, expressing proteins such as alkaline phosphatase (ALP), which provides the necessary phosphate for the mineralization process [6,7].

Recommendations underscore the importance of increased calcium intake as a preventive and therapeutic measure against bone loss, recognizing the pivotal role of calcium in determining bone health [8,9]. Calcium citrate demonstrates distinctive characteristics in absorption, solubility, bioavailability, tolerability, and compatibility with other substances, depending on factors such as a full stomach, fasting, or reduced gastric secretions. Administering calcium citrate between meals alleviates the competition with other nutrients, reduces the risk of renal calculus formation, and prevents abdominal distension and flatulence caused by carbon dioxide production. The suitability of calcium citrate for single-dose administration enhances the therapy flexibility and improves the potential for therapeutic compliance [10].

Probiotics and isoflavones have the potential to enhance calcium absorption and influence bone metabolism. Isoflavones are known to mobilize calcium from skeletal muscles, while probiotics impact calcium absorption through paracellular and transcellular mechanisms. *Lactobacillus* and *Bifidobacterium* nourish the gut microbiome, contributing to enhanced immunity and improved bone health. The microbial impact fosters the growth of beneficial microorganisms while inhibiting that of harmful ones. Through the promotion of osteoblast activity, limitation of bone resorption, and control of bone remodeling, isoflavones and their metabolites contribute to the improvement of bone mineral density [11].

Furthermore, research indicated that *Lactobacillus acidophilus* possesses osteoprotective properties that contribute to bone health. Ovariectomized mice administered *L. acidophilus* demonstrated improved bone microarchitecture, mineral density, and heterogeneity [12]. Daidzein is present in various soy products, especially in fermented soy like tempeh, where the concentration of isoflavones is notably higher compared to that in unfermented soy [13]. The gut microbiota metabolizes daidzein through fermentation, leading to the production of equol [14]. Equol provides protection against the development of osteoporosis in mice subjected to ovariectomy [15].

Intestinal cell culture models, such as Caco-2 cell lines derived from human colon adenocarcinoma, provide a valuable platform for enterocytic differentiation. These cells demonstrate increased levels of lactase, sucrase, and ALP, affirming their credibility as model systems. Morphological differentiation is marked by distinctive brush border membranes, and the existence of tight junctions signifies cellular polarization. Caco-2 cell monolayers function as effective models for studying the calcium paracellular transport pathway [16].

On the flip side, osteosarcoma, constituting approximately 20% of primary bone sarcomas, stands as the most prevalent malignant bone tumor. Various conventional subtypes, namely, osteoblastic, chondroblastic, and fibroblastic, alongside those with nonconventional morphologies like the telangiectatic and small cell subtypes, display variations in their predominant histologic features [17]. Saos-2 (sarcoma osteogenic), a non-transformed cell line derived from primary osteosarcoma cells with the ability to differentiate, has been employed in experimental studies due to its distinctive osteoblastic characteristics [18,19].

Numerous prior investigations scrutinized the bioavailability of calcium using in vitro cell models [20–23]. Concerns regarding the beneficial impacts of probiotics and isoflavones on calcium bioavailability have emerged as a rapidly expanding area of study. The inclusion of Saos-2 cells, as an osteoblast-like model, complements the use of Caco-2 cells, which simulate the absorption phase in an enterocyte-like fashion. Our primary objective was to investigate the potential interplay between gastrointestinal digestion products and bone metabolism. While Caco-2 cells simulate the absorptive aspect of the gastrointestinal process, Saos-2 cells offer insights into any downstream effects on bone cells. The rationale for employing Saos-2 cells was grounded in our interest in exploring potential systemic effects arising from the interaction of digested compounds with bone health. By examining the effects of these compounds on osteoblast-like cells, we aim to uncover their implications for bone metabolism and calcium bioaccessibility. This dual-cell model approach facilitates the exploration of the broader physiological impact of digested compounds beyond the gastrointestinal system, contributing to our understanding of potential connections between gastrointestinal digestion, absorption, and bone health. Thus, the present research delved into the impact of probiotics, isoflavones, and tempeh on the bioavailability of calcium. This was achieved by simulating digestion and using a cell model that involved human enterocyte-like Caco-2 and osteoblast-like Saos-2 cells. This is the first study that we are aware of that combines digestion simulation with cell assessment of the effects of probiotics and isoflavone products. A novel aspect of this study involved examining the impact of probiotics and isoflavones on cellular calcium deposition to determine their potential health benefits.

2. Results

2.1. Calcium Bioaccessibility

Table 1 displays the quantitative analysis of calcium content, calcium release, and potential bioaccessibility of calcium derived from soybean, tempeh, and a probiotic. Tempeh's calcium content was found to be 72% higher than that of soybean, with tempeh also exhibiting a calcium release 2.5 times greater than soybean. Consequently, the percentage of potential calcium bioaccessibility for tempeh was approximately 110% higher compared to that for soybean. Despite having a lower calcium concentration compared to soybean products, the probiotic showed the highest calcium release and potential calcium bioaccessibility.

Table 1. Calcium content in soybean, tempeh, and a probiotic.

Native Sample	Calcium Content (mg/100 g)	Calcium Release (mg/100 g)	Calcium Bioaccessibility (%)
Soybean	234.77 ± 15.83^{b}	4.04 ± 0.04^{a}	1.72 ± 0.02^{a}
Tempeh	402.04 ± 11.31^{c}	14.49 ± 2.57^{b}	3.60 ± 0.64^{b}
Probiotic	115.15 ± 8.02^{a}	20.80 ± 0.02^{c}	18.06 ± 0.02^{c}

Significant differences between means within each column are denoted by distinct letters ($^{a–c}$), and these letters represent comparisons with a significance level of $p < 0.05$. The values are expressed as means ± standard deviation. Calcium release is expressed as mg/100 g, representing the absolute amount of calcium released from the digested samples. Calcium bioaccessibility is expressed as a percentage, reflecting the relative availability of calcium for absorption.

Table 2 presents the results of calcium release and potential bioaccessibility from two different sources: a combination of pure isoflavones (daidzein, calcium citrate, and probiotic) and a combination of tempeh (tempeh, calcium citrate, and probiotic). The combination of pure daidzein demonstrated a notable increase in both calcium release and calcium bioaccessibility when compared with tempeh-based combinations. Particularly, the use of a 1:1:1 formulation, whether comprising pure daidzein or tempeh, resulted in substantially elevated levels of calcium release and calcium bioaccessibility.

Table 2. Calcium bioaccessibility in the digested combination samples.

Digested Sample	Calcium Release (mg/100 g)	Calcium Bioaccessibility (%)
D1:1:1	17.38 ± 0.37 [b]	15.28 ± 0.33 [b]
T1:1:1	6.12 ± 0.83 [a]	1.19 ± 0.16 [a]

Significant differences between means within each column are denoted by distinct letters ([a,b]), and these letters represent comparisons with a significance level of $p < 0.05$. The values are expressed as means ± standard deviation. Calcium release is expressed as mg/100 g, representing the absolute amount of calcium released from the digested samples. Calcium bioaccessibility is expressed as a percentage, reflecting the relative availability of calcium for absorption.

2.2. Effect on Cellular Calcium Deposition

Figure 1 explores the impact of several products, i.e., calcium citrate (CaCt), the probiotic *Lactobacillus acidophilus* (LA), daidzein (D), tempeh (T), and their combinations (D1:1:1 and T1:1:1), on the process of calcium deposition in Saos-2 cells. Subfigures (A,B) specifically demonstrate the effects on extracellular calcium transport. Subfigures (C,D) illustrate the dynamics of intracellular calcium transport, providing insights into the effects of the various treatments. Subfigures (E,F) examine ALP activity, accentuating variations in enzymatic activity associated with each treatment. Additionally, subfigures (G,H) offer an examination of ALP mRNA expression, elucidating the regulatory aspects of transcription in differentiated Saos-2 cells. The results suggest that the analyzed products did not influence extracellular calcium deposition in Saos-2 cells cultured without mineralization stimulators (Figure 1A,B).

Intracellular calcium assessment revealed that only CaCt increased the calcium content in Saos-2 cells cultured without mineralization inducers. D, D1:1:1, and LA did not impact intracellular calcium accumulation, unlike T and T1:1:1, which reduced the calcium deposits in Saos-2 cells (Figure 1C,D). Additionally, the analyzed products did not affect mRNA transcripts and ALP activity levels in Saos-2 cells cultured without inducing mineralization (Figure 1E–H).

Figure 2, visually representing Saos-2 cell cultures stained with alizarin red, highlights differences in cell monolayer morphology based on the supplemented product. The images reveal that intestinally permeable fractions from digested CaCt, LA, D, T, and their combined formulations could enhance Saos-2 cell proliferation. When compared to the non-treated control Saos-2 cell culture, monolayers with increased cell density were observed after treating Saos-2 cells with all analyzed products. Extracellular calcium deposition was detected in Saos-2 cell cultures stimulated by reference mineralization inducers (Figure 2). The promoting effect of the analyzed products on Saos-2 cell proliferation and viability was also evident in the cytotoxicity MTT test (Figure 3).

Figure 3 illustrates the effects on Saos-2 cell proliferation observed in Figure 2. These effects were noted for Saos-2 cells, CaCt, LA, D, T, and the combined formulations D1:1:1 and T1:1:1, with concentrations ranging from 0.05 to 1 mg/mL. Remarkably, these concentrations were deemed noncytotoxic, indicating that CaCt, LA, D, T, D1:1:1, and T1:1:1 promoted the proliferation of Saos-2 cells. The results emphasize the ability of concentrations within the 0.05 to 1.0 mg/mL range to enhance the proliferation of Saos-2 cells.

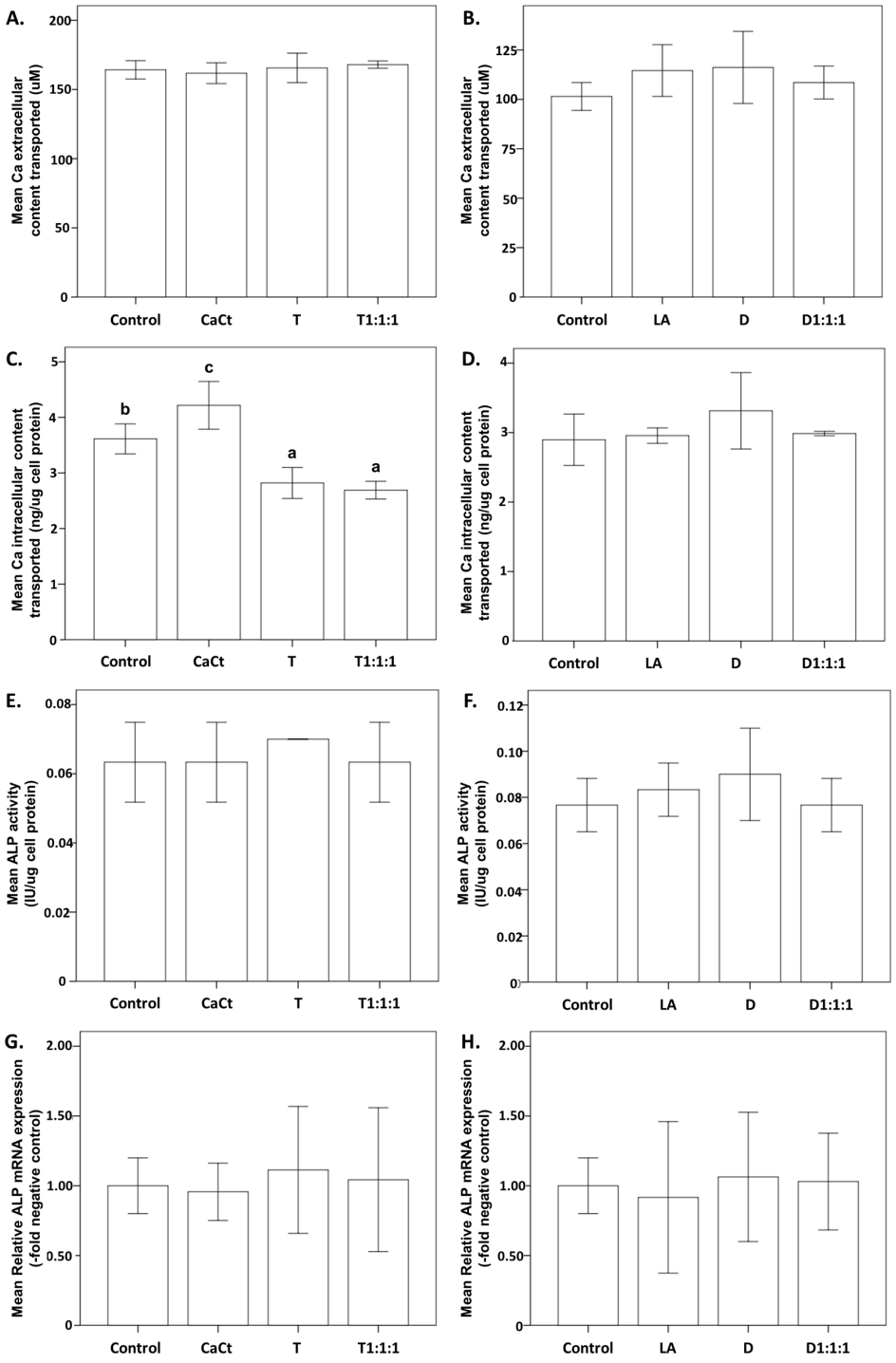

Figure 1. Impact of calcium citrate (CaCt), the probiotic *Lactobacillus acidophilus* (LA), daidzein (D), tempeh (T), and the combinations of tempeh, calcium citrate, and the probiotic *L. acidophilus* (T1:1:1) and of daidzein, calcium citrate, and the probiotic *L. acidophilus* (D1:1:1) on extracellular calcium deposition (**A**,**B**), intracellular calcium content (**C**,**D**), ALP activity (**E**,**F**), and ALP mRNA expression (**G**,**H**) in Saos-2 cells. The analyzed intestinally permeabilized fractions were introduced into Saos-2 cell cultures throughout the culture medium every 3 days for 15 days. Significant differences between means within each figure are denoted by distinct letters (a–c), and these letters represent comparisons with a significance level of $p < 0.05$. The values are expressed as means ± standard deviation. Each figure includes a control group with triplicate analyses. This figure exclusively displays statistically analyzed data, highlighting statistically significant differences.

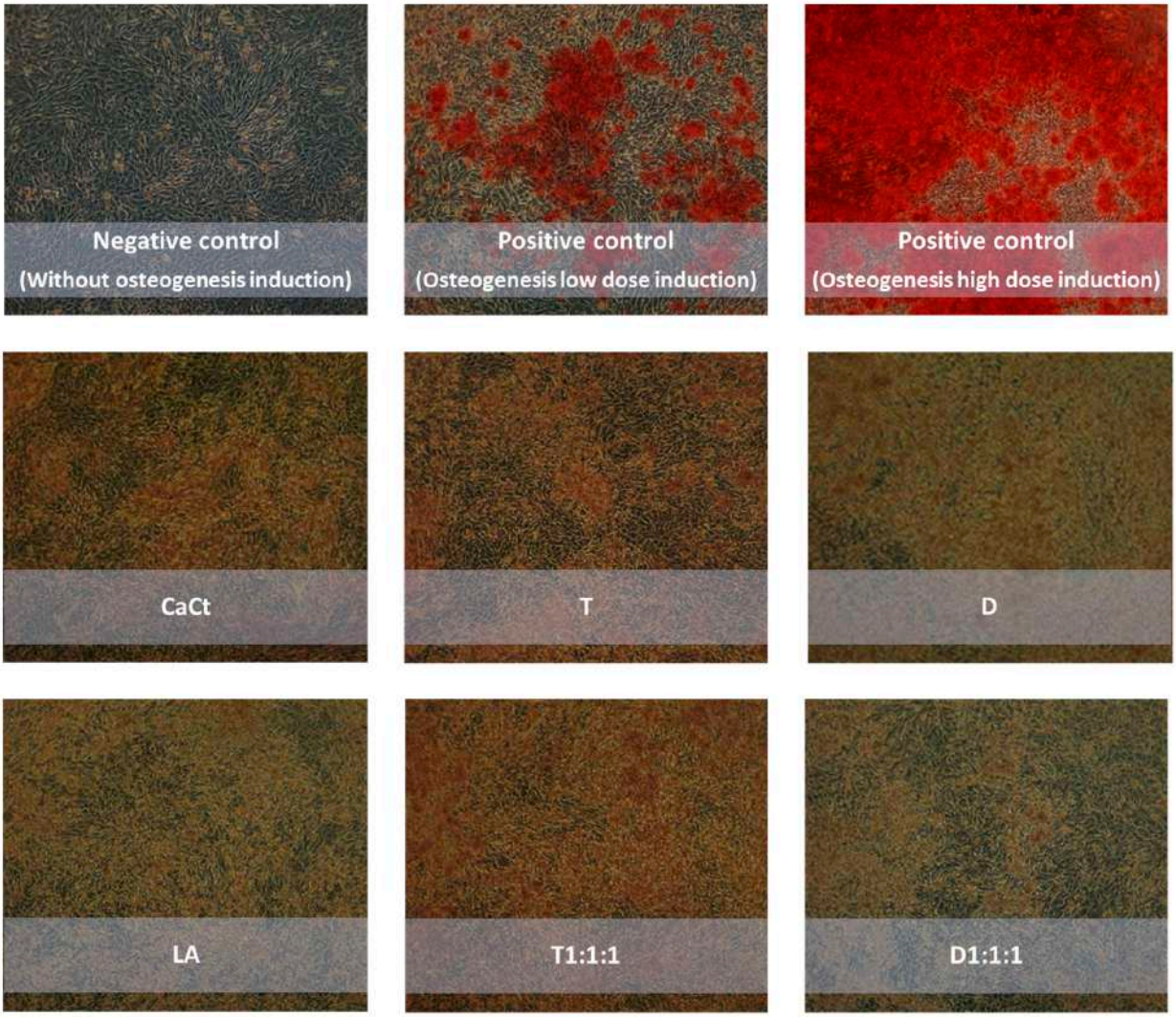

Figure 2. Impact of intestinally permeabilized fractions of calcium citrate (CaCt), tempeh (T), daidzein (D), the probiotic *Lactobacillus acidophilus* (LA), and the combinations of D, CaCt, and LA (D1:1:1) and of T, CaCt, and LA (T1:1:1) on extracellular calcium deposition in Saos-2 cell cultures following a 15-day treatment. In the negative control, cells were cultured without any analyzed fractions and osteogenic mixtures. In the positive controls, the Saos-2 cells were induced by osteogenic medium addition with low and high concentrations of osteogenesis inducers. Extracellular calcium deposits in the treated Saos-2 cells were determined by the alizarin red staining method. Photos were taken at 100× magnification.

2.3. Effect on the Osteogenic Differentiation Process

Figure 4 illustrates the impact of CaCt, LA, D, T, D1:1:1, and T1:1:1 on the process of osteogenic differentiation. This figure provides a detailed exploration of the effects of various components, including calcium citrate (CaCt), the probiotic *L. acidophilus* (LA), daidzein (D), tempeh (T), and their combinations (D1:1:1 and T1:1:1), on the osteogenic differentiation process in Saos-2 cells. Subfigures (A,B) showcase the influence on extracellular calcium transport, revealing a significant increase induced by CaCt compared to the control group. Subfigures (C,D) illustrate the intracellular calcium transport dynamics, offering insights into the impact of the different treatments. Subfigures (E,F) display ALP activity, highlighting the variations in enzymatic activity associated with each treatment. Additionally, subfigures (G,H) provide a closer look at ALP mRNA expression, shedding light on its transcriptional regulation in differentiated Saos-2 cells.

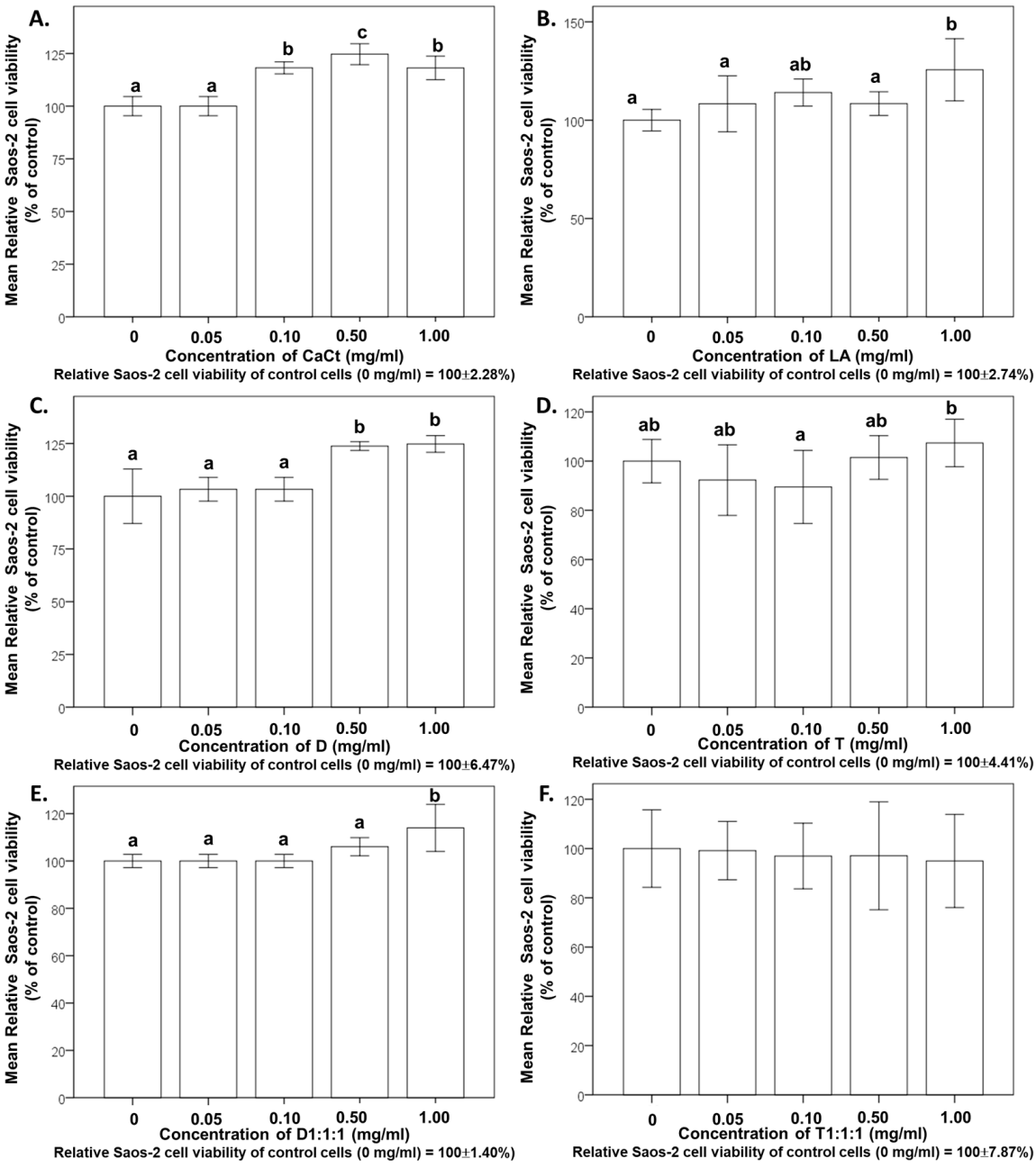

Figure 3. Impact of calcium citrate (CaCt) (**A**), the probiotic *Lactobacillus acidophilus* (LA) (**B**), daidzein (D) (**C**), tempeh (T) (**D**), and the combinations of D, CaCt, and LA (D1:1:1) (**E**) and of T, CaCt, and LA (T1:1:1) (**F**) on Saos-2 cell proliferation, viability, and metabolic activity determined using the MTT test. The cells were treated with CaCt, LA, D, T, T1:1:1, and D1:1:1 at concentrations ranging from 0.05 mg/mL to 10 mg/mL for 48 h. Significant differences between means within each figure are denoted by distinct letters (a,b or a–c), and these letters represent comparisons with a significance level of $p < 0.05$. The values are expressed as means ± standard deviation. Statistical analyses were conducted with triplicate measurements for each figure, resulting in different letter notations for significant differences. This figure exclusively displays statistically analyzed data, highlighting statistically significant differences.

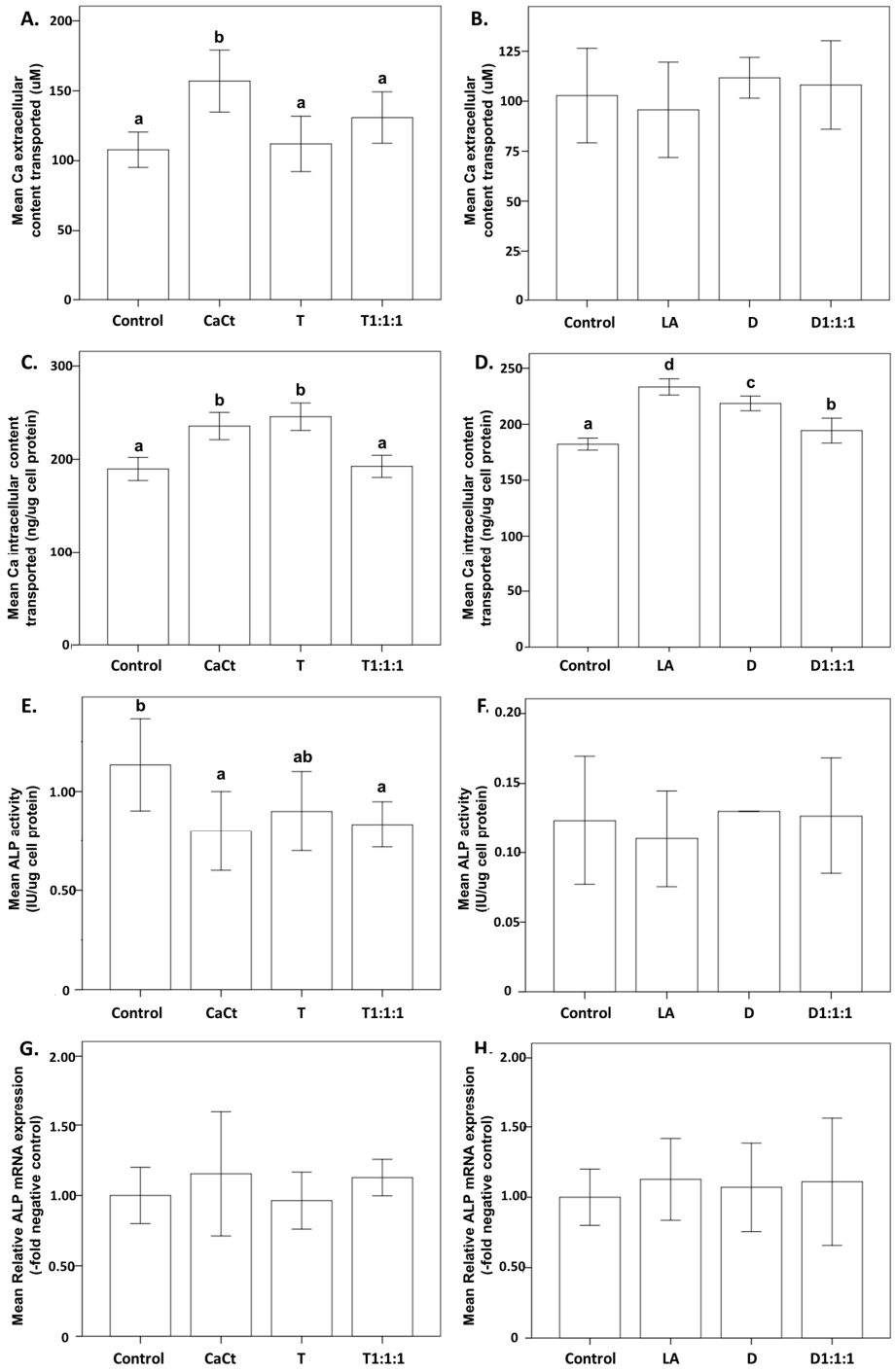

Figure 4. Impact of gastrointestinally digested and intestinally transported calcium citrate (CaCt), probiotic *Lactobacillus acidophilus* (LA), daidzein (D), tempeh (T), and combinations of T, CaCt, and LA (T1:1:1) and of D, CaCt, and LA (D1:1:1) on extracellular calcium deposition (**A**,**B**), intracellular calcium content (**C**,**D**), ALP activity (**E**,**F**), and ALP mRNA expression (**G**,**H**) in Saos-2 cells. The analyzed products were introduced into Saos-2 cell cultures during the osteogenesis process induced by the osteogenesis induction medium, which was refreshed every 3 days for 15 days. Significant differences between means within each figure are denoted by distinct letters (a,b or a–d), and these letters represent comparisons with a significance level of $p < 0.05$. The values are expressed as means ± standard deviation. Statistical analyses were conducted with triplicate measurements for each figure, resulting in different letter notations for significant differences. This figure exclusively displays statistically analyzed data, highlighting statistically significant differences.

Figure 5 visually presents the influence of calcium citrate, tempeh, daidzein, probiotic, and their combinations on Saos-2 cell cultures treated with an osteogenesis induction mixture (OIM). CaCt, T, LA, D, and D1:1:1 significantly increased the intracellular calcium content. Lower ALP activity was observed in differentiated Saos-2 cells under treatment with CaCt and T1:1:1. However, the ALP mRNA expression analysis did not confirm this effect (Figure 4G,H). The other analyzed products did not impact ALP activity following Saos-2 cell differentiation. Similarly, their possible effects on ALP mRNA expression were not detected.

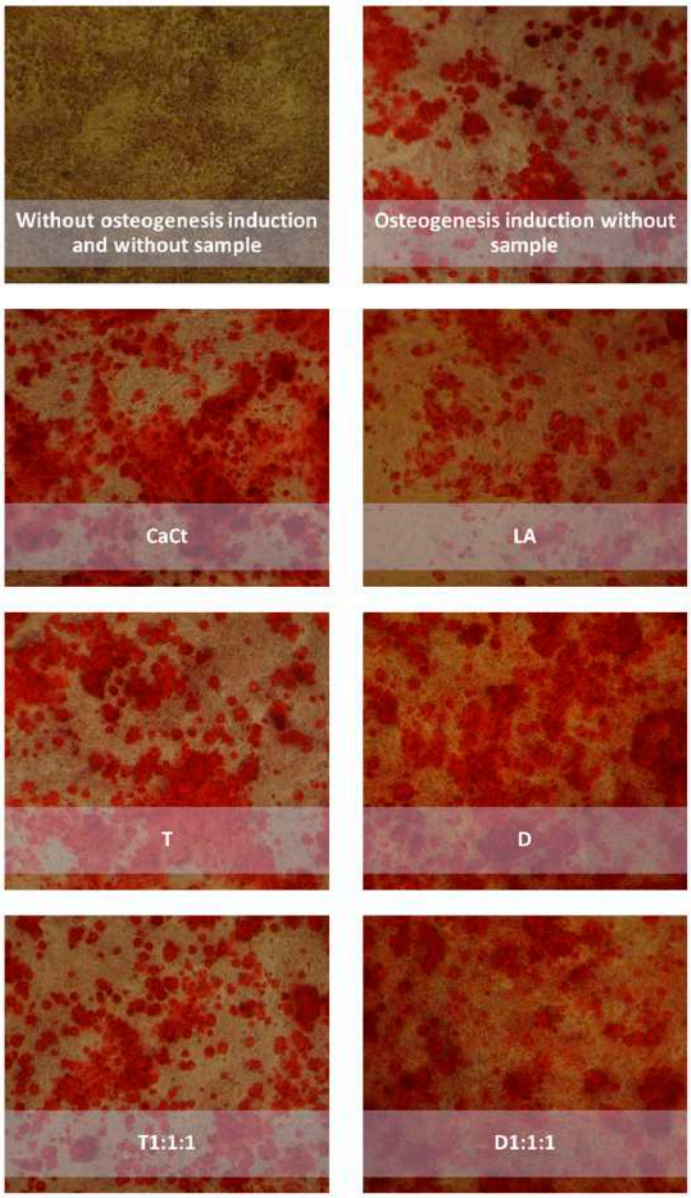

Figure 5. Impact of gastrointestinally digested and intestinally transported calcium citrate (CaCt), probiotic *Lactobacillus acidophilus* (LA), daidzein (D), tempeh (T), and tempeh (T1:1:1) or daidzein (D1:1:1) mixtures (combinations of tempeh or daidzein, calcium citrate, and probiotic *L. acidophilus*) on extracellular calcium deposition in Saos-2 cell cultures following a 15-day treatment with osteogenesis inducers. In the negative control, the cells were cultured without any analyzed fractions and osteogenic mixtures. In the positive controls, Saos-2 cells were induced by osteogenic medium addition. Extracellular calcium deposits in the treated Saos-2 cells were determined by the alizarin red staining method. Photos were taken at 100× magnification.

3. Discussion

In this investigation, we explored the impact of tempeh, isoflavones, and a probiotic on calcium uptake using a cellular model. The significantly higher uptake of calcium induced by the D1:1:1 combination suggests its potential as a calcium source promoting osteogenesis. The elevated calcium content observed in tempeh compared to soybean positions it as a potentially valuable calcium source [24]. However, despite this potential, the current study revealed a relatively low bioaccessibility of calcium from tempeh. Meanwhile, our study did not uncover a positive influence of tempeh, daidzein, and *L. acidophilus*, either individually or in combination, on calcium deposition or ALP activity—a marker of osteogenesis. The observed increase in Saos-2 cell proliferation was evident only when osteogenesis was induced for all nutritional factors analyzed.

Despite the positive impact of tempeh on calcium release and bioaccessibility, it is crucial to note that both pure tempeh and the combination of tempeh with the probiotic and calcium citrate led to a significant reduction in intracellular calcium content during cellular calcium deposition, as illustrated in Figure 1. This phenomenon can be explained by the presence of antinutrient compounds, which have the potential to affect calcium availability [25]. In addition to these nutritional components, soybeans harbor antinutritional factors such as phytic acid [26], oxalate [27], fiber [28], tannins [29], and saponin [30]. These antinutritional factors influence the bioavailability of micronutrients, such as calcium, iron, copper, and zinc [31], acting as chelating agents in the gastrointestinal tract [32]. Results of other studies indicated that isoflavones, including daidzein, exhibit chelation activity towards metal ions [33]. The chelation activity may explain the relatively low calcium bioaccessibility and low calcium intracellular content in Saos-2 cells in the presence of tempeh. The chelation activity of isoflavonoids is a notable aspect that requires further research in light of our findings. Moreover, it is worth noticing that our samples were subjected to enzymatic digestion and might contain more isoflavone aglycones [34]. Isoflavone aglycones are absorbed faster and in greater amounts than their glucosides and may be more effective. The enzymatic process may have influenced the chelating activity of isoflavones and the calcium uptake. Fermentation results in a significant decrease in antinutritional factors [35], and tempeh was the focus of this study. The reduced calcium deposition in Saos-2 cells treated with tempeh was likely influenced by fermentation-induced changes in tempeh's composition. For instance, fermentation leads to a reduction in phytic acid content, a compound known to decrease the bioavailable calcium [36,37]. It confirms that phytic acid has the potential ability to exert an inhibitory effect on calcium absorption [38–40].

While no statistically significant differences were observed, the products containing isoflavones, i.e., tempeh and pure daidzein, demonstrated the capacity to enhance ALP activity (Figure 1E,F) and ALP mRNA expression (Figure 1G,H) during cellular calcium deposition in Saos-2 cells. Our findings align with prior research, exemplified by studies indicating that soy isoflavones can elevate ALP activity after 7 and 14 days of treatment. Furthermore, in rat primary osteoblasts, treatment for 10 days resulted in increased mineralized nodule formation and calcium content within the mineralized nodules [41]. Moreover, isoflavone-enriched whole soymilk powder exhibited the ability to induce significant ALP activity in osteoprogenitors, even at an early treatment time of 48 h [42].

The observed lack of a significant improvement in ALP activity in response to daidzein, tempeh, and the probiotic in our cell study necessitates a careful consideration of the experimental context. The isolated cellular environment may not fully replicate the intricate interactions present in a living organism [43]. Notably, the absence of microbiota and isoflavone metabolites, such as equol, in our cell cultures may have contributed to the observed results, as these factors play a vital role in influencing ALP activity [44,45]. Additionally, the simplified conditions of cell studies may not fully capture the systemic situation present in vivo, including the interplay of endogenous factors [46], hormonal regulation [47], and growth factors [48], which collectively influence ALP activity. Moreover, variations in the concentration and exposure duration of the studied compounds could impact the outcome [49,50].

Our current study revealed that tempeh, a probiotic, and calcium citrate resulted in an increase in calcium intracellular deposition during the osteogenic differentiation process in Saos-2 cells (Figure 4). The observed effects might be linked to the formation of calcium phosphate facilitated by ALP activity in the medium. Moreover, the difference between ALP mRNA expression and ALP activity prompts a deeper investigation. This difference could stem from several biological factors, such as post-transcriptional modifications, translational regulation, or temporal variations in gene expression dynamics. This observed inconsistency underscores the complex and multifaceted nature inherent in cellular processes. In bone and calcifying cartilage, ALP is expressed early in development and is localized on the cell surface and within matrix vesicles. As the mineralized tissue matures, both ALP expression and activity typically decrease [6,7,51].

In the assessment of the effects of daidzein, calcium citrate, and *L. acidophilus* within the three-stage experimental model (Digestion-Caco2-Saos2), a thorough examination of potential interactions is crucial to comprehend the observed outcomes. Interestingly, our current study established that *L. acidophilus* did not manifest a synergistic effect with isoflavone products regarding cellular calcium deposition (Figure 1) and the osteogenic differentiation process (Figure 4). The dynamic interplay among these components is pivotal, as their collective impact on cellular processes may be influenced by antagonistic relationships. A conceivable antagonistic effect of probiotics involves a competition for nutrients and binding sites [52,53]. This competitive interaction introduces a layer of complexity that could contribute to the effects observed in the experimental setting.

As a result, a synergistic effect between isoflavones and probiotics in bone cellular metabolism was not evident in our current investigation. This outcome aligns with our earlier findings in healthy female rats [54–56]. This phenomenon can be attributed to the specific probiotic strain. For instance, in an in vitro study, Raveschot et al. [57] evaluated 174 *Lactobacillus* strains from Mongolian dairy products for their probiotic properties and impact on intestinal calcium absorption. Among the strains, *L. casei* 9b, *L. kefiranofaciens* 15b, *L. plantarum* 46a, *L. helveticus* 49d, and *L. delbrueckii* 50b displayed probiotic characteristics, influencing calcium transport in Caco-2 cells. Notably, *L. casei* 9b, *L. kefiranofaciens* 15b, and *L. helveticus* 49d enhanced the total calcium transport, likely through improved calcium solubility, while *L. delbrueckii* 50b impacted the paracellular pathway. *L. plantarum* 46a improved the calcium uptake via the transcellular pathway involving vitamin D receptor (VDR) and transient receptor potential cation channel subfamily V member 6 (TRPV6). Meanwhile, in an animal study by Scholz-Ahrens et al. [58], the combined supplementation of a specific prebiotic (oligofructose + acacia gum) with *L. acidophilus* NCC90 significantly prevented bone mineral loss in ovariectomized rats, highlighting the potential of synbiotics for maintaining bone health. Moreover, in a prior study involving postmenopausal women, the inclusion of *Bifidobacterium animalis* DN-173010 in a diet rich in isoflavones for an 8-week period demonstrated the capability to enhance parameters related to bone turnover during early menopause [59].

Moreover, the evaluated substances—specifically, tempeh, daidzein, *L. acidophilus*, and calcium citrate—were determined to be noncytotoxic (Figure 3). These results underscore the capacity of concentrations ranging from 0.05 to 1.0 mg/mL to enhance the proliferation of Saos-2 cells. Other studies support the nontoxic nature of daidzein when administered in high doses during experimental investigations [60,61]. Daidzein at a dose exceeding 5000 mg/kg did not exhibit adverse effects in a study of acute oral toxicity and, in a 28-day study with repeated oral doses of 25, 50, and 100 mg/kg, did not induce changes in hematology parameters, clinical biochemistry, or kidney function parameters [61]. The findings in this study lay the groundwork for further exploration in both experimental and clinical investigations.

A limitation of this study is that the in vitro digestion treatment utilized only pepsin and pancreatic enzymes. While this method represents a simplified digestive process, it has been employed in numerous studies [62,63]. Recent standardized in vitro digestion methods aim to simulate the physicochemical processes occurring in the human gastrointestinal

tract (mouth, stomach, and small intestine) during food digestion [64–67]. Additionally, our study did not assess the expression of other metabolites. For instance, osteoblast differentiation requires the expression of bone morphogenetic protein 2 (BMP2), a crucial cytokine in bone formation and regeneration. Tartrate-resistant acid phosphatase (TRAP), a modulator of bone resorption, expressed at low levels in Saos-2 cells, is associated with conditions such as osteoporosis, osteoclastoma, and metabolic bone diseases when its expression is elevated [18].

4. Materials and Methods

4.1. Materials

Soybeans of the Augusta variety were acquired from the Department of Genetics and Plant Breeding at Poznań University of Life Sciences, Poland. *Rhizopus oligosporus* NRRL 2710 was obtained from the Agricultural Research Service Culture Collection (Peoria, IL, USA). Potato dextrose agar (PDA), skim milk, and maltodextrin were procured from Merck, Darmstadt, Germany. *Lactobacillus acidophilus* DSM20079 was sourced from the Leibniz-Institut Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ; German Collection of Microorganisms and Cell Cultures). De Man, Rogosa, and Sharpe (MRS) broth were obtained from Oxoid (Hampshire, UK). Porcine gastric mucosa-derived pepsin enzyme, porcine pancreas-derived pancreatin enzyme, hydrochloric acid (HCl), sodium bicarbonate ($NaHCO_3$), and lanthanum chloride ($LaCl_3$) were purchased from Sigma-Aldrich (Steinheim, Germany). Additionally, calcium citrate tetrahydrate was acquired from Warchem Sp. z o.o., (Warsaw, Poland). The materials used for cell assessments are thoroughly explained and outlined in the method descriptions, with all remaining chemicals adhering to analytical grade standards.

4.2. Tempeh Preparation

Tempeh was prepared following a methodology derived from our previous research [63], with certain adjustments for optimization. In summary, soybeans were dehulled before boiling for 40 min, followed by cooling. An inoculation period of 72 h was then implemented in PDA medium, during which *Rhizopus oligosporus* NRRL 2710 was introduced in the soybean preparation in disposable Petri dishes (15 cm in diameter). The fermentation proceeded for precisely 24 ± 1 h at a controlled temperature of 37 ± 1 °C. After completing the fermentation phase, the tempeh samples were frozen, subjected to freeze-drying, and subsequently processed into a powdered form.

4.3. Probiotic Preparation

Lactobacillus acidophilus DSM20079 was revived from a freeze-dried stock by inoculation into De Man, Rogosa, and Sharpe (MRS) broth, followed by a 1 h incubation at room temperature. The resulting suspension was spread onto MRS agar and cultivated at 37 °C for 24 h. A single colony from this culture was then subcultured in 10 mL of fresh MRS broth and incubated for 18 h at 37 °C. Subsequently, the culture volume was increased, and a portion was transferred into fresh MRS broth, obtaining a 2% culture using the inoculum from the overnight culture. The culture was later centrifuged at 4500× *g* for 10 min at 4 °C to harvest the cells, which were washed once with cold sterilized distilled water. The cells were resuspended at 10^{11} CFU/mL in a mixture containing 10% skim milk powder and 20% maltodextrin, both cold-sterilized.

The cell mixture was poured into sterile plates, frozen at −80 °C, and subsequently freeze-dried at room temperature with the condenser temperature set at 55 °C. Random samples of freeze-dried material were taken, and 1 g of each sample was suspended in 9 mL of 0.85% normal saline. These suspensions were manually homogenized under aseptic conditions to enumerate the viable cells. A 1 mL aliquot was taken from each suspension, and serial dilutions were prepared in 0.85% sterile saline. The appropriate dilutions were then pour-plated onto sterile MRS agar and anaerobically incubated at 37 °C. The resulting

colonies were counted. The final formulation of the probiotic preparation incorporated corn starch and maintained a concentration of 10^{10} CFU/gram.

4.4. Experimental Design

To accomplish the objectives of this study, we implemented a comprehensive methodology comprising two distinct steps. The visual representation of the research design workflow is illustrated in Figure 6. In the initial step, we commenced the investigation by performing digestion simulations to evaluate the bioaccessibility of calcium sourced from three entities: calcium citrate, a probiotic, daidzein, and their combinations. Following this, the calcium content in both the native and the digested samples was quantified using atomic absorption spectrometry (AAS).

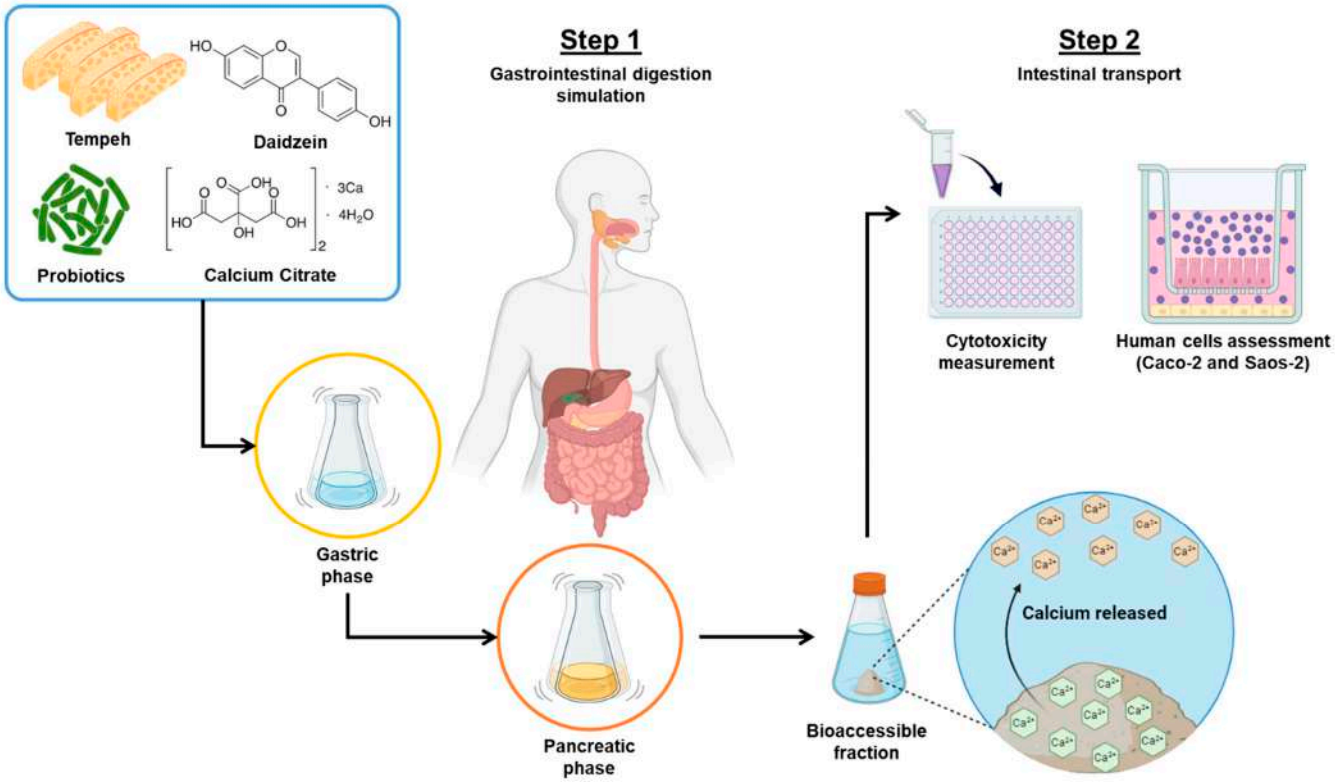

Figure 6. Experimental design of in vitro digestion with human cell assessments.

In the second phase, the gastrointestinally digested samples were administered to differentiated enterocyte-like Caco-2 cells to simulate intestinal absorption. Following this, we examined the fractions that had undergone intestinal permeation in the culture of osteoblast-like Saos-2 cells. In the initial cell experiments, we conducted cytotoxicity assessments using the MTT assay. This segment of our study aimed to evaluate the impact of the selected samples within the simulated intestinal environment provided by the Caco-2 epithelium and to assess their effects on Saos-2 cells, particularly concerning bone mineralization processes. The analyzed samples in this study were formulated individually and in combination. The specific formulations for the individual and combined samples are detailed in Table 3.

Table 3. Composition of the samples for calcium content and calcium bioaccessibility analyses.

Sample Code	Formula			
	Daidzein	Tempeh	Calcium Citrate	Probiotic
CaCt	-	-	1	-
LA	-	-	-	1
D	1	-	-	-
T	-	1	-	-
D1:1:1	1	-	1	1
T1:1:1	-	1	1	1

CaCt: calcium citrate; LA: probiotic *Lactobacillus acidophilus*; D: daidzein; T: tempeh. The amount of added calcium citrate was standardized to 1 mg of calcium. Daidzein and tempeh were included at doses corresponding to the recommended daily intake of isoflavones, set at 100 mg. The probiotic *L. acidophilus* was introduced at a dose aligning with the recommended daily probiotic intake range, falling between 10^8 and 10^{10} CFU/g. The numerical value "1" represents a single quantity.

4.5. Step 1: Digestion Simulation

The in vitro digestion study was designed to evaluate the bioaccessibility of calcium from various sources, including calcium citrate, probiotics, daidzein, tempeh, and their combined formulations. The digestion simulation followed a modified version of our established methodology. Briefly, the samples under investigation were placed in conical flasks, and 20 mL of deionized water was added to each flask. The samples underwent 10 min of homogenization. The digestion procedure included the following steps. First, the pH of the samples was adjusted to 2 using a 0.1 M HCl aqueous solution to activate the pepsin enzyme. Subsequently, a pepsin solution (0.5 mL/100 mL) was added. The samples were incubated in a thermostat-controlled shaker (Benchmark Scientific, Sayreville, NJ, USA) at 37 °C for 2 h, with pH adjustments using a 6 M HCl aqueous solution as needed during this incubation phase. Second, after the initial 2 h incubation, the digested samples underwent pH adjustment to 6.8–7.0 with a 6% $NaHCO_3$ aqueous solution, followed by the addition of a pancreatin solution (10 mL/40 mL homogenate). The samples were then placed back into the thermostat-controlled shaker at 37 °C for 4 h. Third, the digested samples were carefully transferred into conical centrifuge tubes (MPW Med. Instruments, Warsaw, Poland). Finally, the samples were centrifuged at 3800 rpm for 10 min. The calcium concentration of each sample was determined by comparison to a blank sample consisting of deionized water and reagents.

4.6. Determination of Calcium Content in Native Samples and Digested Samples

The quantification of calcium content in both the native and the in vitro digested samples was conducted using atomic absorption spectrometry (AAS-3, Carl Zeiss, Jena, Germany). To determine the total calcium content in the native samples, a dry mineralization process was employed. Specifically, each powdered sample, totaling 2 g, underwent ashing in a muffle furnace set at 450 °C, continuing until complete mineralization was achieved. The resulting ashes were then solubilized in 1 N nitric acid (Suprapure, Merck KGaA, Darmstadt, Germany).

Simultaneously, wet mineralization was performed by introducing 65% nitric acid into the digested samples, which were then subjected to mineralization within a Speedwave XPERT Microwave Digestion System (Berghof, Eningen, Germany). The spectrometer was configured to the calcium wavelength of 422.7 nm. The accuracy of the analytical method for calcium determination was established to be 92% and further validated through the simultaneous analysis of reference material (soya bean flour, INCT-SBF-4, LGC standards, Teddington, UK). The calcium content of the samples is expressed as mg/100 g, while calcium bioaccessibility is indicated as a percentage of the total content released.

4.7. Step 2: Intestinal Transport

4.7.1. Intestinal Caco-2 Epithelium Model

The human intestinal epithelial Caco-2 cell line (HTB-37™) was procured from the American Type Culture Collection (ATCC, Manassas, VA, USA). The cells were cultivated in Dulbecco's Modified Eagle Medium (DMEM, Sigma-Aldrich), supplemented with 1% nonessential amino acids (100× NEAA, Sigma-Aldrich), 20% fetal bovine serum (FBS, Gibco BRL, Grand Island, NY, USA), and gentamicin (50 mg/L), and maintained at 37 °C in a humidified atmosphere of 95% air and 5% CO_2.

For the formation of the intestinal barrier, Caco-2 cells were seeded on polycarbonate membranes with a pore size of 0.4 µm (3.14 cm^2) (Nunc™ polycarbonate cell culture inserts) at an initial density of 4×10^5 cells/cm^2. The cells were cultured for 21–22 days, changing the medium three times a week. The integrity of the Caco-2 cell monolayers was assessed through transepithelial electrical resistance (TEER) measurements using the Millicell Electrical Resistance System (ERS-2, MilliporeSigma, Burlington, MA, USA). Caco-2 cell cultures with TEER values $\geq 600\ \Omega \times cm^2$ were utilized in the transport experiments.

4.7.2. Cytotoxicity Analysis

Caco-2 cells were initially seeded at a density of 2×10^4 cells/cm^2 and cultured under standard conditions. After 24 h, the cells were treated for 48 h with digested calcium citrate, probiotic, daidzein, and their combinations at concentrations ranging from 0 to 1 mg/mL. Cytotoxicity was assessed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay. In essence, the MTT reagent was introduced into the Caco-2 cell culture to achieve a final concentration of 0.5 mg/mL. Following a 3 h incubation, formazan crystals were extracted from the cells using acidic isopropanol. Absorbance was measured using a Tecan Infinite M200 microplate reader (Tecan Group Ltd., Männedorf, Switzerland) at the wavelengths of 570 nm and 690 nm.

4.7.3. Sample Preparation and Transport Experiment

Figure 7 illustrates the cell experiments conducted on human intestinal epithelial Caco-2 cells and osteoblast-like Saos-2 cells. The samples were dispersed in a transport medium (HBSS buffer w/o Ca and Mg, pH 7.2–7.4) at a maximum concentration of 5 mg/mL, determined to be noncytotoxic to Caco-2 cells based on preliminary cytotoxicity tests. The suspensions were sterilized through filtration using 0.22 µm pore-size membranes. The sterile samples were introduced onto the apical (donor) side, representing the intestinal lumen, of the two-compartment intestinal barrier Caco-2 model. Saos-2 cell culture medium (McCoy's medium with 15% FBS) was placed on the basolateral (acceptor) side. The calcium extracellular content explicitly represents the average amount of calcium that has successfully traversed the cellular barrier, specifically referring to the quantity of calcium present in the basolateral compartment.

Intestinal transport was carried out at 37 °C under shaking (100 rpm). Following a 2 h duration, the sample fraction transported across the Caco-2 barrier was analyzed in Saos-2 cell cultures. The TEER parameter was monitored both before and after the transport experiment to ensure the integrity of the intestinal barrier.

4.8. Osteogenic Experiments Using Saos-2 Cells

4.8.1. Osteoblast-like Saos-2 Cell Culture

Saos-2 cells (HTB-85), from a human osteosarcoma cell line, were cultured in McCoy's medium formulated by the ATCC, supplemented with 15% FBS and gentamicin (50 mg/L), and maintained at 37 °C in a humidified atmosphere of 95% air and 5% CO_2. The impact of the intestinally permeable fractions of the digested samples on cellular calcium deposition was assessed in Saos-2 cells cultured under standard conditions (without osteogenesis inducers) or in osteogenesis induction medium (OIM), following established procedures.

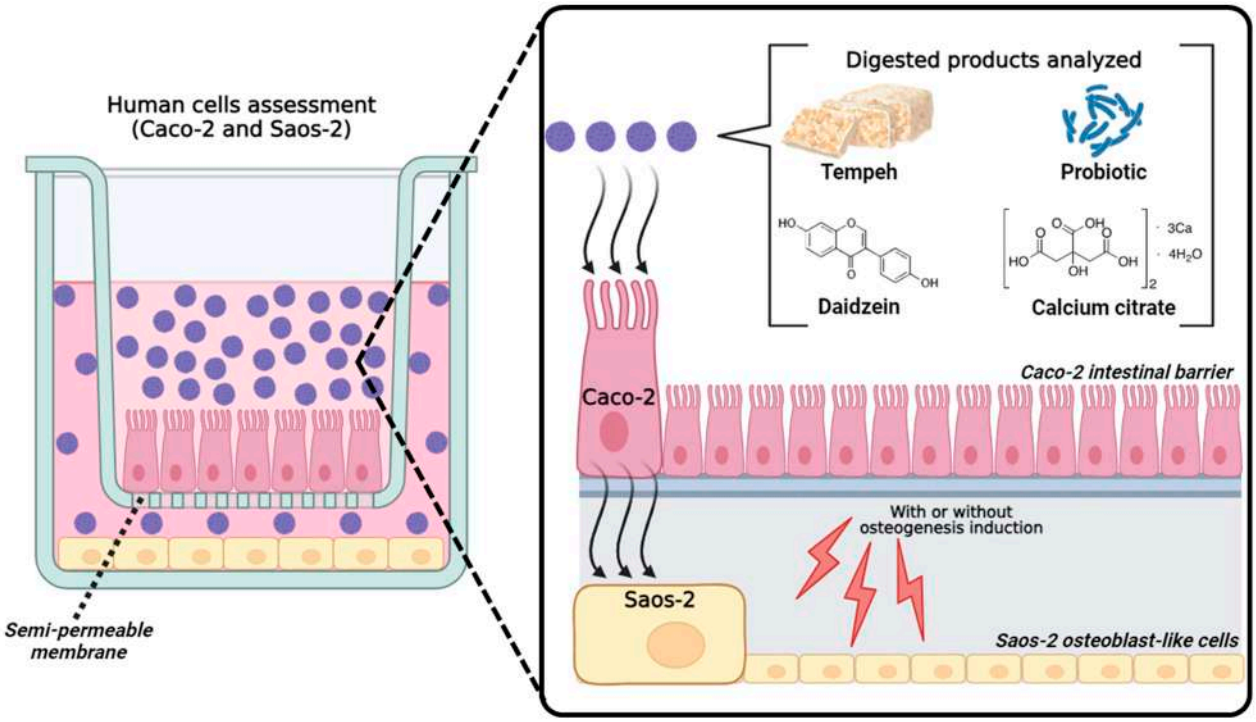

Figure 7. Illustration of the cell experiments performed using the human intestinal epithelial Caco-2 cells and the osteoblast-like Saos-2 cells.

Saos-2 cells were initially seeded at a density of 4×10^4 cells/cm^2 and cultured until confluence, with medium replacement every 48 h. Upon reaching confluency, the osteogenesis process was initiated using the osteogenesis induction medium (OIM), consisting of McCoy's medium, 15% FBS, 10 mM β-glycerophosphate, 100 nM dexamethasone, and 50 µg/mL of L-ascorbic acid. OIM was refreshed every 3 days, and the cells were cultured for a duration of 15 days. Each time the culture medium was changed, the analyzed intestinally permeabilized fractions were administered to Saos-2 cells growing in medium without or with differentiation inducers. Following the 15-day culture period, cellular calcium accumulation was quantified, encompassing extracellular calcium deposition, determined through the alizarin red assay, and intracellular calcium content, obtained via a calcium colorimetric assay. Additionally, the activity and mRNA expression of ALP, an early osteogenic marker, were determined using an ALP assay kit and real-time PCR. All experiments and analyses were conducted in triplicate, and the presented results are expressed as means with standard deviation (±SD).

4.8.2. Alizarin Red Staining and Quantification Assay

Following the 15-day culture period, the cells underwent PBS (phosphate-buffered saline) washing and fixation with 10% formaldehyde at room temperature for 15 min. Subsequently, the cells were rinsed thrice with ddH_2O and stained with a 40 mM alizarin red solution (Sigma-Aldrich, Merck Group) at room temperature for 30 min. After staining, the cells were washed four times with ddH_2O. For quantification, the stained mineralized nodules were subjected to a 30 min incubation with 10% cetylpyridinium chloride (Sigma-Aldrich). The resulting solutions were collected, and absorbance at 562 nm was measured using a Tecan Infinite M200 microplate reader. Sample quantification was performed based on the alizarin red standard curve. The inclusion of alizarin red staining, a widely accepted method for assessing calcium deposition, complemented the quantitative measures by providing a qualitative and visual evaluation of mineralization [68].

4.8.3. Intracellular Calcium Assay

After a 15-day culture, the cells were washed with PBS and lysed with RIPA (radioimmunoprecipitation assay) lysis buffer at room temperature for 30 min, and the lysates were combined with an equal volume of 1 M HCl. The RIPA lysis buffer consists of a detergent, salts, and protease inhibitors, facilitating the efficient extraction of proteins from cells for subsequent analysis. This mixture was then incubated at 4 °C overnight.

The calcium content in the lysates was determined using a colorimetric calcium assay kit (Sigma-Aldrich) suitable for calcium measurements in tissue homogenates and cell lysates. The calcium ion concentration was determined by quantifying the chromogenic complex formed between calcium ions and o-cresolphthalein, which was measured at 575 nm (Tecan Infinite M200 microplate reader). The assay was performed according to the protocol recommended by the manufacturer.

4.8.4. Alkaline Phosphatase Activity Assay

The quantitative assessment of ALP activity in Saos-2 cells was conducted using an ALP assay kit (Sigma-Aldrich), following the manufacturer's instructions. Post treatment, the cells were washed with PBS and then lysed in a 0.2% Triton X-100 solution at room temperature for 20 min. For the ALP activity measurements, *p*-nitrophenyl phosphate was employed as the substrate. This substrate undergoes hydrolysis by ALP, resulting in a yellow-colored product with a maximum absorbance at 405 nm. Since this assay is based on a kinetic reaction, absorbance was measured immediately (T = 0 min) and again after 4 min (T = 4 min) using a Tecan Infinite M200 microplate reader. The obtained data were normalized to the cellular protein content, with total protein quantification carried out through the BCA assay (Pierce® BCA Protein Assay Kit, Thermo Scientific Inc., Waltham, MA, USA) following the manufacturer's protocol.

4.8.5. Alkaline Phosphatase mRNA Expression Analysis

Gene expression analysis followed a previously outlined protocol [69]. The TRI reagent (Sigma-Aldrich) facilitated total RNA isolation, the cDNA Transcriptor First-Strand kit (Roche Diagnostics GmbH, Mannheim, Germany) was employed for initial cDNA synthesis, and SYBR® Select Master Mix (Life Technologies, Carlsbad, CA, USA) was used for real-time PCR. The primers for cDNA amplification were as follows: forward, 5′-GACCCTTGACCCCCACAAT-3′ and reverse, 5′-GCTCGTACTGCATGTCCCCT-3′ (product size 68). The transcript levels were normalized using glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as an internal control, with the following primer sequences: forward, 5′-TGCACCACCAACTGCTTAGC-3′ and reverse, 5′-GGCATGGACTGTGGTCATGAG-3′ (product size 87). The relative mRNA expression is presented as fold change, calculated using the $2^{-\Delta\Delta Ct}$ method, compared to control cells.

4.9. Statistical Analysis

The normality of the data distributions was evaluated using the Shapiro–Wilk test. Following this, analysis of variance (ANOVA) was employed to ascertain statistical significance, with subsequent Tukey's post hoc honest significant difference test. For data with non-Gaussian distribution, we used the Kruskal–Wallis test. All identified differences reached statistical significance at a significance level of 5%. The statistical analysis was executed using SPSS version 22 on the Windows operating system.

5. Conclusions

In summary, this study aimed to assess the impact of tempeh, pure daidzein, and *Lactobacillus acidophilus* on calcium uptake and deposition in Saos-2 cells, with which the bone mineralization process was simulated. In the initial phase, we evaluated calcium bioaccessibility from these nutrients and their combinations through digestion in an artificial gastrointestinal tract. Subsequently, the digested products were subjected to simulated intestinal absorption in the intestinal epithelial Caco-2 cell model, and the intestinal perme-

able fractions were scrutinized in a culture of osteoblast-like Saos-2 cells. This methodology allowed for exploring the interactions and effects of the studied products on calcium bioavailability and bone-related cellular processes in our experimental models.

Our findings suggest that daidzein, tempeh, and *L. acidophilus* do not have a beneficial effect on cellular calcium deposition in Saos-2 cells. However, tempeh, daidzein, and their combinations, along with *L. acidophilus*, may enhance the osteogenic differentiation process in Saos-2 cells. Notably, no synergistic effect on calcium deposition and the osteogenic differentiation process in Saos-2 cells between the studied isoflavones and probiotic was observed in this study.

Future investigations should delve into the intricate molecular pathways that underlie the observed effects of daidzein, tempeh, and *L. acidophilus* on the osteogenic differentiation process in Saos-2 cells. Long-term studies are essential to evaluate the sustained effects of these nutrients, considering factors such as prolonged exposure, cellular adaptation, and potential cumulative benefits. Additionally, employing in vivo models and clinical trials could validate and extrapolate to more biologically significant conditions these findings from cell culture, providing a more comprehensive understanding of their translational potential and of safety considerations for incorporating these elements into interventions aimed at preserving or enhancing bone health.

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Effect of Tempeh and Daidzein on Calcium Status, Calcium Transporters, and Bone Metabolism Biomarkers in Ovariectomized Rats

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Abstract: Menopause marks a critical life stage characterized by hormonal changes that significantly impact bone health, leading to a heightened susceptibility to bone fractures. This research seeks to elucidate the impact of daidzein and tempeh on calcium status, calcium transporters, and bone metabolism in an ovariectomized rat model. Forty female Wistar rats, aged 3 months, participated in a two-phase experiment. The initial phase involved inducing a calcium deficit, while the second phase comprised dietary interventions across five groups: Sham (S) and Ovariectomy (O) with a standard diet, O with bisphosphonate (OB), O with pure daidzein (OD), and O with tempeh (OT). Multiple parameters, encompassing calcium levels, calcium transporters, bone histopathology, and serum bone metabolism markers, were evaluated. The findings revealed that the OT group showcased heightened levels of bone turnover markers, such as pyridinoline, C-telopeptide of type I collagen, bone alkaline phosphatase, and procollagen type I N-terminal propeptide, in contrast to S and O groups, with statistical significance ($p < 0.05$). Histopathologically, both the OD and OT groups exhibited effects akin to the OB group, indicating a decrease in the surface area occupied by adipocytes in the femoral bone structure, although statistically non-equivalent, supporting the directionally similar trends. Although TRPV5 and TRPV6 mRNA expression levels in the jejunum and duodenum did not display statistically significant differences ($p > 0.05$), the OD and OT groups exhibited increased expression compared to the O group. We hypothesized that obtained results may be related to the effect of isoflavones on estrogen pathways because of their structurally similar to endogenous estrogen and weak estrogenic properties. In conclusion, the daily consumption of pure daidzein and tempeh could potentially improve and reinstate calcium status, calcium transport, and bone metabolism in ovariectomized rats. Additionally, isoflavone products demonstrate effects similar to bisphosphonate drugs on these parameters in ovariectomized rats.

Keywords: isoflavones; calcium; bone metabolism; menopause; osteoporosis

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1. Introduction

Menopause signifies a crucial life stage for women, often marked by a decline in estrogen hormone levels, leading to various health challenges [1]. Among these challenges, osteoporosis emerges as a major concern. The decrease in estrogen levels during menopause contributes to calcium deficiency, ultimately elevating the risk of bone fractures. Women, particularly those in postmenopausal stages, are highly vulnerable to the detrimental

effects of osteoporosis, underscoring the necessity for effective interventions to uphold bone health [2].

While addressing osteoporosis is paramount, the current treatments, such as bisphosphonates, come with notable drawbacks. Despite being widely used for managing bone fractures, bisphosphonates are associated with adverse effects, prompting the exploration of alternative, safe, and long-term solutions. Discovering innovative approaches to improve bone health and prevent fractures is crucial, especially considering the limitations of existing therapeutic options [3]. In light of ongoing debates and controversies surrounding bone health and menopause management [4], it is imperative to explore alternative treatment modalities that address the multifaceted aspects of osteoporosis while minimizing adverse effects.

Bone metabolism constitutes a dynamic process crucial for maintaining skeletal integrity throughout an individual's life [5–9]. Two primary facets governing bone metabolism are bone resorption and bone formation [10]. Bone resorption involves the breakdown of bone tissue, with key markers of this process including pyridinoline, deoxypyridinoline, and C-telopeptide of type I collagen. Conversely, bone formation is characterized by the synthesis of new bone tissue, with notable markers being bone alkaline phosphatase, osteocalcin, and procollagen type I N-terminal propeptide [10]. The delicate equilibrium between resorption and formation is vital for overall bone health [11,12].

At the core of bone metabolism lies the intricate regulation of calcium homeostasis. Calcium plays a pivotal role in bone mineralization. Within bone tissue, calcium ions (Ca^{2+}), mediated by the calcium-sensing receptor, are instrumental in initiating processes like the proliferation of preosteoblasts. Moreover, calcium signaling influences the differentiation of preosteoblasts into mature osteoblasts, along with the synthesis and mineralization of essential bone proteins [13]. Maintaining an optimal balance of calcium levels is crucial for preventing bone disorders and ensuring proper cellular functions [14]. A critical aspect of calcium regulation unfolds in the small intestines, where epithelial calcium transporters, specifically TRPV5 and TRPV6, play a vital role in facilitating Ca^{2+} absorption [15–18]. Epithelial calcium transporters, such as TRPV5 and TRPV6, are specialized proteins located in the cells lining the small intestine. They are responsible for transporting calcium ions from the intestine into the bloodstream [19,20].

Dysregulation in bone metabolism, especially disruptions in the delicate balance between resorption and formation, can lead to various skeletal disorders, including osteoporosis [11]. Consequently, investigating factors influencing this equilibrium is crucial for developing interventions aimed at preventing or managing bone-related conditions. This includes exploring dietary components and their potential impact on bone health, forming the basis for our current study.

Isoflavones, acknowledged as phytoestrogens, have demonstrated promise in influencing calcium regulation, absorption, and bone metabolism [21]. Phytoestrogens are natural compounds found in plants that have estrogen-like effects in the body. Daidzein and genistein are specific types of isoflavones abundant in soybean. Among the sources of isoflavones, soy tempeh stands out as a noteworthy candidate. The fermentation process amplifies the levels of daidzein and genistein in tempeh, enhancing its nutritional content [22–24].

Astawan et al. [25] conducted a 90-day intervention study investigating the impact of various protein sources and levels on calcium absorption and retention, as well as serum and bone calcium content. Interestingly, their findings indicated no significant differences in these parameters across different protein sources and levels in the diet. Similarly, Yoo et al. [26] demonstrated increased bone mineral density and bone mineral content in ovariectomized rats fed fermented soybeans. These results suggest a potential beneficial effect of fermented soybeans on bone health. Furthermore, while our previous study did not utilize an ovariectomized rat model, we found that a diet with daidzein and genistein improves calcium transport in the duodenum and reduces serum concentrations of pyridinoline in healthy female rats [27]. However, it is noteworthy that despite these studies,

there remains a lack of detailed discussion on the specific effects of tempeh and daidzein on calcium status, calcium transporters, and bone metabolism markers in ovariectomized rats. The ovariectomized rat model is well-established and widely used for studying menopausal osteoporosis due to its relevance in mimicking postmenopausal bone loss and its advantages in providing controlled experimental conditions. Therefore, our study aims to address this gap by systematically investigating the impacts of tempeh and pure daidzein, contrasting them with a conventional bisphosphonate drug. We hypothesize that tempeh and daidzein intake will lead to improvements in calcium status, calcium transporters, and bone metabolism in the ovariectomized rat model of menopausal osteoporosis. Gaining insights into the influence of isoflavones, especially in the context of soy tempeh, on bone health can offer valuable information for potential dietary interventions in managing menopausal osteoporosis.

This study presents a unique approach by examining the impacts of tempeh and pure daidzein in contrast to the conventional bisphosphonate drug. Employing an ovariectomized rat model of menopausal osteoporosis, our goal is to elucidate the effects of daidzein and tempeh on calcium status, calcium transporters, and bone metabolism. Through this comparative analysis, we aim to contribute to the identification of dietary strategies that could provide safe and effective alternatives for managing bone health in the context of menopausal osteoporosis.

2. Materials and Methods

2.1. Materials

The study involved 40 female Wistar rats aged 3 months, obtained from the Nencki Institute of Experimental Biology—Polish Academy of Sciences in Warsaw, Poland. The rats were fed the AIN 93M food, purchased from Zoolab in Sędziszów, Poland, as their standard diet. This diet comprised various components, including Augusta variety soybeans obtained from Poznań University of Life Sciences, Poland, pure daidzein procured from Gentaur Molecular Products BVBA in Kampenhout, Belgium, and alendronate sodium trihydrate purchased from TCI Europe N.V. in Zwijndrecht, Belgium.

Additionally, the crucial component, calcium citrate tetrahydrate, was acquired from Warchem Sp. z o.o. in Warsaw, Poland. The strain *Rhizopus oligosporus* NRRL 2710 used in the fermentation process was obtained from the Agricultural Research Service Culture Collection located in Illinois, United States of America. Moreover, we produced tempeh, a fermented soybean product, using *R. oligosporus* NRRL 2710, following the methodology described in our previous investigation [28].

2.2. Ethics of Animal Research

Ethical approval was obtained from the Lokalna Komisja Etyczna (Local Ethical Committee) in Poznań, Poland. Registration number 21/2021 was issued on 21 May 2021. The study adhered rigorously to various guidelines and regulations, including the National Institutes of Health's Handbook for the Care and Use of Laboratory Animals (NIH Publication No. 80-23, Revised 1978), Directive 2010/63/EU of the European Parliament and the Council of 22nd September 2010 on the Protection of Animals Used for Scientific Purposes, and relevant Polish legislation. It is crucial to note that all animal experiments were conducted strictly following the rules established by Poznań University of Life Sciences, Poland, as outlined in the Animal Research: Reporting of In Vivo Experiments (ARRIVE) guidelines.

2.3. Adaptation and Conditioning the Animal Lab Environment

The 3-month-old female Wistar rats were housed in the secure and well-regulated conditions of the Animal Laboratory at Poznań University of Life Sciences, Poland. Wistar rats were chosen as the experimental model for this study due to their established sensitivity to estrogen and consistent sexual cycle stability [29]. The rats were accommodated in a chamber maintained at a constant temperature of 21 $\pm$ 2 °C, with a relative humidity range of 55–65%, and subjected to a 12-h light/dark cycle. Throughout the adaptation and

intervention periods, the rats were paired and housed in stainless steel cages with enamel coating to minimize electromagnetic interference.

A 1-week acclimatization phase was provided for the rats to adjust to the laboratory environment before the commencement of the trial. Labofeed B (Żurawia, Kcynia, Poland) and tap water were freely available to the rats during this phase. In terms of the nutritional requirements for adult rats, Labofeed B adheres to the guidelines established by the National Research Council of Poland.

Stress was minimized by limiting the number of individuals interacting with the animals and the duration of their contact during the trial. Moreover, throughout the experiment, the rats had continuous access to veterinary care. Figure 1 is a research flowchart that visually outlines the research process.

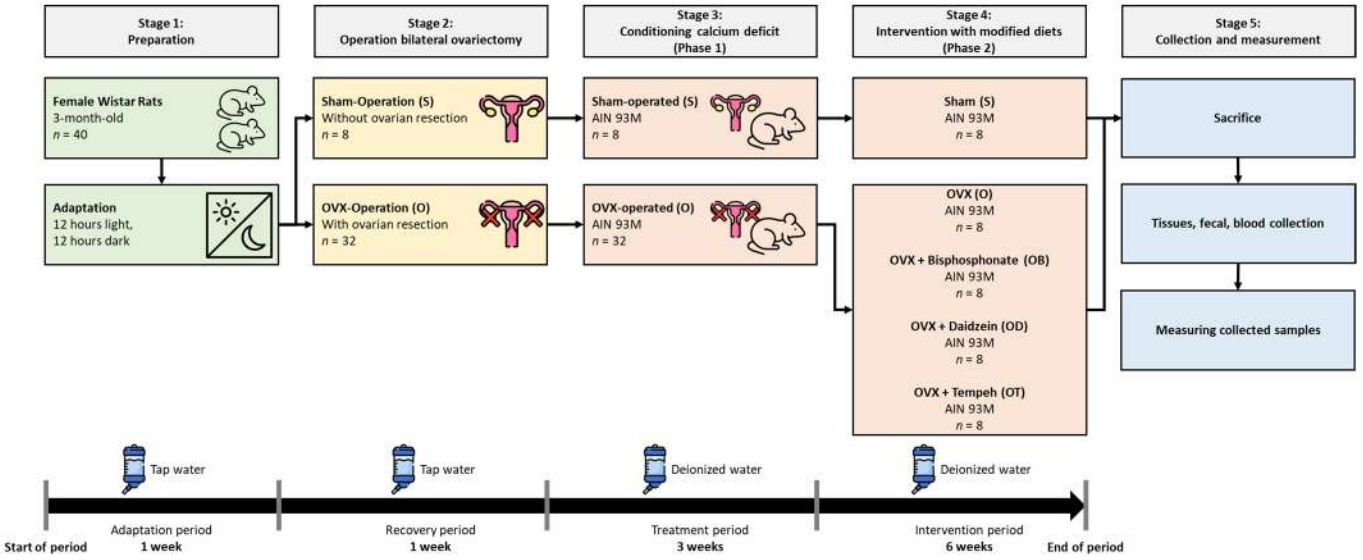

Figure 1. Experimental design of adaptation, treatment, intervention, and measurement periods.

2.4. Ovariectomized Operation

After the adaptation period, the rats were divided into three distinct groups: a sham operation group (n = 8) and a group that underwent bilateral ovariectomy (n = 32). A sham control group has been incorporated into this study to replicate a procedure or treatment experience in a manner that does not involve the actual administration of the procedure or test substance [30]. This group allows for the comparison of outcomes between rats undergoing ovariectomy and those undergoing a sham operation, providing valuable insights into the specific effects of only ovariectomy (without the effect of surgery) on the variables under investigation. All ovariectomy procedures were conducted by highly qualified experts in animal ovariectomy. During the surgical procedures, a mixture of Ketamine and Cepetor was administered for anesthesia induction. Additionally, meticulous attention was given to maintaining stringent cleanliness practices and sterile conditions throughout the surgical interventions. To enhance surgical accessibility, the dorsal region of each rat was depilated, and a linear incision measuring approximately 10–15 mm was made within the designated operative site. Following the surgical procedure, each rat was placed on a heated mat that was carefully regulated to maintain a temperature of roughly 30 ± 1 °C, creating a favorable and soothing atmosphere to facilitate recuperation. Throughout the post-operative period, the rats were closely monitored for any signs of distress, pain, or complications, including changes in behavior, appetite, and mobility. Any observed abnormalities were promptly addressed by the veterinary team to ensure the welfare of the animals and minimize any potential impact on the study outcomes. A period of 7 days was dedicated to the attentive implementation of continuous monitoring to safeguard the welfare of all rats that underwent surgery. After the completion of the surgical

operations, the rats were provided with a semi-synthetic meal formulated according to the AIN-93M guidelines [31]. Additionally, they were given free access to tap water throughout this stage.

2.5. Grouping the Rats

After a 7-day recovery period, the initial body weights of the rats were measured using a calibrated scale. Implementing this process is crucial to properly randomize the experimental groups, ensuring that each group begins the investigation from a comparable initial state. Subsequently, the rats that underwent ovariectomy were randomly assigned to seven groups, each comprising eight rats, based on their body weight. The inclusion of randomization of animals holds utmost significance in the context of experimental design, as it minimizes the potential for bias and ensures the comparability of groups at the commencement of the study [32]. We acknowledge the importance of ensuring adequate statistical power in our study design. Previous studies have indicated that a minimum of eight rats in each group is sufficient to achieve appropriate statistical power for detecting a significant effect of the intervention [33]. Therefore, we utilized eight rats in each group to ensure the study's design had adequate power to detect significant differences.

2.6. Conditioning the Calcium Deficiency

Group 1 comprised rats assigned to the sham group (S; n = 8) and were fed a standard diet with a calcium deficiency. Group 2 consisted of ovariectomized rats (O; n = 32) who were subsequently provided with a standard diet exhibiting a calcium deficiency. The calcium-deficient diet was formulated by removing calcium from the mineral composition of the regular diet. Throughout the investigation, the researchers meticulously observed and recorded the rats' daily dietary consumption over 3 weeks. Additionally, the rats had unrestricted access to deionized water. Based on a prior investigation, it has been determined that 3 weeks is sufficient for the induction of calcium deficiency [34]. Our objective was to establish the impact of daidzein and tempeh intakes on bone health parameters. To achieve this, we commenced the study with a calcium-deficient diet. By employing this methodology, we were able to simulate a practically significant scenario and establish a strong basis for examining the possible therapeutic effectiveness of our interventions in reducing osteoporosis caused by calcium deficiency.

2.7. Modified Diet Intervention

After inducing calcium deficiency, both the sham (S) and ovariectomized (O) groups were given a standard diet with calcium citrate tetrahydrate. The ovariectomized (O) group was further divided into four subgroups, each comprising eight rats, and these subgroups received a standard diet enriched with calcium citrate tetrahydrate. Table 1 displays the diet formulas. Throughout the 6-week intervention period, the rats had unrestricted access to the diets and deionized water.

The selection of daidzein and tempeh doses was informed by prior studies that established their beneficial impacts on the skeletal well-being of rodents and humans. Through a comprehensive analysis of aglycon content, specifically daidzein, glycitein, and genistein, it has been demonstrated that the optimal daily consumption of tempeh should be set at 250 g [35]. The dosage of daidzein at 10 mg/kg diet was chosen based on our previous research [27,28,36–38], and the quantity of tempeh corresponds to the daidzein content. In this study, the amount of tempeh was adjusted to the appropriate amount of pure daidzein. To incorporate 250 g/kg of tempeh flour and 10 mg/kg of daidzein into the AIN93M diets, we carefully adjusted the diets by replacing starch with 250 g and 10 milligrams of the corresponding ingredients. This approach ensured that the diets had the correct amount of tempeh flour or daidzein while being nutritionally consistent.

We determined the dosages of isoflavones by referencing prior studies that have documented their positive impacts on bone health in both rodents and humans [35]. Furthermore, long-term therapy with alendronate bisphosphonate at a dosage of 3 mg/kg/day

has been shown to benefit fracture healing and bone remodeling in ovariectomized rats [39]. The dose of alendronate bisphosphonate was adjusted weekly after measuring the rats' body weight.

Table 1. List of formula diets.

Phase	Code	Group	Number of Rats	Diet Formula
Phase 1— Conditioning calcium deficit	AIN	Sham	8	AIN 93M
	AIN_CaDef	OVX	32	AIN 93M with calcium deficit
Phase 2— Intervention modified diets	S	Sham	8	AIN 93M
	O	OVX	8	AIN 93M
	OB	OVX + Bisphosphonate	8	AIN 93M + Bisphosphonate
	OD	OVX + Daidzein	8	AIN 93M + Daidzein
	OT	OVX + Tempeh	8	AIN 93M + Tempeh flour

AIN 93M: a formulated diet designed to provide the necessary nutrients required to maintain rats in this study; AIN_CaDef: an AIN 93M diet without calcium content; OVX: ovariectomized rats.

2.8. Body Weight and Food Intake Monitoring

The rats in each group underwent weekly weighing using a calibrated scale throughout the intervention phase. Throughout the experiment, researchers meticulously documented the daily diet consumption for each group. To ensure continuous access to sustenance, the rats received a daily supply of fresh food and deionized water, with any remnants from the previous day promptly removed. This standardized procedure guaranteed consistent food and water conditions, crucial for preserving the rats' well-being and supporting their regular development [40]. Monitoring weight and food intake was deemed crucial, as any alterations in these variables could signal potential health concerns, underscoring the necessity for ensuring the reliability and validity of the experiment. Moreover, the daily provision of fresh food and water, coupled with the removal of any remaining food, effectively prevented spoilage, ensuring that the rats consumed an untainted and fresh diet and water throughout the experiment.

2.9. Body Composition Analysis

Three days prior to decapitation, the body composition of rats in each experimental group was assessed using Bruker's All New 2nd Generation Minispec LF90II Body Composition Analyzer. This analysis enabled the measurement of fat mass (in grams).

2.10. Decapitating the Rats

Upon completion of the intervention phase, a fasting period lasting 4–6 h was imposed on the rats, and their body weight was measured on a calibrated scale. This fasting protocol aimed to minimize the potential impact of recent food intake on subsequent weight measurements. Following the weight assessment, euthanasia was performed on the rats through decapitation, and a blood sample was collected for subsequent blood morphology analysis.

2.11. Blood, Serum, Bone, and Fecal Collection

After a 6-week dietary intervention, rats underwent a 12-h fasting period. Following the measurement of their body mass, the rats were decapitated, and tissue and blood samples were collected. Whole blood was obtained via cardiac puncture using anticoagulant-treated tubes, while serum samples were collected using serum-separated tubes. Additionally, serum samples were transferred to sterilized tubes, and the blood was allowed to clot at room temperature for 30 min. Subsequently, the samples underwent centrifugation at 4 °C for 15 min at 2000 rpm to separate blood cells. The resulting supernatants were collected and stored at −80 °C.

2.12. Blood Morphology and Bone Histopathology Measurements

Whole-blood morphological and bone histopathology measurements were performed in a certified commercial laboratory (Alab Laboratories, Poznań, Poland). The methodology employed in this study followed a rigorous step-by-step process for handling and preparing tissue samples, ensuring their optimal quality and suitability for subsequent histopathological examination. Upon arrival in the laboratory, tissue specimens underwent an initial macroscopic evaluation to assess the degree of fixation. If any tissues were inadequately fixed, they were bisected sagittally and then further fixed to ensure preservation and stability.

Following the fixation assessment, the tissue samples underwent decalcification for 14 h using an ionic decalcifier ethylenediaminetetraacetic acid (EDTA) solution. They were then immersed in 70% ethanol for a minimum of 24 h to complete the fixation process. After fixation and decalcification, the tissues underwent another round of macroscopic evaluation to ascertain the level of preparation and suitability for further histopathological examination. Trimmed sections of the specimens were carefully sealed in appropriately labeled histology cassettes to maintain their integrity and proper identification throughout the process. Histological preparation followed established protocols for standard histological paraffin techniques. The sealed cassettes containing tissue sections were processed in a tissue processor, undergoing a series of alcohol solutions with increasing concentrations, culminating in xylene treatment, all following established histological paraffin techniques. Once embedded in paraffin blocks, the tissue specimens were precisely sectioned using a histological microtome, resulting in thin slices suitable for subsequent staining procedures. For histological staining, standard haematoxylin-eosin topographic staining was employed to reveal cellular and tissue structures.

The methodology utilized for microscopy assessments and consultations in this study was executed with meticulous precision to ensure precise histopathological evaluation and documentation. A team of experienced veterinarian-pathologists conducted the histopathological assessments using Axiolab 5 microscopes from Zeiss in Halle, Germany. The criteria for histopathological evaluation were derived from a thorough review of scientific literature and recommendations provided by The Global Editorial and Steering Committee (GESC) in their International Harmonization of Nomenclature and Diagnostic Criteria (INHAND) guidelines.

The evaluation of tissue specimens was conducted at various magnifications, specifically 5×, 10×, and 40× objective magnification, enabling pathologists to thoroughly scrutinize the location, nature, and severity of pathological changes within the samples. To standardize the grading assessment, a catalog of histopathological changes was established, drawing upon literature, team expertise, and the initial review of preparations. Each identified change underwent evaluation on a scale ranging from 0 (none) to 4 (severe), providing a quantitative measure of the extent of pathology. Additionally, three distinct B scales were employed to assess the severity of each specific lesion.

In the morphometry aspect of the study, measurements of the surface area and width of trabeculae within spongy bone were undertaken. This process was conducted in two compartments: the spongy bone of the distal epiphysis below the epiphyseal line and the spongy bone of the distal epiphysis above the epiphyseal line, situated within the medullary area. Three random areas within each compartment were chosen for assessment at a 10× high-power field, with a total area of 1,687,500 μm^2 per compartment. Trabecular surface area measurements were recorded and subsequently presented as both total and relative areas (percentage). Additionally, the width of trabeculae was extensively measured, encompassing 80–120 measurements per compartment, ensuring a comprehensive understanding of trabecular structure.

To document the microscopic findings, images of the most representative areas in all study groups were captured using a 3DHISTECH PANNORAMIC 250 Flash III microscope with a 10× objective. These images were saved in JPG format with a resolution of 3840 × 2160 pixels, and a scale was incorporated for reference. Furthermore, all slides

were digitized as Whole Slide Images (WSI) through the Grundium Ocus®20 microscope slide scanner, stored in the SVS Aperio format, comprising single-file pyramidal tiled TIFF images with nonstandard metadata and compression. This comprehensive methodology ensured precise histopathological assessment and meticulous documentation of the findings through high-quality photographic and digital records.

2.13. Calcium Content Measurement

The calcium concentration in the diet, serum, bone, and fecal samples was determined using flame atomic absorption spectrometry (AAS-3, Carl Zeiss, Jena, Germany) after appropriate dilution with deionized water and 0.5% Lanthanum (III) chloride (Merck KGaA, Darmstadt, Germany). The calcium content in these samples was quantified at a specific wavelength of 422.7 nm. To assess the precision and reliability of this analytical technique, we employed a certified reference material, specifically Bovine Liver 1577C (Sigma-Aldrich, St. Louis, MO, USA). The results obtained from the analysis of this reference material demonstrated a notably high level of method accuracy, with a calculated accuracy rate of 92% for calcium quantification.

2.14. Bone Biomarkers Measurement

We utilized commercial enzyme-linked immunosorbent assay (ELISA) kits obtained from Qayee Bio-Technology Co., Ltd., Shanghai, China, in conjunction with absorption spectrophotometry (LEDetect96, Labexim, Lengau, Austria) to quantify serum levels of markers associated with bone metabolism. Specifically, pyridinoline, deoxypyridinoline, and C-telopeptide of type I collagen were measured as biomarkers of bone resorption, while bone alkaline phosphatase, osteocalcin, and procollagen type I N-terminal propeptide were measured as biomarkers of bone formation.

2.15. Calcium Transporters Analysis

The presence of calcium transporters was assessed through quantitative real-time polymerase chain reaction (RT-PCR). Total RNA extraction from duodenum and jejunum tissues was performed using EXTRAzol (Cytogen, Zgierz, Poland). Subsequently, EXTRAzol was introduced into separate PCR tubes, and mechanical homogenization of the sample material was achieved using TissueLyser II (Qiagen, Germantown, MD, USA). The High-Capacity cDNA Reverse Transcription Kit (Life Technologies, Grand Island, NY, USA) was employed to reverse-transcribe 1 µg of total RNA into cDNA. RT-PCR of the collected DNA was carried out on QuantStudio 12K Flex™ using gene-specific primers and 5× HOT FIREPol® Eva-Green® qPCR Mix Plus (ROX). Melting points of the DNA were measured (transition rate of 0.1 C/s) to assess specificity. Relative gene expression was analyzed using the $^{\Delta\Delta}$CT method, with Gapdh serving as a standard. TRPV5 and TRPV6 mRNA levels were expressed as arbitrary units relative to Gapdh mRNA levels. The complete list of PCR primers is provided in Table 2.

Table 2. Primer sequences.

Target	Forward Primer	Reverse Primer
Gapdh	TGACTTCAACAGCGACACCCA	CACCCTGTTGCTGTAGCCAAA
TRPV5	CGAGGATTCCAGATGC	GACCATAGCCATTAGCC
TRPV6	GCACCTTCGAGCTGTTCC	CAGTGAGTGTCGCCCATC

Gapdh: Glyceraldehyde-3-phosphate dehydrogenase; TRPV5: Transient Receptor Potential channel family Vanilloid subgroup 5; TRPV6: Transient Receptor Potential channel family Vanilloid subgroup 6.

2.16. Statistical Analysis

Statistical significance for identified differences, as determined through analysis of variance, was evaluated using Tukey's post hoc test for multiple comparisons among the groups. Significance was set at a 5% probability level for all observed distinctions. Pearson's correlation analysis was employed to assess the relationships among serum calcium levels,

bone biomarkers, and calcium transporters. ANOVA was chosen due to its suitability for comparing means across multiple groups, followed by Tukey's post hoc test to identify specific differences between pairs of groups. Statistical analysis and figure generation were conducted using SPSS version 22 for Windows. All measurements were carried out in duplicate, and the data were presented as mean values along with their corresponding standard deviations. It was calculated that a sample size of 8 rats in each group would yield 80% power of detecting statistical significance at the 0.05 α level.

3. Results

Table 3 displays the nutritional content of diets used in phase 1 to induce a calcium deficit and phase 2 for administering modified diets to the rats. When compared with the standard diet with a calcium deficit, no significant differences were observed in the nutritional compositions of the modified diets (S, O, OB, OD, and OT), including dry matter, organic matter, protein, fiber, and carbohydrates. However, a modified diet with tempeh (OT) showed a significantly higher nutritional content, specifically in protein, fat, energy, and calcium.

Table 3. Nutritional content of diets.

Parameter	Type of Diet				
	AIN_CaDef	**S & O**	**OB**	**OD**	**OT**
	Phase 1	**Phase 2**	**Phase 2**	**Phase 2**	**Phase 2**
Dry matter (mg/g dry mass)	941.30 ± 1.41 ^{ab}	940.15 ± 2.19 ^{a}	939.75 ± 0.92 ^{a}	938.30 ± 0.14 ^{a}	947.45 ± 2.90 ^{b}
Organic matter (mg/g dry mass)	939.80 ± 1.34 ^{ab}	937.48 ± 2.51 ^{ab}	936.82 ± 1.22 ^{ab}	935.49 ± 0.04 ^{a}	943.95 ± 2.98 ^{b}
Protein (mg/g dry mass)	136.04 ± 0.88 ^{a}	132.16 ± 0.68 ^{a}	133.12 ± 3.59 ^{a}	134.23 ± 0.51 ^{a}	221.38 ± 1.64 ^{b}
Fiber (mg/g dry mass)	40.05 ± 0.69 ^{a}	38.61 ± 0.54 ^{a}	39.48 ± 1.46 ^{a}	46.04 ± 1.65 ^{b}	41.69 ± 1.77 ^{ab}
Fat (mg/g dry mass)	44.88 ± 1.06 ^{b}	43.29 ± 0.10 ^{ab}	41.93 ± 0.26 ^{ab}	41.41 ± 0.08 ^{a}	83.70 ± 1.24 ^{c}
Carbohydrates (mg/g dry mass)	826.41 ± 3.20 ^{b}	822.85 ± 1.99 ^{b}	820.27 ± 3.79 ^{b}	816.00 ± 4.12 ^{b}	673.66 ± 3.48 ^{a}
Energy (Kcal/g)	4333.85 ± 5.39 ^{b}	4286.87 ± 3.25 ^{a}	4269.86 ± 6.06 ^{a}	4265.65 ± 11.87 ^{a}	4416.81 ± 0.22 ^{c}
Calcium (mg/g dry mass)	0.02 ± 0.01 ^{a}	5.06 ± 0.30 ^{b}	4.66 ± 0.71 ^{b}	4.03 ± 1.12 ^{b}	7.06 ± 0.48 ^{c}

AIN_CaDef: an AIN 93M diet without calcium content; S & O: sham and ovariectomized rats fed AIN; OB: ovariectomized rats fed AIN with bisphosphonate; OD: ovariectomized rats fed AIN with daidzein; OT: ovariectomized rats fed AIN with tempeh. Phase 1: conditioning calcium deficit; Phase 2: intervention modified diets. Results of ANOVA analysis followed by Tukey's post hoc honestly significant difference test showing significant differences between types of diet. Data are presented as mean ± standard deviation. $^{a, b, c}$ represent significantly different mean values ± SD at $p < 0.05$.

3.1. Body Mass Gain and Body Composition

Due to the common association of menopause with increases in body mass and alterations in body composition, we systematically analyzed these parameters in our study. Table 4 presents the outcomes related to body mass gain and body composition in rats throughout the study. Significant increases in final body mass were observed in all ovariectomized groups (O, OB, OD, and OT) compared to the sham group (S). During the calcium deficit period (Phase 1), there was no statistically significant increase in body mass gain in the ovariectomized groups fed with modified diets (OB, OD, and OT) when compared to the S group. However, it is noteworthy that a significant increase in body mass gain during the intervention-modified diet period (Phase 2) was observed between the S and O groups. Furthermore, two distinct scenarios emerged during the second phase. First, the OB group exhibited a body loss of 45% compared to the O group. Second, the OD

and OT groups demonstrated body losses of 31% and 34%, respectively, compared to the O group.

Table 4. Final body mass, body mass gain, and fat mass in the rats fed the modified diets during the experimental study.

Parameter	Group				
	S	O	OB	OD	OT
Initial body mass (g)	275.88 ± 18.79	294.50 ± 20.63	292.38 ± 20.32	294.63 ± 20.42	294.50 ± 20.36
Body mass gain in Phase 1 (g)	22.38 ± 13.06	33.00 ± 21.84	39.13 ± 16.50	37.50 ± 5.95	34.63 ± 15.50
Body mass gain in Phase 2 (g)	7.25 ± 7.89 ^{a}	27.88 ± 16.98 ^{b}	15.25 ± 10.87 ^{ab}	19.13 ± 15.80 ^{ab}	18.38 ± 7.23 ^{ab}
Final body mass (g)	305.50 ± 31.51 ^{a}	355.38 ± 23.54 ^{b}	346.75 ± 28.41 ^{b}	351.25 ± 20.80 ^{b}	347.50 ± 33.15 ^{b}
Fat mass (g)	62.94 ± 29.63	86.95 ± 14.93	89.53 ± 24.69	88.64 ± 21.43	62.28 ± 22.07
Food intake (g/day)	16.75 ± 1.10	17.78 ± 1.14	17.27 ± 0.96	17.98 ± 1.20	16.80 ± 0.90
FER (%)	43.25 ± 46.96 ^{a}	156.88 ± 95.64 ^{b}	88.38 ± 63.02 ^{ab}	106.25 ± 87.70 ^{ab}	109.38 ± 43.07 ^{ab}
Calcium intake (mg/day)	85.28 ± 5.60 ^{bc}	90.52 ± 5.79 ^{c}	80.96 ± 4.52 ^{ab}	72.97 ± 4.86 ^{a}	119.50 ± 6.42 ^{d}

S: sham rats fed AIN, serving as the reference group; O: ovariectomized rats fed AIN; OB: ovariectomized rats fed AIN with bisphosphonate; OD: ovariectomized rats fed AIN with daidzein; OT: ovariectomized rats fed AIN with tempeh. Body mass gain was calculated as the difference in body mass between the end and the beginning of each phase. Phase 1: conditioning calcium deficit; Phase 2: intervention modified diets. FER: Food Efficiency Ratio (%) = weight gain (g)/food intake (g) × 100. Results of ANOVA analysis followed by Tukey's post hoc honestly significant difference test showing significant differences between types of diet. Data are presented as mean ± standard deviation. $^{a, b, c, d}$ represent significantly different mean values ± SD at $p < 0.05$.

Furthermore, these findings illustrate that ovariectomy led to an increase in fat mass. It is noteworthy that, although no significant differences were observed among the various ovariectomized groups, the OT group displayed reduced levels of body fat mass compared to the other ovariectomized groups (O, OB, and OD).

In addition to these findings, Table 4 provides insights into the results concerning food intake, food efficiency ratio (FER), and calcium intake in rats subjected to modified diets during the intervention stage. The FER was significantly higher in the O group compared to the S group, while no differences were noted between the OB, OD, and OT groups when compared to the O and S groups. Furthermore, in contrast to the S and O groups, the OD group exhibited a significantly lower calcium intake, while the OT group demonstrated a significantly higher calcium intake.

3.2. Impact on Blood Morphology

Given the typical association of menopause with changes in blood morphology, we analyzed these parameters in our study to discern the alterations. Table 5 provides insights into blood morphology among rats exposed to modified diets. In the comparison between the S and O groups, ovariectomy resulted in a significant increase in leukocytes, neutrophils, lymphocytes, and cholesterol by 86%, 83%, 96%, and 31%, respectively. The results indicated a substantial elevation in the concentration of leukocytes, neutrophils, lymphocytes, and cholesterol following ovariectomy. However, the inclusion of modified diets (OB, OD, and OT) did not induce significant alterations in the levels of these parameters. The OD group demonstrated a notable increase in the levels of monocytes and eosinophils compared to group S, whereas the OB group exhibited significantly higher eosinophil

levels than group S. Interestingly, the OT group displayed lower levels of neutrophils and cholesterol among ovariectomized rat groups.

Table 5. Blood morphology in rats fed modified diets.

Parameter	Group				
	S	O	OB	OD	OT
Erythrocytes (T/L)	7.94 ± 0.40	8.18 ± 0.32	8.09 ± 0.33	8.09 ± 0.51	8.28 ± 0.29
Hemoglobin (g/dL)	14.99 ± 0.65	15.25 ± 0.41	15.44 ± 0.50	15.46 ± 0.59	15.63 ± 0.50
Hematocrit (%)	42.80 ± 1.51	43.55 ± 1.30	43.75 ± 1.87	43.76 ± 2.30	43.80 ± 1.85
MCV (fL)	53.98 ± 1.61	53.40 ± 1.83	54.20 ± 1.24	54.20 ± 1.73	52.99 ± 2.18
MCH (pg)	18.90 ± 0.96	18.69 ± 0.51	19.15 ± 0.67	19.19 ± 0.67	18.88 ± 0.60
MCHC (g/dL)	35.01 ± 1.01	35.03 ± 0.65	35.31 ± 1.10	35.38 ± 0.76	35.65 ± 1.05
Platelets (G/L)	836.25 ± 74.53	838.75 ± 123.39	861.88 ± 144.46	815.63 ± 86.88	852.63 ± 73.45
RDW-CV (%)	12.60 ± 0.50	12.83 ± 0.42	12.83 ± 0.71	12.65 ± 0.58	13.18 ± 0.43
Leukocytes (G/L)	7.34 ± 2.20 ^{a}	13.65 ± 2.79 ^{b}	13.91 ± 2.10 ^{b}	13.07 ± 1.87 ^{b}	12.93 ± 1.91 ^{b}
Neutrophils (G/L)	0.98 ± 0.28 ^{a}	1.79 ± 0.58 ^{b}	1.88 ± 0.53 ^{b}	1.84 ± 0.55 ^{b}	1.64 ± 0.41 ^{ab}
Lymphocytes (G/L)	5.45 ± 1.96 ^{a}	10.67 ± 2.44 ^{b}	10.61 ± 1.82 ^{b}	9.56 ± 1.98 ^{b}	10.11 ± 1.90 ^{b}
Monocytes (G/L)	0.70 ± 0.25 ^{a}	0.85 ± 0.32 ^{ab}	1.00 ± 0.44 ^{ab}	1.26 ± 0.49 ^{b}	0.84 ± 0.34 ^{ab}
Eosinophils (G/L)	0.20 ± 0.05 ^{a}	0.30 ± 0.05 ^{ab}	0.38 ± 0.12 ^{b}	0.37 ± 0.10 ^{b}	0.30 ± 0.00 ^{ab}
Basophils %	0.45 ± 0.15	0.29 ± 0.10	0.26 ± 0.09	0.33 ± 0.15	0.31 ± 0.08
ALT (U/L)	37.02 ± 4.96	44.44 ± 8.23	45.79 ± 6.66	54.70 ± 29.77	47.46 ± 7.81
AST (U/L)	155.01 ± 41.32	184.36 ± 40.92	167.86 ± 41.36	251.65 ± 194.95	152.99 ± 45.13
Cholesterol (mg/dL)	81.03 ± 20.85 ^{a}	106.51 ± 12.24 ^{b}	110.08 ± 16.40 ^{b}	115.42 ± 12.75 ^{b}	97.35 ± 16.13 ^{ab}
Glucose (mg/dL)	115.99 ± 12.96	132.30 ± 15.53	132.59 ± 15.53	135.44 ± 20.36	126.08 ± 10.36
Triglycerides (mg/dL)	210.70 ± 100.72	166.03 ± 71.82	164.48 ± 73.72	163.89 ± 47.69	130.40 ± 34.39

MCV: Mean Corpuscular Volume; MCH: Mean Corpuscular Hemoglobin; MCHC: Mean Corpuscular Hemoglobin Concentration; RDW-CV: Red Cell Distribution Width; ALT: Alanine Transaminase; AST: Aspartate Aminotransferase. S: sham rats fed AIN, serving as the reference group; O: ovariectomized rats fed AIN; OB: ovariectomized rats fed AIN with bisphosphonate; OD: ovariectomized rats fed AIN with daidzein; OT: ovariectomized rats fed AIN with tempeh. Results of ANOVA analysis followed by Tukey's post hoc honestly significant difference test show significant differences between types of diet. Data are presented as mean ± standard deviation. $^{a, b}$ represent significantly different mean values ± SD at $p < 0.05$.

3.3. Impact on Calcium in Serum, Calcium in Bone, Calcium in Fecal, and Bone Metabolism Biomarkers

In our study, we performed an analysis of calcium levels in serum, bone, and calcium fecal as well as bone metabolism biomarkers in light of the known correlation between menopause and changes in bone metabolism and calcium status. Table 6 presents the outcomes concerning serum calcium, fecal calcium, and biomarkers associated with bone resorption (pyridinoline, deoxypyridinoline, and C-telopeptide of type I collagen) and bone formation (bone alkaline phosphatase, osteocalcin, and procollagen type I N-terminal propeptide) in rats receiving modified diets.

Table 6. Calcium in serum, calcium in bone, calcium in fecal, and femoral bone metabolism biomarkers.

Parameter	Group				
	S	O	OB	OD	OT
Calcium in serum (mmol/L)	1.94 ± 0.13 ^{c}	1.78 ± 0.07 ^{bc}	1.82 ± 0.07 ^{c}	1.61 ± 0.18 ^{b}	1.42 ± 0.11 ^{a}
Calcium in femur (mg/g dry mass)	255.73 ± 63.87 ^{ab}	206.98 ± 74.95 ^{a}	345.70 ± 41.69 ^{c}	331.74 ± 66.53 ^{bc}	273.57 ± 54.87 ^{abc}
Calcium in faecal (mg/g dry mass)	45.49 ± 9.32	40.14 ± 10.30	38.57 ± 7.65	45.43 ± 4.29	42.20 ± 4.96
Pyridinoline (ng/L)	71.41 ± 14.69 ^{a}	80.12 ± 13.19 ^{ab}	84.64 ± 9.61 ^{ab}	81.15 ± 5.16 ^{ab}	91.17 ± 11.73 ^{b}

Table 6. *Cont.*

Parameter	Group				
	S	O	OB	OD	OT
Deoxypyridinoline (ng/mL)	57.99 ± 16.60	59.08 ± 7.25	62.57 ± 8.25	62.63 ± 8.39	68.75 ± 5.44
C-telopeptide of Type I Collagen (ng/mL)	84.06 ± 4.81 [a]	84.06 ± 4.81 [a]	87.43 ± 4.81 [ab]	86.28 ± 9.42 [a]	97.87 ± 11.55 [b]
Bone Alkaline Phosphatase (ng/mL)	42.11 ± 4.51 [a]	42.11 ± 4.51 [a]	47.34 ± 8.11 [ab]	41.46 ± 7.55 [a]	51.37 ± 5.51 [b]
Osteocalcin (pg/mL)	232.12 ± 60.98	213.33 ± 29.78	235.09 ± 34.95	251.31 ± 40.39	262.52 ± 52.47
Procollagen Type I N-Terminal Propeptide (ng/mL)	8.13 ± 1.30 [a]	8.52 ± 1.53 [ab]	8.54 ± 0.80 [ab]	9.75 ± 1.60 [ab]	10.46 ± 1.58 [b]

S: sham rats fed AIN, serving as the reference group; O: ovariectomized rats fed AIN; OB: ovariectomized rats fed AIN with bisphosphonate; OD: ovariectomized rats fed AIN with daidzein; OT: ovariectomized rats fed AIN with tempeh. Results of ANOVA analysis followed by Tukey's post hoc honestly significant difference test showing significant differences between types of diet. Data are presented as mean ± standard deviation. [a, b, c] represent significantly different mean values ± SD at $p < 0.05$.

In the comparison between the S and O groups, ovariectomy led to a slight decrease in calcium content in serum, femur, and feces, although this decline did not reach statistical significance. Notably, within the ovariectomized rat groups, the OB group displayed a significant increase in calcium content in femur bones compared to the O group. Furthermore, despite the significant reduction in serum calcium content observed in the OD group compared to the S group and the OT group compared to the O group, the OD and OT groups exhibited an increased calcium content in feces compared to the other ovariectomized groups.

Additionally, in the comparison between the S and O groups, ovariectomy did not induce alterations in pyridinoline, C-telopeptide of type I collagen, bone alkaline phosphatase, and procollagen type I N-terminal propeptide levels. Intriguingly, within the ovariectomized rat groups, the OT group exhibited a significant elevation in pyridinoline, C-telopeptide of type I collagen, bone alkaline phosphatase, and procollagen type I N-terminal propeptide levels compared to both the S and O groups.

3.4. Impact on Histopathological Changes in Femoral Bone

Our study analyzed bone histopathology in order to identify any changes or abnormalities in bone structure, considering the frequent incidence of such changes during menopause. Table 7 details the histopathological alterations observed in various formations of the femoral bone following a 6-week intervention, and the histopathological changes are depicted in Figure 2. The observations reveal that the O group displayed an increased presence of medullary spaces, with a larger surface area occupied by adipocytes and a corresponding area devoid of any bone marrow components, as compared to the S group. This image of the O group indicates an osteoporotic condition. Conversely, the OD and OT groups demonstrated effects comparable to the OB group, illustrating a reduction in the surface area occupied by adipocytes within the femoral bone structure compared to the O group.

3.5. Impact on Calcium Transporters

Our study examined calcium transporter expression in ovariectomized rats to understand calcium homeostasis in menopause, which is associated with altered calcium uptake. Table 8 details the mRNA expression of calcium transporters (TRPV5 and TRPV6) in the duodenum and jejunum. Although no statistically significant differences were observed in all groups, the O group showed a slight reduction in TRPV5 and TRPV6 mRNA expression

levels in the jejunum and duodenum compared to the S group. Importantly, in both the jejunum and duodenum, the OD and OT groups exhibited elevated TRPV5 and TRPV6 mRNA expression levels when compared to the O group.

Table 7. Histopathological changes in several different formation of femoral bone after a 6-week intervention.

Location	Name of Histopathological Change		Group				
			S	O	OB	OD	OT
Methaphyseal trabeculae	Decreased bone	Median	0	1	2	3	2
		Quartile deviation	0	0.25	0	0.5	0
Methaphyseal trabeculae	Trabecular anisotropy	Median	0	0	2	2	1
		Quartile deviation	0.25	0.25	0.25	0.5	0.25
Methaphyseal trabeculae	Trabecular fracture	Median	1	1	1	1	2
		Quartile deviation	0.25	0	0.5	0.5	0.25
Methaphyseal trabeculae	Endosteum resorption	Median	0	0	1	1	2
		Quartile deviation	0.25	0.25	0.25	0	0.25
Bone marrow	Enlargement of the medullary spaces	Median	0	2	3	2	2
		Quartile deviation	0	0.25	0.25	0.25	0
Median sum			1	4	9	9	9

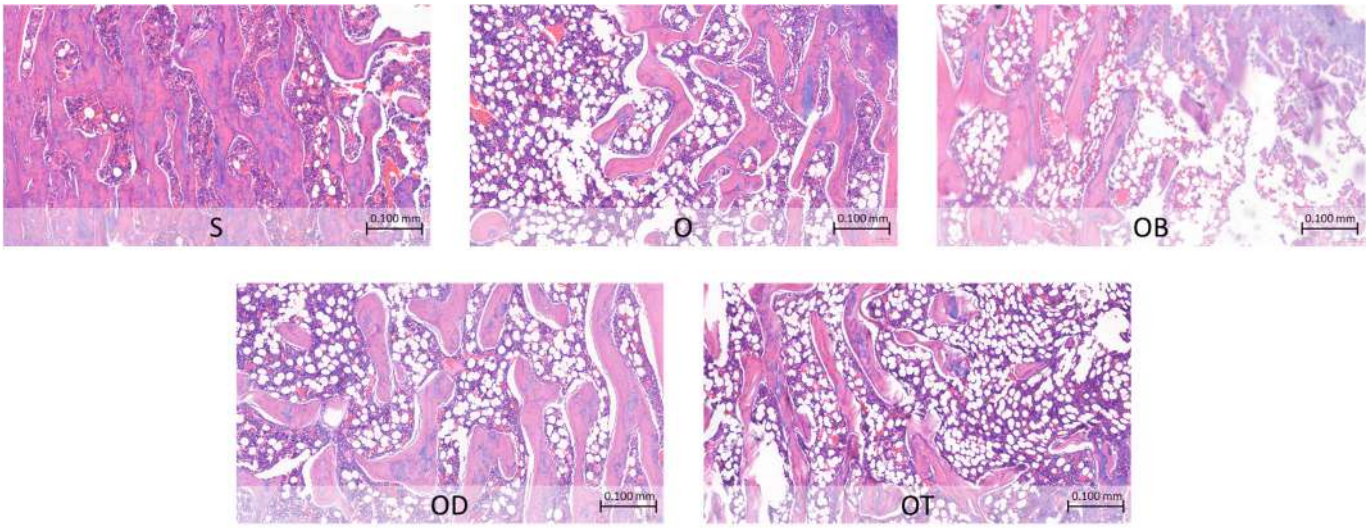

Figure 2. Histopathological changes in femoral bone after a 6-week intervention. S: sham rats fed AIN, serving as the reference group; O: ovariectomized rats fed AIN; OB: ovariectomized rats fed AIN with bisphosphonate; OD: ovariectomized rats fed AIN with daidzein; OT: ovariectomized rats fed AIN with tempeh. Photos were taken under objective 10× and a scale of 0.100 mm.

3.6. Correlation between Calcium Status, Calcium Transporters, and Bone Metabolism Biomarkers

Table 9 provides results from Pearson's correlation analysis, exploring the associations among calcium status, calcium transporters, selected blood morphology parameters, and bone metabolism biomarkers. The analysis unveiled a positive correlation (r = 0.333) between calcium levels in serum and TRPV5 in the jejunum, signifying a positive connection between these variables. Conversely, the most significant negative correlation (r = −0.544) was observed between serum calcium and procollagen type I N-terminal propeptide, indicating an inversely proportional relationship.

Table 8. TRPV5 and TRPV6 mRNA expression in the duodenum and jejunum.

Parameter	Tissue	Group				
		S	**O**	**OB**	**OD**	**OT**
TRPV5	Duodenum	1.45 ± 0.60	1.83 ± 0.86	0.95 ± 0.78	0.95 ± 0.68	0.94 ± 0.42
TRPV5	Jejunum	0.68 ± 0.46	0.45 ± 0.36	0.32 ± 0.25	0.56 ± 0.47	0.33 ± 0.10
TRPV6	Duodenum	0.49 ± 0.53	0.27 ± 0.30	0.38 ± 0.31	0.54 ± 1.02	0.31 ± 0.39
TRPV6	Jejunum	2.00 ± 2.94	0.99 ± 1.01	0.52 ± 0.28	0.63 ± 0.39	1.25 ± 1.09

TRPV5: Transient Receptor Potential channel family Vanilloid subgroup 5; TRPV6: Transient Receptor Potential channel family Vanilloid subgroup 6. S: sham rats fed AIN, serving as the reference group; O: ovariectomized rats fed AIN; OB: ovariectomized rats fed AIN with bisphosphonate; OD: ovariectomized rats fed AIN with daidzein; OT: ovariectomized rats fed AIN with tempeh. Values (means ± SD) are expressed as arbitrary units, representing the relative abundance of TRPV5 and TRPV6 proteins compared to the reference protein Gapdh.

Table 9. Pearson's correlation between calcium status, calcium transporters, and bone metabolism biomarkers.

	Correlations	Correlation Coefficient	Significance
Calcium status and calcium transporters	Calcium in femur—TRPV5 in duodenum	−0.322	0.043
	Calcium in serum—TRPV5 in jejunum	0.333	0.047
Calcium status and bone metabolism biomarkers	Calcium in serum—Pyridinoline	−0.358	0.023
	Calcium in serum—Deoxypyridinoline	−0.317	0.046
	Calcium in serum—C-telopeptide of Type I Collagen	−0.363	0.021
	Calcium in serum—Bone Alkaline Phosphatase	−0.362	0.022
	Calcium in serum—Procollagen Type I N-Terminal Propeptide	−0.544	0.000
Calcium transporters and bone metabolism biomarkers	TRPV6 in jejunum—Pyridinoline	−0.374	0.023
	TRPV6 in jejunum—Procollagen Type I N-Terminal Propeptide	−0.339	0.040

TRPV5: Transient Receptor Potential channel family Vanilloid subgroup 5; TRPV6: Transient Receptor Potential channel family Vanilloid subgroup 6. The table depicts statistically significant relationships observed in the correlations between calcium status, calcium transporters, and bone metabolism biomarkers.

Moreover, our study unveiled negative correlations between serum calcium levels and biomarkers linked to bone resorption and bone formation. Furthermore, the calcium transporter TRPV6 in the jejunum displayed negative correlations with specific markers representing both bone resorption and bone formation, as outlined in Table 9. These findings emphasize the interconnected relationship between calcium distribution, intestinal calcium transporters, and bone metabolism.

4. Discussion

In our meticulously designed experiment, a rat group underwent a sham condition, entailing the administration of a simulated treatment or procedure to account for nonspecific effects. This sham group played a pivotal role as a stringent control, aiding in the differentiation between the authentic effects of the experimental intervention and any potential confounding factors or placebo responses. Simultaneously, we utilized ovariectomized rats, a model involving the surgical removal of ovaries to induce hormonal depletion. This approach facilitated a focused exploration into the precise influences of hormonal fluctuations on key parameters, including blood morphology, calcium status, calcium transport, and bone metabolism.

In comparison to the sham (S) group, our investigation unveiled significant alterations in various body metabolism parameters during menopause. The ovariectomy (O) group exhibited increased body mass gain, fat mass, white blood cells, and cholesterol levels (Tables 4 and 5). Prior research has extensively documented changes in body composition, weight, and lipid profiles during menopause [41–43]. The menopausal transition is intricately linked to heightened adiposity, particularly in the abdominal region. Hypoestrogenism and an imbalanced androgen/estrogen ratio are prominent factors explaining

this phenomenon, although other hormonal influences likely contribute [44]. Furthermore, menopause accelerates the process of biological aging [45], reflected in blood white cell composition, providing an indicator of inflammatory and immune status [46]. Menopause is associated with an increase in systemic inflammation and a reduction in T-cell levels. The interplay of heightened fat mass and reduced hormone levels may contribute to inflammation postmenopause [47]. Changes in total cholesterol are independently influenced by menopausal status and are linked to amino acids such as glutamine, tyrosine, isoleucine, and atherogenic lipoproteins [41,48]. From a mechanistic standpoint, the metabolism of glutamine and glutamic acid is proposed to play a role in regulating glucose metabolism and insulin secretion. Additionally, there was a noted increase in leucine concentration, and the hormonal shift during menopause was correlated with elevated tyrosine levels. The elevation in aromatic amino acids and branched-chain amino acid concentrations aligns with insulin resistance. Collectively, these findings suggest intricate connections between lipid profiles, amino acid metabolism, hormonal changes, and insulin-related processes, contributing to a comprehensive understanding of the mechanisms underlying metabolic shifts during menopausal transitions [48–51].

Furthermore, the ovariectomy condition is observed to diminish calcium status, calcium transporters, and bone formation (Tables 6 and 8), ultimately resulting in osteoporosis (Figure 2). Notably, in the pursuit of mitigating the effects of ovariectomy, our study represents a pioneering effort to illustrate the significant influence of daidzein and tempeh on calcium status, calcium transporters, as well as bone metabolism and structure in a menopausal osteoporotic animal model. These findings underscore the potential of dietary interventions utilizing daidzein and tempeh to offer novel therapeutic strategies for managing menopausal osteoporosis, warranting further investigation in both preclinical and clinical settings.

In light of the findings presented, it is essential to present the implications of these results within the broader context of bone health, especially concerning menopausal osteoporosis. The observed effects of tempeh and daidzein intake on serum calcium levels, bone biomarkers, and calcium transporters provide valuable insights into potential dietary interventions for managing menopausal osteoporosis. Specifically, the improvements in these parameters suggest that tempeh and daidzein intake may offer promising alternatives to conventional bisphosphonate drugs in promoting bone health and preventing fractures in menopausal women. Additionally, the correlation analyses conducted shed light on the relationships between serum calcium levels, bone biomarkers, and calcium transporters, further emphasizing the complex interplay of factors influencing bone metabolism in the context of menopausal osteoporosis. These below subsections exhibit specifically the results of our current study.

4.1. Isoflavone Products and Body Composition in Postmenopausal Osteoporotic

Our findings indicate that the inclusion of pure daidzein and tempeh in the diet for a 6-week duration among ovariectomized rats resulted in a reduction in body mass gain compared to an ovariectomized group receiving a standard diet. Additionally, our investigation revealed that tempeh intake in ovariectomized rats led to a decrease in body fat mass when contrasted with the ovariectomized group subjected to the standard diet, as shown in Table 4. This reduction in body fat mass may have practical implications for bone health, as excess body fat is known to negatively impact bone density and increase the risk of osteoporosis. By promoting a reduction in body fat mass, tempeh intake could potentially contribute to improved bone health outcomes, including enhanced bone density and reduced fracture risk, particularly in postmenopausal women susceptible to osteoporosis. These results underscore the potential of tempeh consumption to positively influence both body mass regulation and composition during the menopausal period.

Similarly, existing literature supports our findings, suggesting that the consumption of tempeh may lead to favorable changes in body weight and adiposity. For instance, studies by Watanabe et al. [52] and Ali et al. [53] observed comparable trends in improvements

in body composition associated with tempeh intake, further underscoring the consistent positive influence of tempeh on obesity treatment.

Within the groups of ovariectomized rats, a subgroup receiving tempeh exhibited the lowest levels of neutrophils, along with reduced levels of aspartate aminotransferase, cholesterol, glucose, and triglycerides, as outlined in Table 5. These results suggest that the consumption of tempeh may have favorable effects on immune response and lipid metabolism. Consistent with our findings, previous studies have also indicated that tempeh consumption is associated with enhanced immune parameters and improved lipid profiles. Notably, the works of Suarsana et al. [6] and Afifah et al. [54] offer additional insights into the specific mechanisms underlying these observed benefits, shedding light on the potential pathways through which tempeh positively modulates immune and metabolic parameters. While our findings align with previous studies indicating similar benefits of tempeh consumption on immune parameters and lipid profiles, it is important to consider potential mechanisms underlying these effects. Variability in experimental methodologies, such as differences in animal models, tempeh compositions, and duration of intervention, may contribute to inconsistencies in outcomes across studies.

4.2. Isoflavone Products and Calcium Status in Postmenopausal Osteoporotic

Our findings suggest that including pure daidzein and tempeh in the diet for a 6-week duration among ovariectomized rats resulted in decreased serum calcium levels and increased calcium content in the femur compared to the ovariectomized group receiving a standard diet, as depicted in Table 6. Increased calcium levels in the femoral bone due to tempeh or daidzein intake can have significant practical implications for bone health. Higher levels of calcium in the bone contribute to improved bone mineral density and strength, which are crucial factors in reducing the risk of fractures and maintaining overall skeletal integrity. This practical outcome suggests that incorporating tempeh or daidzein into the diet could potentially enhance bone health and reduce the likelihood of osteoporosis-related complications. Remarkably, the observed effects on femoral calcium levels were comparable to the impact of bisphosphonate treatment. These results underscore the potential of isoflavone products, including pure daidzein and tempeh, to positively influence calcium homeostasis during the menopausal period. It appears that isoflavone products may contribute to restoring calcium balance in the menopausal condition across serum, femoral bone, and fecal matters.

This study highlights the potential pathways through which soy isoflavones modulate calcium homeostasis in the context of menopausal osteoporosis. Our current investigation provides compelling evidence suggesting that isoflavones, particularly the abundance of daidzein found in fermented soy foods [28], contribute to restoring calcium balance during the menopausal condition. Through their estrogenic activity, these isoflavones may act as partial substitutes for declining endogenous estrogen levels, potentially regulating bone turnover and calcium homeostasis [55,56]. Furthermore, the observed enhancement in intestinal calcium absorption associated with isoflavone consumption could play a pivotal role in countering the diminished calcium absorption commonly encountered during menopause [57]. These multifaceted effects collectively underscore the potential of isoflavones to positively influence calcium metabolism. Moreover, tempeh serves as a notable source of dietary calcium, surpassing the calcium content found in the standard diets utilized in this study and raw soybeans [58].

4.3. Isoflavone Products and Calcium Transporters in Postmenopausal Osteoporotic

In contrast to our prior discovery, which demonstrated that isoflavone products improved TRPV6 levels in the duodenum and reduced TRPV5 levels in the jejunum in healthy female rats [27], our current investigation reveals an intriguing correlation. In this study, tempeh exhibited a high calcium content, contributing to increased calcium intake in the OT group. Despite no significant changes in fecal calcium levels, the OT group displayed potentially elevated calcium absorption. Our findings indicate heightened

activity of epithelial calcium transporter channels, TRPV5 and TRPV6, in both the jejunum and duodenum compared to other ovariectomized rat groups (Table 8). This increase in calcium transporter activity suggests a potential improvement in intestinal calcium absorption, which is crucial for maintaining calcium homeostasis and bone health. These changes in calcium transporter activity may have practical implications for bone health, potentially reducing the risk of osteoporosis and related fractures. This suggests that the consumption of isoflavone products, specifically tempeh, may contribute to the restoration of calcium balance during menopause. These results underscore the potential of isoflavones to positively influence calcium absorption and transporter regulation in the gastrointestinal tract. This phenomenon can be explained by the beneficial effects of soy isoflavones on intestinal function, including improvements in secretory capacity, enhancements in the integrity of the intestinal epithelial barrier through the upregulation of tight junction proteins, modulation of intestinal immune or inflammatory responses, and attenuation of histomorphological damage [59].

4.4. Isoflavone Products and Bone Metabolism in Postmenopausal Osteoporotic

An intriguing revelation in our current study is that incorporating tempeh into the diet for a 6-week duration among ovariectomized rats led to elevated levels of all serum bone metabolism biomarkers compared to both sham and ovariectomized groups, as illustrated in Table 6. Tempeh intake indicates enhanced bone formation and suggests heightened bone resorption. The observed alterations in bone turnover markers may contribute to improved bone density and strength. Consequently, these findings suggest that tempeh intake could have practical implications for enhancing bone health and reducing the risk of osteoporosis-related fractures. These findings underscore the potential of tempeh to positively impact both bone absorption and formation during the menopausal period, suggesting that tempeh products may play a role in restoring bone metabolism in the context of menopausal conditions.

Our study observed a significant increase of calcium levels in femoral bone following intervention, aligning with previous research indicating the potential of daidzein and tempeh to positively influence calcium metabolism [60]. Similarly, the alterations in bone biomarkers, such as increased bone alkaline phosphatase [61] and procollagen type I N-terminal propeptide [62] levels, are consistent with studies highlighting the bone-forming properties of these phytoestrogens. Although our research did not show the expected impact on TRPV5 and TRPV6 mRNA expression, but these results confirmed several studies [63,64]. Thus, our results contribute valuable insights into the complex mechanisms underlying the bone-protective effects of daidzein and tempeh, emphasizing the need for further investigation to elucidate their full therapeutic potential in managing bone health, particularly in menopausal osteoporosis.

The novel insight into tempeh's influence on serum bone resorption and formation levels is supported by histopathology results depicting bone microstructure, as shown in Figure 2. The ovariectomized group fed a standard diet exhibited an enlarged surface area dominated by adipocytes, with regions devoid of any bone marrow components. Such an increase in bone marrow adipocytes is known to impede bone formation and fracture healing [65,66]. Furthermore, this enlargement is correlated with endosteum resorption. The endosteum, comprising flattened osteoprogenitor cells and collagenous fibers, plays a crucial role in bone growth and development [67–69]. In contrast, the addition of tempeh appears to ameliorate this condition, resulting in a narrower area occupied by bone marrow adipocytes compared to the ovariectomized group fed only a standard diet. This significant finding suggests that a tempeh-rich diet may contribute to the improvement of osteoporotic bone fractures.

It is imperative to underscore the pivotal role of estrogen pathways in bone metabolism [70], given their significance in the pathophysiology of osteoporosis and the rationale behind the use of isoflavones in its prevention and therapy. Isoflavones are known to exert their effects by interacting with estrogen receptors and modulating estrogenic pathways.

Specifically, they act as selective estrogen receptor modulators, exhibiting both estrogenic and antiestrogenic properties depending on the tissue and cellular context [71,72].

Regarding their mechanisms of action in bone metabolism, isoflavones can effectively replace endogenous estrogens in metabolic pathways crucial for bone health. One such pathway is the RANKL/RANK/OPG system, which plays a central role in regulating osteoclast differentiation and bone resorption [73]. Additionally, isoflavones enhance the production of osteoprotegerin (OPG), a decoy receptor that binds to RANKL and prevents its interaction with RANK, thus further inhibiting osteoclast formation and activity [74].

By targeting these estrogenic pathways, isoflavones contribute to the enhancement of bone metabolism and a concurrent decrease in bone resorption, ultimately promoting bone health. Furthermore, their ability to modulate other pathways involved in calcium homeostasis [56], such as vitamin D metabolism [75] and calcium transport [64], further underscores their potential as therapeutic agents for osteoporosis.

Furthermore, our study focused on tempeh, which undergoes soybean fermentation inoculated by *R. oligosporus*, leading to an increase in isoflavone content, particularly in the form of aglycone [76]. It is noteworthy that isoflavones in the aglycone form exhibit greater lipid solubility, which facilitates enhanced absorption by the intestines and represents the most bioactive form [77]. Through these mechanisms, isoflavones contribute to the enhancement of bone metabolism and may offer therapeutic potential in the management of osteoporosis.

4.5. Isoflavone Products versus Current Osteoporosis Drug

Bisphosphonates play a central role in the treatment of osteoporosis [78]. They function by reducing the risk of fractures through the suppression of bone resorption and improvement of bone strength. However, the clinical use of bisphosphonates for osteoporosis management presents challenges [79], with reported unforeseen adverse effects such as osteonecrosis of the jaw, atypical femur fractures, atrial fibrillation, and esophageal cancer [80]. The primary action of bisphosphonates is the inhibition of bone resorption. Their inherent affinity for bone tissue, particularly osteoclasts, is attributed to the acidic pH within the resorption lacuna during bone resorption, facilitating intracellular uptake [81]. As chemically stable analogs of inorganic pyrophosphate, bisphosphonates serve as potent inhibitors of calcification [82]. By inhibiting bone resorption, bisphosphonates effectively reduce the efflux of calcium from bone, leading to a brief and minor decrease in serum calcium levels [80].

In our comparison between the addition of isoflavone products (pure daidzein and tempeh) and bisphosphonates, our results reveal no significant differences in body composition, blood morphology profiles, calcium status, calcium transporters, and bone metabolism biomarkers. This finding underscores the potential of isoflavone products as safer alternatives to traditional bisphosphonate therapies for managing menopausal osteoporosis. To provide a deeper understanding of these comparisons, we acknowledge the importance of exploring the molecular basis underlying the differential effects of isoflavone products and bisphosphonates on bone health. While bisphosphonates primarily inhibit bone resorption by targeting osteoclast activity, isoflavone products may exert their effects through modulation of various cellular pathways involved in bone metabolism, such as the RANKL/RANK/OPG system and Wnt signaling pathway [12]. Further elucidating these mechanisms could offer valuable insights into the comparative efficacy and safety profiles of these therapeutic approaches.

Additionally, considering other osteoporosis treatments, such as hormone replacement therapy, selective estrogen receptor modulators, and denosumab, alongside isoflavone products and bisphosphonates, could provide a comprehensive overview of available therapeutic options. Future research comparing the effectiveness, safety, and long-term outcomes of these treatments in diverse patient populations is warranted to inform evidence-based clinical decision-making. Overall, our current study suggests that daidzein and tempeh

could serve as viable daily alternatives for preventing menopausal osteoporotic risks and are considered safe for long-term consumption.

4.6. Isoflavone Products and Their Correlation with Calcium Status, Calcium Transporters, and Bone Metabolism

Aligned with previous research, our study confirms the association between calcium transport and serum calcium levels [27]. Furthermore, our current investigation reveals a reciprocal correlation between serum calcium levels and both bone resorption and formation, emphasizing their involvement in maintaining overall bone metabolism. Calcium homeostasis is intricately governed by processes related to bone. The interaction between cells involved in bone formation and resorption incorporates calcium signals into their differentiation and activation [83]. The dynamic control of calcium signaling, involving the release of calcium from internal stores and its entry into the extracellular fluid, oversees a range of cellular processes. Particularly in osteoclasts, calcium signals play a crucial role in regulating gene transcription, differentiation, and bone resorption [84].

The plausible mechanism behind this phenomenon is attributed to genistein, an isoflavonoid phytoestrogen found in Leguminosae, which may counteract osteoporosis through anabolic bone metabolism. In vitro investigations demonstrate its ability to stimulate protein synthesis in osteoblastic cells, promoting bone formation. Genistein intervenes in osteoblastic bone resorption by hindering the genesis and differentiation of osteoclast-like cells, inducing apoptosis in mature osteoclasts through the Ca^{2+} signaling pathway. This results in a decrease in osteoblastic bone resorption. Additionally, the modulation of protein kinase and tyrosine phosphatase contributes to the reduction in rat bone osteoclast activity. Isoflavones like genistein and daidzein show potential in mitigating bone loss in ovariectomized rats, serving as a model for osteoporosis [60]. Our current findings suggest a dynamic interplay among calcium distribution, intestinal calcium transporters, and bone metabolism in ovariectomized rats.

4.7. Study Strengths, Limitations, Future Perspective

This study encompasses several strengths that bolster the scientific rigor and validity of our findings. Firstly, the inclusion of a sham group in our investigation enables the elimination of the impact of ovariectomy on changes in parameters. Additionally, the comparison with the current drug used in osteoporosis management adds valuable context to our research, providing insights into the potential efficacy of daidzein and tempeh as alternative interventions. This meticulous care enhances the reliability of the collected data. The systematic approach in the assessment of calcium and bone metabolism further fortifies the methodological foundation of our study. The integration of these robust methodologies contributes to a comprehensive understanding of the intricate interactions between hormonal fluctuations and physiological parameters, elucidating the potential effects of daidzein and tempeh in the context of menopausal osteoporosis management.

In examining the impact of daidzein and tempeh intake on bone health parameters, it is imperative to acknowledge the inherent limitations of our study design. While our findings reveal promising trends in calcium metabolism and bone biomarkers, it is essential to interpret them within the context of the chosen animal model and its extrapolation to human populations, particularly in postmenopausal bone metabolism.

The utilization of an animal model, although well-established and widely used for studying menopausal osteoporosis, may not fully replicate the intricacies of bone metabolism observed in humans. Variations in physiology, hormonal regulation, and response to interventions between rodents and humans underscore the need for cautious interpretation of our results in clinical settings. Furthermore, while our study provides valuable insights into the potential bone-protective effects of daidzein and tempeh, the extrapolation of these findings to human populations requires careful consideration of factors such as dosage, bioavailability, and long-term effects.

In light of these limitations, our study underscores the necessity for continued research to elucidate the full therapeutic potential of dietary interventions in managing bone health, particularly in menopausal osteoporosis. Future studies employing diverse models, including human clinical trials, and comprehensive analyses of the molecular mechanisms underlying phytoestrogen action will be essential for advancing our understanding and translation of these findings into clinical practice.

While our current study provides valuable insights into the short-term effects of daidzein and tempeh intake on bone health parameters in an animal model, there are indeed several avenues for future research that warrant exploration. For instance, investigating the long-term effects of these diets on bone health would be of great interest. Longitudinal studies could provide a comprehensive understanding of how sustained daidzein and tempeh consumption impacts bone metabolism and fracture risk over extended periods. Additionally, exploring other bone health markers beyond those examined in this study, such as bone mineral density and microarchitecture, could offer a more comprehensive assessment of bone health outcomes.

In addition, it is crucial to recognize the inherent limitations that could impact the interpretation of our findings, despite the fact that our research offers valuable insights into particular parameters associated with calcium transport, calcium status, and bone metabolism. Notably, it is essential to acknowledge the limitations associated with variations in the composition of the diets used. Specifically, the significant differences observed in protein, fat, carbohydrate, and calcium content in the OT diet introduce the possibility that these nutrients could potentially underlie the effects observed in the OT group. Additionally, a notable shortcoming is the lack of measures for vitamin D and vitamin K, which are essential for bone health. Vitamin D, particularly calcitriol, is involved in calcium regulation by diffusing into cells and forming complexes with vitamin D receptors [85]. The absence of vitamin D measurements in our study limits our understanding of its potential influence on calcium metabolism and bone health.

Similarly, the lack of vitamin K measurements is another notable limitation. Vitamin K, comprising fat-soluble vitamers including phylloquinone (K1) and menaquinones (K2), plays a pivotal role in modulating the expression and synthesis of crucial biomarkers associated with bone metabolism [86]. The absence of vitamin K measurements in our study precludes a comprehensive assessment of its impact on bone health parameters. Additionally, the omission of measurements for calcium deficiency biomarkers after the first stage of the study with calcium deficit diet represents a gap in our understanding, as it could have provided valuable insights into the calcium status of the rats and confirmed their potential calcium deficit condition [87].

To address these limitations and strengthen future studies, we propose including measurements for vitamin D, vitamin K, and calcium deficiency biomarkers in subsequent research. These additional analyses would provide a more comprehensive understanding of the complex interplay between these factors and their influence on bone health outcomes. By addressing these gaps in knowledge, future studies can build upon our findings and contribute to a more robust understanding of dietary interventions for bone health.

In addition to these limitations, it is important to address the absence of quantitative bone histomorphometric measurements in our investigation, a methodology exemplified by Dempster et al. [88] and Behets et al. [89]. These studies have demonstrated the utility of quantitative assessments in providing specific measurements of bone formation and resorption parameters. However, our research design opted for a semi-quantitative and qualitative approach, focusing on bone histopathology, aligning with methodologies employed in prior studies [90–92]. This choice was motivated by the aim to visually capture microarchitectural changes and pathological conditions in bone tissue induced by tempeh and daidzein in ovariectomized rats. While quantitative measurements offer precise numerical data, qualitative histopathology provides valuable insights into the overall bone microstructure, potentially identifying subtle alterations that may not be captured solely through quantitative means. Recognizing these limitations underscores the need for future

research endeavors to address these unexplored facets and further refine our understanding of the interplay between calcium, vitamin D, and bone metabolism.

Our study has significant implications for public health by addressing menopausal osteoporosis through dietary interventions. As the aging population grows, safe and sustainable strategies for managing bone health are increasingly important. Our findings suggest promising alternatives to pharmaceutical drugs, which may have safety concerns with prolonged use. Furthermore, future research endeavours should focus on investigating the long-term effects of daidzein and tempeh intake on bone health, exploring additional bone health markers, and conducting human studies to validate our findings in animal models. These efforts will contribute to a deeper understanding of the potential therapeutic benefits of dietary interventions in managing osteoporosis and promoting bone health in clinical settings. To translate our findings, future research should explore optimal dosages, durations, and modes of administration for daidzein and tempeh. Investigating synergistic effects with lifestyle modifications like exercise could enhance bone health outcomes. Implementing these dietary strategies may face challenges, including cultural preferences, accessibility to tempeh and isoflavone-based food sources, and individual responses to interventions. Interdisciplinary collaborations involving nutritionists, clinicians, and policymakers will be essential to develop tailored, culturally sensitive recommendations. Overall, further research in this area can lead to holistic, evidence-based approaches for long-term bone health in aging populations.

5. Conclusions

In summary, our findings indicate that the daily consumption of daidzein and tempeh may improve and restore calcium status, calcium transport, and bone metabolism in ovariectomized rats. Additionally, isoflavone products exhibit effects comparable to bisphosphonate drugs on calcium status, calcium transport, and bone metabolism in ovariectomized rats.

Future inquiries should involve clinical trials to validate and extrapolate findings from animal models, providing a more thorough understanding of the translational potential and safety considerations linked to incorporating isoflavone products into interventions aimed at preserving or enhancing bone health. Clinical trials involving postmenopausal osteoporotic women can offer valuable insights into the efficacy, safety profile, and potential mechanisms of action of isoflavones in human subjects. This, in turn, contributes to the evidence base for informed dietary recommendations and therapeutic strategies in the domain of bone health.

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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

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Abstract: Osteoporosis in menopausal women requires alternatives to current medications, considering their adverse effects. In this context, probiotics and isoflavone products are promising dietary interventions. The objective of our study was to examine the impacts of *Lactobacillus acidophilus* and its combination with daidzein and tempeh on calcium status, calcium transporters, and bone metabolism biomarkers in a post-menopausal osteoporotic rat model. A total of 48 female Wistar rats were exposed to a two-stage experiment involving calcium deficit induction and subsequent dietary interventions across six groups. Calcium levels, the gene expression of TRPV5 and TRPV6 calcium transporters, bone histopathology, serum bone metabolism markers, and blood biochemistry were evaluated. The results revealed that, while decreasing serum calcium levels, the groups that received the probiotic *L. acidophilus* and isoflavone combination exhibited increased bone metabolism biomarkers and decreased calcium transporter expressions, akin to the effects of bisphosphonate. Additionally, significant improvements in bone histopathology were observed in these groups. However, the group receiving probiotic *L. acidophilus* alone did not exhibit significant changes in bone resorption biomarkers, calcium transporter expression, or various blood parameters. Meanwhile, the combination of probiotic *L. acidophilus* with tempeh positively influenced hematological parameters and reduced cholesterol and triglyceride levels, but it led to elevated blood glucose levels. Correlation analyses highlighted associations between serum calcium levels, calcium transporter expression, and bone metabolism biomarkers. In conclusion, our findings suggest that the daily consumption of probiotic *L. acidophilus* in combination with isoflavone products may improve bone health in ovariectomized rats, warranting further research to elucidate potential interactions with other nutrients.

Keywords: isoflavones; probiotics; calcium; bone health; postmenopausal; osteoporosis

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1. Introduction

Probiotics are live micro-organisms that offer health benefits when consumed in adequate amounts [1]. Research has indicated that specific probiotic strains, such as *Lactobacillus acidophilus*, can influence the composition of the gut microbiota [2] and improve nutrient absorption [3], including calcium [4], which may contribute to better bone health. *L. acidophilus*, known for its positive effects on gastrointestinal health, has gained attention for its potential role in promoting bone health through the modulation of the gut microbiota. While the exact mechanisms by which *L. acidophilus* affects bone health are

still being studied, it is believed that they may impact bone homeostasis through various pathways, such as regulating inflammatory cytokines, improving nutrient absorption, and influencing immune responses that indirectly affect bone remodeling processes [5]. Moreover, an asymmetry in the composition of the gut microbiota, referred to as dysbiosis, has been associated with a range of health issues, including osteoporosis. The idea of using microbiome-based therapies for common human diseases is fascinating [6]. The concept of the gut–bone axis highlights the intricate relationship between the gut microbiota and bone metabolism [7], suggesting that microbial-derived metabolites and signaling molecules produced in the gut can affect bone remodeling processes through systemic circulation or local interactions with bone cells. Understanding how probiotics, gut dysbiosis, and the gut–bone axis interact is crucial for understanding their potential therapeutic applications in preventing and managing osteoporosis.

However, combining probiotics with isoflavones has emerged as a promising dietary component for enhancing calcium levels and supporting bone health [8]. These bioactive compounds, notably found in soybeans and soy-based products, have received considerable attention due to their potential benefits for bone metabolism. Isoflavones possess phytoestrogenic properties that mimic the effects of estrogen in the body, which can positively impact bone mineral density [9]. Together, probiotics and isoflavones present a promising nutritional strategy for preventing osteoporosis and reducing the risk of fractures.

Isoflavones, classified as phytoestrogens, are plant-derived compounds that structurally resemble estrogen and exhibit estrogenic effects in the body [10–12]. These compounds have gained significant attention due to their potential role in improving calcium balance and bone health. Mechanistically, isoflavones influence bone metabolism through various pathways, including regulation of the RANKL/RANK/OPG system and the modulation of osteoblast and osteoclast activity [13]. Rich sources of isoflavones include fermented soybean products such as tempeh, which undergo microbial fermentation processes that enhance the bioavailability and activity of these compounds [14,15]. In addition to tempeh, the effects of daidzein—a prominent isoflavone found in soy products—are particularly known due to its potential health benefits in post-menopausal women. Daidzein acts as a weak estrogen agonist and is believed to contribute to the protective effects of soy against osteoporosis. The unique chemical composition of isoflavones, combined with their ability to mimic estrogenic effects, position them as promising dietary agents for preserving bone health and reducing the risk of osteoporosis. Understanding the mechanisms underlying the bone-protective effects of isoflavones and their natural dietary sources is crucial for elucidating their therapeutic potential in managing skeletal disorders.

A comprehensive understanding of bone metabolism relies on assessing various biomarkers that reflect the processes of bone resorption and formation. Pyridinoline (PYD) and deoxypyridinoline (DPD) are well-established biomarkers of bone resorption, reflecting the breakdown of collagen fibers in bone tissue. Similarly, *C*-telopeptide of type I collagen (CTX) serves as a reliable indicator of bone resorption activity, providing insights into the rate of bone turnover [16]. Conversely, biomarkers of bone formation, such as Bone Alkaline Phosphatase (BALP), Osteocalcin (OC), and Procollagen Type I *N*-Terminal Propeptide (PINP), offer valuable information regarding the processes involved in the synthesis and mineralization of new bone tissue [17]. These biomarkers serve as proxies for the activity of osteoblasts—the cells responsible for bone formation—and provide crucial insights into bone health and remodeling dynamics.

In addition to these biomarkers, calcium transporters play a pivotal role in maintaining calcium homeostasis, a fundamental aspect of bone metabolism. TRPV5 and TRPV6 are key calcium transporters involved in this process. These calcium channels, found in epithelial cells, exhibit a notable preference for calcium ions (Ca^{2+}). Research has indicated that these channels play a vital role in maintaining the body's calcium equilibrium through facilitating the absorption of Ca^{2+} in the intestines and the re-absorption of Ca^{2+} in the kidneys. Dysregulation of this process has been implicated in conditions such as osteoporosis and calcium metabolism disorders. Furthermore, TRPV channels modulate

osteoblast and osteoclast differentiation, which are pivotal processes in bone remodeling. Notably, TRPV5/TRPV6 channels have been identified on the surface of osteoclasts, where TRPV5 functions as a negative regulator, modulating bone resorption triggered by RANKL signaling to maintain bone homeostasis [18]. Therefore, investigating the expression and regulation of TRPV5 and TRPV6 provides valuable insights into the mechanisms underlying calcium homeostasis and its impact on bone physiology.

Our previous investigation in a healthy female rat model yielded promising findings regarding calcium status [19], calcium transporter expression, and bone metabolism biomarkers [20]. However, a notable research gap exists regarding the effects of probiotics and their combination with isoflavone products on these parameters in a post-menopausal osteoporotic context. Despite the fact that our previous study provided valuable insights into the baseline physiology of bone health, it is imperative to evaluate the impacts of dietary interventions—particularly those involving the combination of probiotics and isoflavone products—on bone health. In our previous study, we investigated the impact of tempeh and daidzein on calcium metabolism and bone biomarkers in ovariectomized rats [21]. Expanding upon these findings, the current study explores the effects of *L. acidophilus* and its combination with isoflavone products (including tempeh and daidzein) on calcium status, calcium transporters, and bone metabolism biomarkers in a rat model of post-menopausal osteoporosis. Incorporating our previous research outcomes, the objective of the present study was designed to bridge this gap through evaluating the influence of a combined intervention involving probiotic *L. acidophilus* combined with isoflavones on calcium status, calcium transporter expression, and bone metabolism biomarkers in a rat model of post-menopausal osteoporosis. Thus, the purpose of this study was to evaluate the effects of the combination of the probiotic *L. acidophilus* and isoflavones on calcium status, calcium transporter expression, and bone metabolic biomarkers in an ovariectomized rat model. Additionally, we aimed to assess the impact of this combination on various hematological parameters in order to obtain a comprehensive understanding of its effects on overall health. For this study, we selected *L. acidophilus* DSM20079 due to its well-documented probiotic properties, such as surviving gastrointestinal transit, adhering to intestinal epithelial cells, and modulating immune responses [22,23], which are essential for enhancing calcium absorption and improving bone metabolism. This strain has been extensively studied for its safety and efficacy [24], making it a reliable choice for investigating the potential benefits in post-menopausal osteoporotic rat models. We hypothesized that this intervention would positively influence these parameters, potentially offering a novel therapeutic approach for managing post-menopausal osteoporosis. Through this investigation, we expected to elucidate the effects of these dietary components on bone health in menopausal individuals, providing valuable insights for the development of novel osteoporosis management strategies.

2. Materials and Methods

2.1. Materials and Ethical Considerations

This study used 48 female Wistar rats, aged 3 months, obtained from the Nencki Institute of Experimental Biology (Warsaw, Poland). The preparation of tempeh and probiotic powder followed the methodology described in our previous work [20]. The AIN 93M diet, Augusta variety soybeans, pure daidzein, alendronate sodium trihydrate, calcium citrate tetrahydrate, and other chemicals used have been detailed in our prior study [21].

Ethical approval was granted by the Local Ethical Committee in Poznań, Poland (registration number 21/2021, 21 May 2021). This study adhered to national and international guidelines, including the NIH Guide for the Care and Use of Laboratory Animals, Directive 2010/63/EU, and relevant Polish legislation, with procedures conducted in compliance with the ARRIVE guidelines.

2.2. Animal Housing and Surgical Procedures

Female Wistar rats, aged 3 months, were housed in a controlled environment at the Department of Human Nutrition and Dietetics, Poznań University of Life Sciences, Poland. They were kept at 21 ± 2 °C with 55–65% relative humidity, on a 12 h light/dark cycle, in pairs within stainless steel cages. The rats were acclimatized for 1 week with ad libitum access to Labofeed B and tap water.

Following acclimatization, the rats were divided into two groups: a sham operation group (S, n = 8) and a bilateral ovariectomy group (OVX, n = 40). Sham operations served as controls to compare against the ovariectomy group. The inclusion of a sham control group in this study was aimed at simulating a procedure or treatment experience without the actual application of the procedure or test substance [25]. All surgeries were performed under anesthesia with ketamine and Cepetor, adhering to sterile techniques. Post-surgery, rats were placed on a heated mat for recovery and monitored for distress, with veterinary care available as needed. A 7-day observation period followed, with rats receiving a semi-synthetic diet (AIN-93M) [26] and unrestricted access to tap water.

Following a 7-day recovery, the initial body weights of the rats were measured to ensure proper randomization. The 40 OVX rats were then randomly assigned to five groups of eight based on body weight, which is crucial for reducing bias and ensuring group comparability [27]. Previous research supports that eight rats per group provides adequate statistical power to detect significant effects [28].

To evaluate the effects of *L. acidophilus* and isoflavone products on bone health, we fed the rats a low-calcium diet to simulate post-menopausal conditions. This method helped to assess the therapeutic potential of our interventions against osteoporosis risks due to decreased estrogen and calcium levels. Table 1 outlines the dietary composition during this period. In our previous study [21], we detailed the composition of the AIN 93M diet. The calcium-to-phosphorus (Ca:P) ratio was calculated based on available data, with a ratio of 2.51 g/g as provided by Zoolab (Sędziszów, Poland). The calcium-deficient diet was provided for 3 weeks (stage 1), followed by standard and modified diets for 6 weeks (stage 2). Group 1 included sham rats (S, n = 8) on a standard diet, and Group 2 consisted of OVX rats (O, n = 40) on a calcium-deficient diet. Daily dietary intake was monitored, with deionized water provided ad libitum. A three-week period is sufficient to induce calcium deficiency [29].

Table 1. Dietary formulas used during the calcium deficiency period.

Code	Group	Number of Rats	Composition
S	Sham	8	AIN 93M
O	OVX	40	AIN 93M with calcium deficit

AIN 93M: A formulated diet that contains essential nutrients for rodents; OVX: ovariectomized rats. The standard diet was based on the AIN-93M formulation [26], which was designed to meet the nutritional requirements of adult rodents.

Following the induction of calcium deficiency, both the S and OVX groups were switched to a standard diet containing calcium citrate tetrahydrate as the source of calcium. The OVX group was further divided into five groups: O group fed with AIN 93M; OB group fed with AIN 93M and bisphosphonate; OL group fed with AIN 93M and probiotic *L. acidophilus*; ODL group fed with AIN 93M, daidzein, and probiotic *L. acidophilus*; and OTL group fed with AIN 93M, tempeh, and *L. acidophilus*. The specific dietary formulations are detailed in Table 2. During the 6-week intervention stage, the diets and deionized water were available to the rats without restriction.

In this study, the doses of daidzein and tempeh were meticulously selected based on their respective isoflavone contents. We conducted a laboratory analysis to determine the isoflavone concentration in the tempeh sample through its preparation. Our analysis revealed that 250 g of tempeh corresponded to an equivalent of 10 mg of daidzein. This information guided our dosage selection, ensuring that appropriate levels of daidzein and

tempeh were chosen. To ensure uniformity, the quantity of pure daidzein was adjusted to match the concentration specified in 250 g of tempeh. To incorporate 250 g/kg of tempeh flour and 10 mg/kg of daidzein into the AIN93M diets, we carefully modified the diets by replacing starch with 250 g and 10 mg of the relevant components. This method ensured that the diets included the appropriate amount of tempeh flour or daidzein while maintaining nutritional consistency. We calculated the isoflavone dosages based on previous research demonstrating their efficacy in improving bone health in both mice and humans [30]. Administering alendronate bisphosphonate at a dosage of 3 mg/kg/day over an extended period of time promoted bone remodeling and facilitated the healing of fractures in rodents that underwent ovariectomy [31]. We used rat body weight measurements to determine weekly dose adjustments for alendronate bisphosphonate.

Table 2. Dietary formulas used during the intervention with modified diets period.

Code	Group	Number of Rats	Composition
S	Sham	8	AIN 93M
O	OVX	8	AIN 93M
OB	OVX + Bisphosphonate	8	AIN 93M + Bisphosphonate
OL	OVX + Probiotic	8	AIN 93M + *L. acidophilus*
ODL	OVX + Daidzein + Probiotic	8	AIN 93M + Daidzein + *L. acidophilus*
OTL	OVX + Tempeh + Probiotic	8	AIN 93M + Tempeh + *L. acidophilus*

AIN 93M: A formulated diet that contains essential nutrients for rodents; OVX: ovariectomized rats.

The selection of probiotic dosage was informed by prior research demonstrating its beneficial impacts on both rodent and human bone health. Specifically, we referred to the study by Dar et al., who investigated ovariectomized female mice fed diets containing *L. acidophilus* with a daily dose of 10^9 CFU/day over 6 weeks [32]. The authors observed a decrease in osteoclastogenic factor expression and an increase in antiosteoclastogenic factor expression with the 10^9 CFU/day dose; however, a more significant effect was noted in postmenopausal women receiving a higher probiotic dose (10^{10} CFU/day), compared to a lower dose (2.5×10^9 CFU/day) [33]. Therefore, we chose to administer *L. acidophilus* at a dosage of 10^{10} CFU/day in our investigation. Figure 1 provides a flowchart depicting the research process from adaptation to intervention periods.

2.3. Monitoring of Body Weight, Dietary Intake, and Decapitating the Rats

During the intervention stage, rats in each group were weighed weekly using a calibrated scale (RADWAG PS 750.X2, Radom, Poland). Meticulous records of daily food consumption were maintained for each group throughout the experiment. Rats received a daily allocation of fresh food and deionized water, with any leftovers from the previous day promptly removed to maintain uninterrupted access to nourishment. This standardized protocol ensured consistent food and water availability, which is crucial for preserving the well-being of rats and facilitating their normal growth [34]. The food efficiency ratio (FER) reflects the efficiency with which animals convert consumed food into body weight, which is calculated by dividing the weight gained by the amount of food consumed during the study period. Moreover, providing fresh food and water daily and removing any remnants effectively prevented spoilage, ensuring that the rats consumed a fresh and untainted diet and water throughout the study. Three days before the end of the intervention period (i.e., three days before euthanasia), all rats in each experimental group underwent body composition analysis using Bruker's All New 2nd Generation Minispec LF90II Body Composition Analyzer, MA, USA. This assessment enabled the quantification of fat mass (in grams).

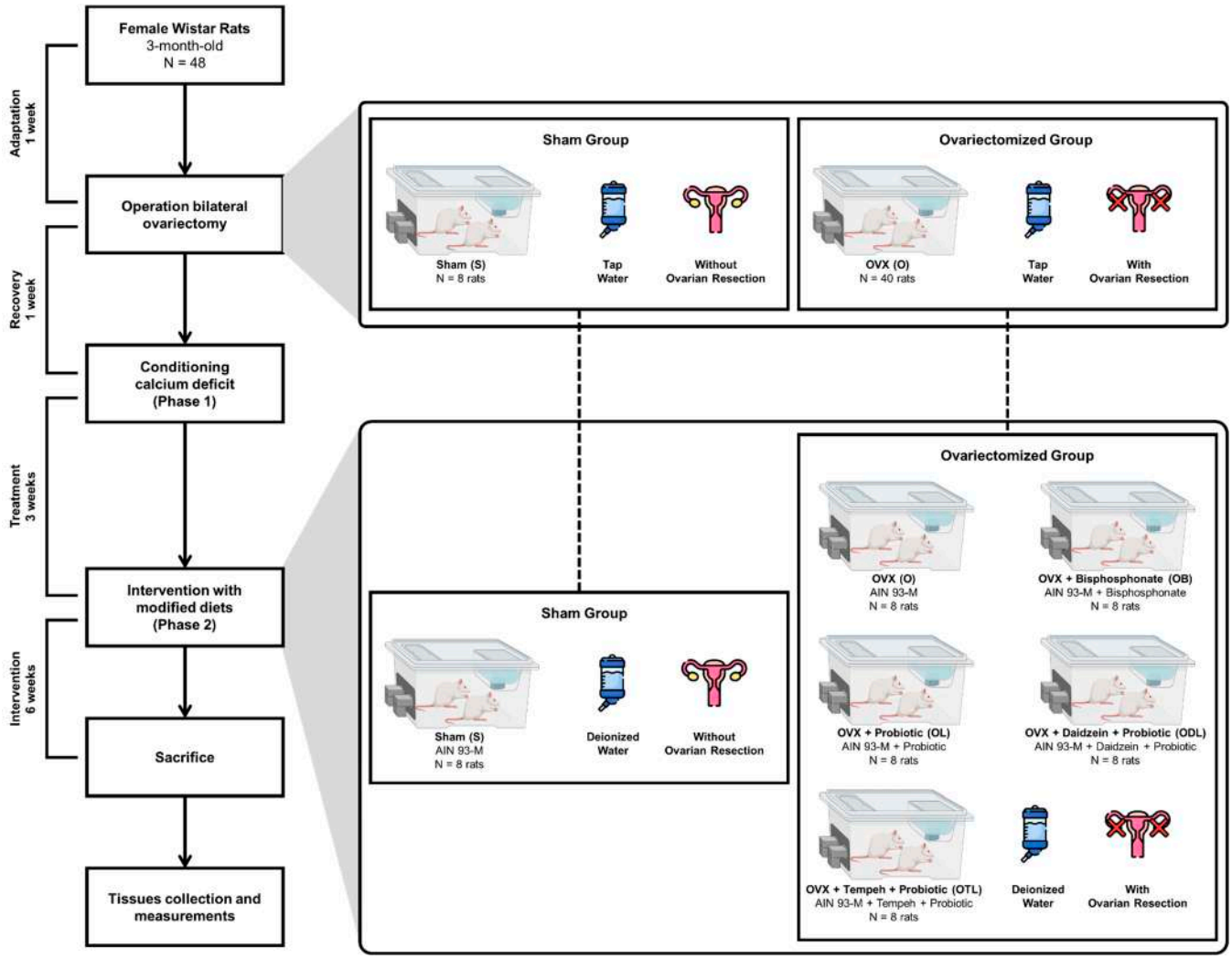

Figure 1. Experimental design from adaptation to intervention periods. AIN 93M: a formulated diet that contains essential nutrients for rodents; OVX: ovariectomized rats.

After the intervention stage, rats underwent a fasting period lasting 4–6 h before their body weight was measured using a calibrated scale. This fasting protocol aimed to mitigate any potential influence of recent food intake on subsequent weight measurements. Following the weight assessment, euthanasia was carried out via decapitation—a well-established and ethically acceptable method in experimental animal research. Decapitation ensures swift and painless termination, thereby minimizing the likelihood of distress.

2.4. Collection of Blood, Serum, Bone, and Feces

Blood samples were collected to analyze blood morphology. The serum was transferred to sterilized tubes and left to clot at room temperature for 30 min. Afterward, the samples were centrifuged at 4 °C for 15 min at 2000 rpm to separate the blood cells. Femoral bone samples were meticulously extracted, removing surrounding tissue. Fecal samples were gathered from each rat's cage. All samples were stored at −80 °C until analysis.

2.5. Analysis of Blood Morphology and Biochemistry Parameters and Femoral Bone Histopathology

Whole-blood morphological and biochemistry parameters, as well as femoral bone histopathology assessments, were conducted at Alab Laboratories in Poznań, Poland. Erythrocytes, hemoglobin, hematocrit, mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), platelets, red cell distribution width–coefficient of variation (RDW-CV), leukocytes, neutrophils, lym-

phocytes, monocytes, eosinophils, basophils percentage, alanine aminotransferase (ALT), aspartate aminotransferase (AST), cholesterol, glucose, and triglycerides were measured using standard laboratory techniques. The measurements were performed according to established protocols using automated analyzers and commercially available assay kits. The units of measurement for each parameter were as follows: erythrocytes (Y/L), hemoglobin (g/dL), hematocrit (%), MCV (fL), MCH (pg), MCHC (g/dL), platelets (G/L), RDW-CV (%), leukocytes (G/L), neutrophils (G/L), lymphocytes (G/L), monocytes (G/L), eosinophils (G/L), basophils percentage, ALT (U/L), AST (U/L), cholesterol (mg/dL), glucose (mg/dL), and triglycerides (mg/dL). For bone histopathology assessment, femoral bone samples underwent rigorous preparation, including evaluation of fixation upon arrival, followed by a 14 h de-calcification in EDTA solution and subsequent immersion in 70% ethanol for over 24 h. After confirming the adequacy of preparation, trimmed sections were sealed in labeled histology cassettes and processed using standard paraffin techniques. Histological staining with hematoxylin–eosin was performed to visualize cellular and tissue structures. Pathological evaluations were carried out by experienced veterinarian pathologists using Zeiss Axiolab 5 microscopes in Halle, Germany at magnifications of 5×, 10×, and 40×. Grading of histopathological changes and morphometric analysis of trabecular bone were conducted using established criteria and protocols. Representative areas were captured using a 3DHISTECH PANNORAMIC 250 Flash III microscope, Budapest, Hungary, and digital slides were generated with the Grundium Ocus®20 microscope slide scanner, Tampere, Finland, ensuring meticulous documentation of findings.

2.6. Analysis of Calcium Concentration

Calcium concentrations in diets and fecal samples were determined by ashing 2 g of each diet in a muffle furnace at 450 °C until complete mineralization, followed by dissolution in 1 N nitric acid (Suprapure, Merck). Meanwhile, calcium levels in femoral bone were assessed after digestion in 65% (*w*/*w*) spectra pure HNO_3 (Merck, Kenilworth, NJ, USA) using a Microwave Digestion system (Speedwave Xpert, Berghof, Eningen, Germany). Calcium concentrations in diet, serum, fecal, and bone samples were determined via flame atomic absorption spectrometry (AAS-3, Carl Zeiss, Jena, Germany) after dilution with Lanthanum (III) chloride (Merck KGaA, Darmstadt, Germany) and deionized water. The calcium content was analyzed at a wavelength of 422.7 nm. The accuracy and dependability of the method were assessed using bovine liver 1577C (Sigma-Aldrich, St. Louis, MO, USA) as a certified reference material. The study of this reference material yielded data that showed a high level of accuracy in the method used. Specifically, the accuracy rate for quantifying calcium was calculated to be 92%.

2.7. Analysis of Bone Metabolism Biomarkers

ELISA kits were acquired from Qayee Bio-Technology Co., Ltd. (Shanghai, China) for the purpose of measuring serum concentrations of bone metabolism indicators. The ELISA kits were utilized in conjunction with the LEDetect96 absorption spectrophotometry instrument from Labexim in Lengau, Austria. Pyridinoline (PYD), deoxypyridinoline (DPD), and *C*-telopeptide of type I collagen (CTX) were specifically quantified as biomarkers to assess bone resorption. Conversely, Bone Alkaline Phosphatase (BALP), Osteocalcin (OC), and Procollagen Type I *N*-Terminal Propeptide (PINP) were measured as biomarkers to evaluate bone formation.

2.8. Analysis of TRPV5 and TRPV6 Calcium Transporters

Quantitative real-time polymerase chain reaction (qRT-PCR) was utilized to evaluate the expression of calcium transporters, following the method outlined in our previous study [21]. The primers used were: Gapdh (forward: TGACTTCAACAGCGACACCCA, reverse: CACCCTGTTGCTGTAGCCAAA), TRPV5 (forward: CGAGGATTCCAGATGC, reverse: GACCATAGCCATTAGCC), and TRPV6 (forward: GCACCTTCGAGCTGTTCC, reverse: CAGTGAGTGTCGCCCATC).

2.9. Statistical Analysis

The variables were evaluated for normal distribution using the Shapiro–Wilk method. The statistical significance of the detected differences was assessed using analysis of variance (ANOVA) and Tukey's post hoc test. A significance level of 5% was used to determine if there were significant differences between the groups. Moreover, it was estimated that a statistical power of 80% could be attained when detecting significance at the 0.05 level by utilizing a sample size of 8 rodents in each group. Pearson's correlation analysis was conducted to evaluate the associations between serum calcium levels, bone metabolism indicators, and calcium transporters. The statistical analysis and creation of figures were conducted using SPSS version 22 for Windows. The data are reported as mean values along with their respective standard deviations.

3. Results

Comparing the calcium deficit (AIN_CaDef) diet to the standard diet with calcium (S & O), no significant differences were noted in their food energy calories, while differences were observed for calcium content (Table 3). The AIN_CaDef group in stage 1 exhibited a significantly lower calcium content compared to the other diet groups in stage 2. No significant differences in calcium content were observed among the S & O, OB, OL, ODL, and OTL diets.

Table 3. Energy and calcium contents in diets.

Parameter	Type of Diet					
	AIN_CaDef	**S & O**	**OB**	**OL**	**ODL**	**OTL**
	Stage 1	**Stage 2**	**Stage 2**	**Stage 2**	**Stage 2**	**Stage 2**
Energy (Kcal/g dry mass)	3947.78 ± 551.38	3883.34 ± 573.92	3850.51 ± 586.98	3936.88 ± 481.91	3924.61 ± 526.70	3865.97 ± 759.89
Calcium (mg/g dry mass)	0.02 ± 0.01 *	5.06 ± 0.30	4.66 ± 0.71	4.87 ± 0.47	5.34 ± 0.54	6.06 ± 0.88

AIN_CaDef: an AIN 93M diet without calcium content; S & O: sham and ovariectomized rats fed AIN 93M; OB: ovariectomized rats fed AIN 93M with bisphosphonate; OL: ovariectomized rats fed AIN 93M with probiotic; ODL: ovariectomized rats fed AIN 93M with daidzein and probiotic; OTL: ovariectomized rats fed AIN 93M with tempeh and probiotic. Stage 1: calcium deficiency treatment; Stage 2: treatment with dietary modifications. Data are presented as mean ± standard deviation. The composition of diets in AIN_CaDef, S & O, and OB groups were as previously described in our paper [21]. The composition diet in the intervention groups was as follows: protein content (mg/g dry mass) = OB: 133.12 ± 3.59, OL: 112.62 ± 0.67, ODL: 122.37 ± 1.45, and OTL: 196.24 ± 0.4; fiber content (mg/g dry mass) = OB: 39.48 ± 1.46, OL: 32.40 ± 0.71, ODL: 34.86 ± 1.28, and OTL: 43.57 ± 0.51; fat content (mg/g dry mass) = OB: 41.93 ± 0.26, OL: 35.23 ± 1.53, ODL: 39.18 ± 0.30, and OTL: 68.53 ± 0.55; and carbohydrate content (mg/g dry mass) = OB: 715.44 ± 144.47, OL: 776.14 ± 124.95, ODL: 753.23 ± 128.91, and OTL: 594.28 ± 188.57. * indicates statistically significant differences ($p < 0.05$) compared to the control group (AIN 93M, S & O groups).

3.1. Body Weight Gain, Body Fat Mass, and Food Intake

Table 4 presents the findings related to body weight gain, body fat mass, food intake, and calcium intake among the experimental rat groups throughout the study. In the comparison between the S and O groups, during stage 1 (aimed at inducing a calcium deficit), no significant differences in body weight gain were observed across all rat groups. However, in the final body weight, the O group had a significantly higher body weight compared to the S group. Moreover, the percentage of FER in the O group was significantly higher than in the S group. No significant differences were observed in fat mass, food intake, and calcium intake between the S and O groups.

In the comparison between the O group and the groups receiving modified diets (OB, OL, ODL, and OTL), no significant differences were found in body weight gain during both stage 1 and final body weight, fat mass, and food intake. However, the OTL group exhibited a significantly higher calcium intake, compared to the S and O groups.

Table 4. Final body weight, body weight gain, and fat mass in the rats.

Parameter	Group					
	S	O	OB	OL	ODL	OTL
Initial body weight (g)	275.88 ± 18.79	294.50 ± 20.63	292.38 ± 20.32	294.50 ± 20.14	294.63 ± 20.52	294.63 ± 20.89
Body weight gain in stage 1 (g)	22.38 ± 13.06	33.00 ± 21.84	39.13 ± 16.50	38.00 ± 9.40	31.63 ± 7.76	32.00 ± 12.63
Final body weight (g)	305.50 ± 31.51	355.38 ± 23.54	346.75 ± 28.41	352.00 ± 27.92 *	340.00 ± 36.77	344.50 ± 33.50
Final fat mass (g)	62.94 ± 29.63	86.95 ± 14.93	89.53 ± 24.69	77.83 ± 19.19	75.88 ± 35.24	56.51 ± 26.54
Food intake (g/day)	16.75 ± 1.10	17.78 ± 1.14	17.27 ± 0.96	17.54 ± 1.31	16.93 ± 1.10	16.67 ± 1.38
FER (%)	43.27 ± 47.08	156.76 ± 95.50	88.33 ± 62.97	111.19 ± 58.15	81.21 ± 87.19	107.24 ± 53.99
Calcium intake (mg/day)	85.28 ± 5.60	90.52 ± 5.79	80.96 ± 4.52	85.86 ± 6.42	90.99 ± 5.90	101.68 ± 8.42 **

S: sham rats fed AIN 93M; O: ovariectomized rats fed AIN 93M; OB: ovariectomized rats fed AIN 93M with bisphosphonate; OL: ovariectomized rats fed AIN 93M with probiotic; ODL: ovariectomized rats fed AIN 93M with daidzein and probiotic; OTL: ovariectomized rats fed AIN 93M with tempeh and probiotic. Body weight gain was calculated as the difference in body weight between the end and the beginning of each stage. FER: food efficiency ratio (weight gain (g)/food intake (g) × 100). Results of ANOVA analysis followed by Tukey's post hoc honestly significant difference test showing significant differences between types of diet. Data are presented as mean ± standard deviation. * indicates statistically significant difference ($p < 0.05$) compared to the control group (S). ** indicates statistically significant difference ($p < 0.05$) compared to both control groups (S & O).

3.2. Impact on Blood Morphological and Biochemical Parameters

Table 5 outlines the blood morphology profiles observed in rats following the 6-week intervention with modified diets. When comparing the S and O groups, the O group exhibited significantly elevated levels of blood parameters including leukocytes, neutrophils, lymphocytes, eosinophils, and cholesterol.

Table 5. Blood morphological and biochemical parameters in rats fed modified diets.

Parameter	Group					
	S	O	OB	OL	ODL	OTL
Erythrocytes (Y/L)	7.94 ± 0.40	8.18 ± 0.32	8.09 ± 0.33	8.01 ± 0.40	8.26 ± 0.30	8.41 ± 0.39
Hemoglobin (g/dL)	14.99 ± 0.65	15.25 ± 0.41	15.44 ± 0.50	15.29 ± 0.60	15.59 ± 0.30	15.98 ± 0.55 *
Hematocrit (%)	42.80 ± 1.51	43.55 ± 1.30	43.75 ± 1.87	42.44 ± 1.81	44.65 ± 0.74	45.34 ± 2.55 *
MCV (fL)	53.98 ± 1.61	53.40 ± 1.83	54.20 ± 1.24	53.05 ± 2.79	54.03 ± 1.71	53.96 ± 1.24
MCH (pg)	18.90 ± 0.96	18.69 ± 0.51	19.15 ± 0.67	19.13 ± 1.13	18.85 ± 0.81	19.03 ± 0.64
MCHC (g/dL)	35.01 ± 1.01	35.03 ± 0.65	35.31 ± 1.10	36.03 ± 0.63	34.90 ± 0.71	35.26 ± 1.06
Platelets (G/L)	836.25 ± 74.53	838.75 ± 123.39	861.88 ± 144.46	798.00 ± 82.32	732.50 ± 115.44	801.25 ± 70.78
RDW-CV (%)	12.60 ± 0.50	12.83 ± 0.42	12.83 ± 0.71	13.16 ± 0.65	12.74 ± 0.52	12.81 ± 0.34
Leukocytes (G/L)	7.34 ± 2.20	13.65 ± 2.79	13.91 ± 2.10 *	16.38 ± 4.43 *	13.35 ± 2.91 *	15.68 ± 4.89 *
Neutrophils (G/L)	0.98 ± 0.28	1.79 ± 0.58	1.88 ± 0.53 *	1.85 ± 0.54 *	1.46 ± 0.43	1.49 ± 0.31
Lymphocytes (G/L)	5.45 ± 1.96	10.67 ± 2.44	10.61 ± 1.82 *	12.98 ± 4.46 *	10.36 ± 2.46 *	13.01 ± 4.76 *
Monocytes (G/L)	0.70 ± 0.25	0.85 ± 0.32	1.00 ± 0.44	1.11 ± 0.36	1.09 ± 0.38	0.85 ± 0.26
Eosinophils (G/L)	0.20 ± 0.05	0.30 ± 0.05	0.38 ± 0.12 *	0.39 ± 0.06 *	0.41 ± 0.08 ***	0.31 ± 0.06 *
Basophils %	0.45 ± 0.15	0.29 ± 0.10	0.26 ± 0.09	0.33 ± 0.10	0.38 ± 0.10	0.26 ± 0.11 *
ALT (U/L)	37.02 ± 4.96	44.44 ± 8.23	45.79 ± 6.66	44.79 ± 13.45	46.33 ± 6.63	49.63 ± 12.31
AST (U/L)	155.01 ± 41.32	184.36 ± 40.92	167.86 ± 41.36	175.48 ± 86.04	180.98 ± 67.96	212.27 ± 112.37
Cholesterol (mg/dL)	81.03 ± 20.85	106.51 ± 12.24	110.08 ± 16.40 *	115.41 ± 17.16 *	106.45 ± 16.08 *	80.67 ± 14.02 **
Glucose (mg/dL)	115.99 ± 12.96	132.30 ± 15.53	132.59 ± 15.53	135.42 ± 8.77 *	131.59 ± 12.08	140.43 ± 11.20 *
Triglycerides (mg/dL)	210.70 ± 100.72	166.03 ± 71.82	164.48 ± 73.72	205.28 ± 114.05	125.47 ± 52.97	89.63 ± 30.74 *

S: sham rats fed AIN 93M; O: ovariectomized rats fed AIN 93M; OB: ovariectomized rats fed AIN 93M with bisphosphonate; OL: ovariectomized rats fed AIN 93M with probiotic; ODL: ovariectomized rats fed AIN 93M with daidzein and probiotic; OTL: ovariectomized rats fed AIN 93M with tempeh and probiotic. Results of ANOVA analysis followed by Tukey's post hoc honestly significant difference test showing significant differences between types of diet. Data are presented as mean ± standard deviation. * indicates statistically significant difference ($p < 0.05$) compared to the control group (S). ** indicates statistically significant difference ($p < 0.05$) compared to the control group (O). *** indicates statistically significant difference ($p < 0.05$) compared to both control groups (S & O).

In the comparison between the O group and the groups receiving modified diets (OB, OL, ODL, and OTL), the ODL group showed a significant increase in eosinophil

levels. Conversely, the OT group displayed a significant decrease in cholesterol levels, compared to the O group. Notably, when compared to the S group, the OTL group had higher hemoglobin and hematocrit levels. Furthermore, the OL and OTL groups exhibited significantly higher glucose levels, and the OTL group showed significantly lower triglyceride levels compared to the S group.

3.3. Impact on Calcium Status and Bone Metabolism Biomarkers

Table 6 presents the serum calcium, fecal calcium, and bone metabolism biomarkers measured in rats fed with modified diets. A notable decrease in the serum calcium level was observed in the O group, compared to the S group. However, no significant differences were noted for calcium levels in bone and fecal samples, as well as bone metabolism biomarkers, between the S and O groups.

Table 6. Calcium status and bone metabolism biomarkers.

Parameter	Group					
	S	**O**	**OB**	**OL**	**ODL**	**OTL**
Calcium in serum (mmol/L)	1.94 ± 0.13	1.80 ± 0.05	1.82 ± 0.07 *	1.55 ± 0.10 ***	1.53 ± 0.02 ***	1.42 ± 0.03 ***
Calcium in femoral bone (mg/g dry mass)	239.51 ± 15.32	212.13 ± 76.82	354.31 ± 42.73 ***	295.62 ± 38.92 **	309.78 ± 50.19 **	318.06 ± 50.64 ***
Calcium in fecal (mg/g dry mass)	46.38 ± 9.50	40.92 ± 10.50	39.32 ± 7.80	46.65 ± 15.30	44.23 ± 16.74	48.29 ± 0.59
PYD (ng/L)	71.41 ± 14.69	75.69 ± 4.42	84.64 ± 9.61	95.47 ± 9.11 ***	91.72 ± 10.48 *	89.65 ± 13.77 *
DPD (ng/mL)	53.59 ± 11.88	59.08 ± 7.25	62.57 ± 8.25	56.36 ± 1.51	61.54 ± 8.83	73.36 ± 5.11 **
CTX (ng/mL)	79.16 ± 3.38	85.46 ± 2.95	87.43 ± 4.81	93.68 ± 8.88 *	90.58 ± 10.29 *	89.78 ± 6.27 *
BALP(ng/mL)	37.56 ± 5.35	40.74 ± 2.51	47.34 ± 8.11 **	40.15 ± 2.20	45.59 ± 8.04	46.36 ± 6.40
OC (pg/mL)	214.54 ± 38.11	203.18 ± 8.53	224.81 ± 20.95	283.10 ± 39.76 ***	275.44 ± 42.84 ***	306.97 ± 43.15 ***
PINP (ng/mL)	8.13 ± 1.30	8.52 ± 1.53	8.54 ± 0.80	11.35 ± 1.27 ***	12.04 ± 1.51 ***	12.80 ± 1.45 ***

PYD: pyridinoline; DPD: deoxypyridinoline; CTX: *C*-telopeptide of type I collagen; BALP: Bone Alkaline Phosphatase; OC: Osteocalcin; PINP: Procollagen Type I *N*-Terminal Propeptide. S: sham rats fed AIN 93M; O: ovariectomized rats fed AIN 93M; OB: ovariectomized rats fed AIN 93M with bisphosphonate; OL: ovariectomized rats fed AIN 93M with probiotic; ODL: ovariectomized rats fed AIN 93M with daidzein and probiotic; OTL: ovariectomized rats fed AIN 93M with tempeh and probiotic. Results of ANOVA analysis followed by Tukey's post hoc honestly significant difference test showing significant differences between types of diet. Data are presented as mean ± standard deviation. * indicates statistically significant difference ($p < 0.05$) compared to the control group (S). ** indicates statistically significant difference ($p < 0.05$) compared to the control group (O). *** indicates statistically significant difference ($p < 0.05$) compared to both control groups (S & O).

Comparing the O group with the groups receiving modified diets (OB, OL, ODL, and OTL), the OL, ODL, and OTL groups showed significant decreases in serum calcium levels. Conversely, the OB, OL, ODL, and OTL groups displayed significant increases in calcium levels in the femoral bone compared to the O group.

Regarding bone metabolism biomarkers, the OL, ODL, and OTL groups exhibited significant increases in PYD and CTX levels compared to the S group. In addition, the OL group showed a significant increase in PYD levels compared with the O group. The OL, ODL, and OTL groups demonstrated significant increases in OC and PINP levels compared to the S and O groups. Furthermore, the OTL group showed a significant increase in DPD levels compared to the S and O groups, whereas the OB group presented a significant increase in BALP levels compared to the O group.

3.4. Impact on Histopathological Changes in Femoral Bone

The histopathological changes depicted in Figure 2 illustrate the significant differences between the experimental groups. Specifically, the O group showed an increased presence of medullary spaces, characterized by a larger surface area occupied by adipocytes and a corresponding area devoid of bone marrow components, indicating an osteoporotic condition compared to the S group. In contrast, the ovariectomized groups fed with different

intervention diets (OL, ODL, and OTL) showed similar effects to the ovariectomized group treated with bisphosphonate (OB), demonstrating a reduction in the surface area occupied by adipocytes within the femoral bone structure.

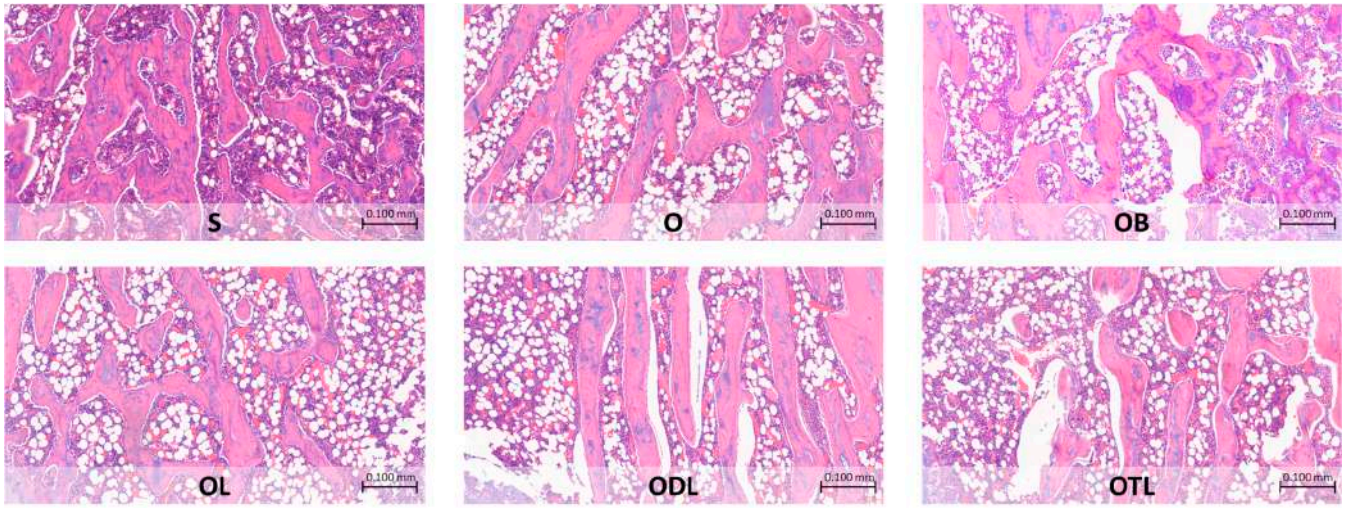

Figure 2. Histopathological changes in femoral bone after a 6-week treatment with dietary modifications. S: sham rats fed AIN 93M; O: ovariectomized rats fed AIN 93M; OB: ovariectomized rats fed AIN 93M with bisphosphonate; OL: ovariectomized rats fed AIN 93M with probiotic; ODL: ovariectomized rats fed AIN 93M with daidzein and probiotic; OTL: ovariectomized rats fed AIN 93M with tempeh and probiotic. Photos were taken under objective 10× and a scale of 0.100 mm.

3.5. Impact on Calcium Transporters

Figure 3 presents the mRNA expression levels of calcium transporters (TRPV5 and TRPV6) in the duodenum and jejunum. Comparison between the S and O groups did not reveal any significant differences in the mRNA expression of TRPV5 and TRPV6 in either the duodenum or jejunum. However, comparison of the O group with the groups receiving modified diets (OB, OL, ODL, and OTL) revealed a significant decrease in the mRNA expression of TRPV5 in the duodenum for OB, OL, ODL, and OTL. Additionally, a significant reduction in the mRNA expression of TRPV5 was observed in the jejunum of the OTL group, compared to the S group.

3.6. Correlation between Calcium Status, Calcium Transporters, and Bone Metabolism Biomarkers

In Figure 4, the Pearson's correlation analysis results are illustrated, revealing the relationships among calcium status, calcium transporters, and bone metabolism biomarkers. Figure 4A shows significant positive correlations, notably between serum calcium levels and TRPV5 expression in the duodenum ($r = 0.475$). Conversely, Figure 4B demonstrates prominent negative correlations, particularly between serum calcium levels and Procollagen Type I *N*-Terminal Propeptide ($r = -0.748$). Figure 4C,D highlight the correlations between calcium transporters and bone metabolism biomarkers, indicating a significant positive correlation between TRPV6 expression in the duodenum and deoxypyridinoline ($r = 0.365$), as well as a prominent negative correlation between TRPV5 expression in the duodenum and Procollagen Type I *N*-Terminal Propeptide ($r = -0.386$).

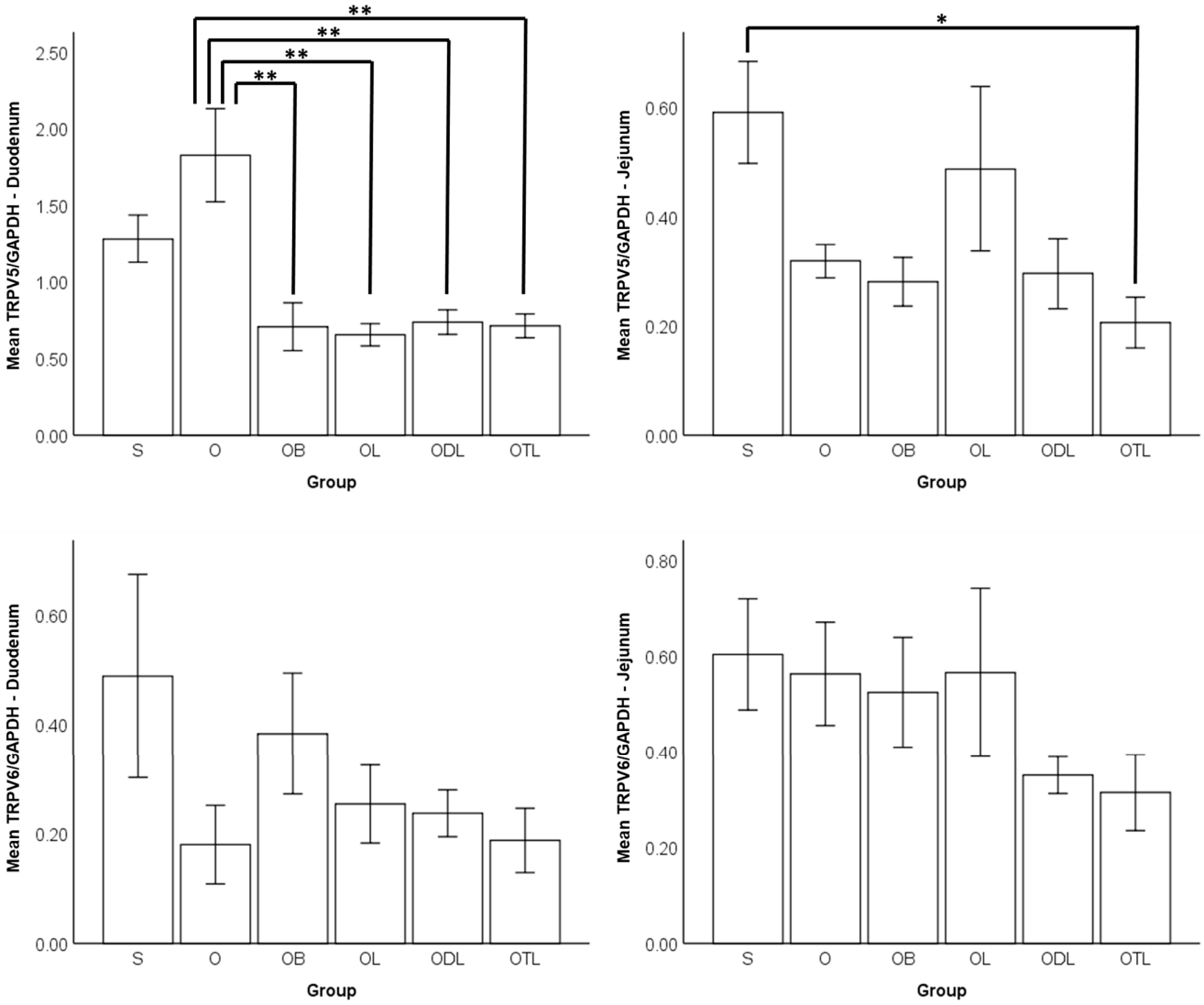

Figure 3. mRNA expression of the calcium transporters TRPV5 and TRPV6 in the duodenum and jejunum. TRPV5: Transient Receptor Potential channel family Vanilloid subgroup 5; TRPV6: Transient Receptor Potential channel family Vanilloid subgroup 6. S: sham rats fed AIN 93M; O: ovariectomized rats fed AIN 93M; OB: ovariectomized rats fed AIN 93M with bisphosphonate; OL: ovariectomized rats fed AIN 93M with probiotic; ODL: ovariectomized rats fed AIN 93M with daidzein and probiotic; OTL: ovariectomized rats fed AIN 93M with tempeh and probiotic. Values (means ± SD) are presented as cycle threshold values for gene expression analysis and as the relative abundance of TRPV5 and TRPV6 proteins normalized to the reference protein GAPDH. Results of ANOVA followed by Tukey's post hoc honestly significant difference test showing significant differences between types of diet. Data are presented as mean ± standard deviation. *: statistically significant difference ($p < 0.05$) compared to the control group (S). **: statistically significant difference ($p < 0.05$) compared to the control group (O).

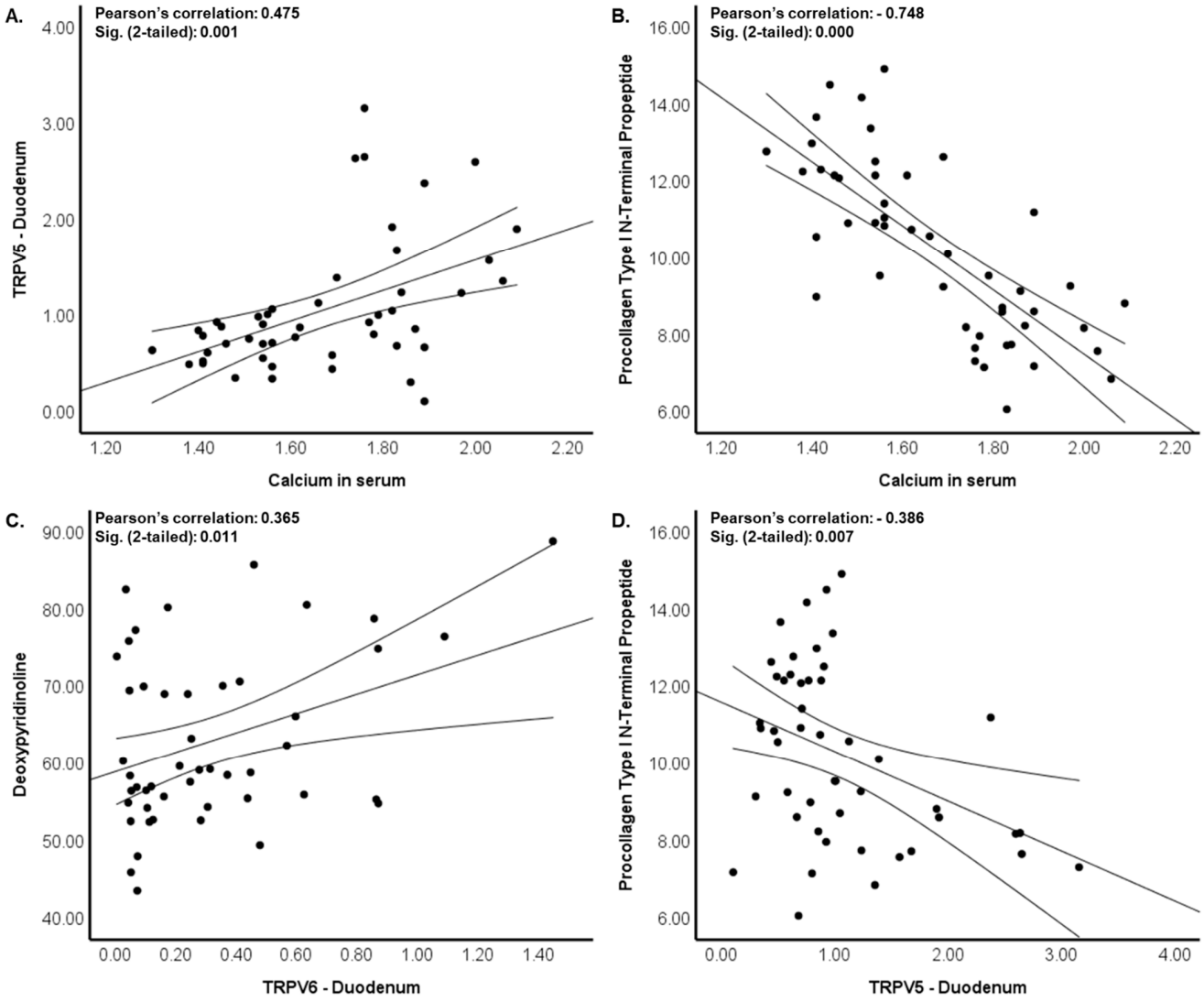

Figure 4. Pearson's correlation between calcium status, calcium transporters, and bone metabolism biomarkers. (**A**) A significant positive correlation between serum calcium levels and TRPV5 expression in the duodenum; (**B**) A significant negative correlation between serum calcium levels and Procollagen Type I N-Terminal Propeptide; (**C**) A significant positive correlation between TRPV6 expression in the duodenum and deoxypyridinoline; (**D**) A significant negative correlation between TRPV5 expression in the duodenum and Procollagen Type I N-Terminal Propeptide. TRPV5: Transient Receptor Potential channel family Vanilloid subgroup 5; TRPV6: Transient Receptor Potential channel family Vanilloid subgroup 6.

4. Discussion

In contrast to our previous studies in healthy female rats, where daily intake of pure daidzein and probiotic *L. acidophilus* did not significantly increase calcium levels in femoral bones [19] or sera [20], our current study in ovariectomized rats showed that the daily intake of a combination of probiotic *L. acidophilus* and tempeh decreased calcium levels in serum while increasing them in femoral bone. This suggests that isoflavones—especially daidzein, which is found in fermented soy foods such as tempeh—may play a role in restoring calcium balance and reducing the risk of developing post-menopausal osteoporosis. Through their estrogenic activity, these isoflavones may act as partial substitutes for declining endogenous estrogen levels, potentially regulating bone turnover and calcium homeostasis [35,36]. Additionally, the relationship between *L. acidophilus* and calcium absorption [37] may contribute to the mechanism underlying the decreasing calcium levels in sera. These

findings suggest that the combination of probiotic *L. acidophilus* and isoflavone products modulates calcium homeostasis, potentially affecting skeletal calcium levels.

A key finding of this study was that daily consumption of probiotic *L. acidophilus* and its combination with isoflavone products led to a significant increase in femoral bone calcium levels, accompanied by a reduction in serum calcium levels. This decrease in serum calcium levels could be attributed to calcium re-distribution from the bloodstream to the bone, resulting in increased calcium levels within the femoral bone. This phenomenon of calcium deposition in bone leading to a decrease in serum calcium levels is in line with previous research, indicating that interventions with probiotics and isoflavones promoting bone health can induce such calcium re-distribution [19]. Therefore, the observed decrease in serum calcium levels could potentially indicate augmented calcium deposition in bone. However, at present, this remains an observational finding without a definitive mechanism elucidated at the molecular level. Further investigations are warranted to delineate the underlying mechanisms by which *L. acidophilus* and its combination with isoflavone products influence bone metabolism and calcium status in post-menopausal osteoporotic rats.

Furthermore, consuming a combination of probiotic *L. acidophilus* and isoflavone products may have beneficial effects on both bone formation and resorption metabolism after menopause, potentially improving overall bone health. Isoflavones, as phytoestrogens found in soy products such as tempeh, have been shown to exert estrogen-like effects on bone cells, promoting osteoblast activity and inhibiting osteoclast-mediated bone resorption [38]. Additionally, probiotics such as *L. acidophilus* may influence taurine metabolism and the composition of the intestinal microbiota. Taurine—an amino sulfonic acid—is involved in modulating calcium signaling and is primarily biosynthesized in the liver. Its antioxidant properties can also improve gastric injury [39]. These combined effects may have contributed to the observed improvements in bone metabolism biomarkers among the intervention groups.

The purpose of analyzing the expression of calcium transporters—specifically, TRPV5 and TRPV6—was to understand how our dietary interventions might influence calcium absorption in post-menopausal osteoporotic rats. TRPV5 and TRPV6 are epithelial Ca^{2+} channels known for their high selectivity for calcium absorption. Our findings indicate that bisphosphonates, probiotic *L. acidophilus*, and the latter's combination with isoflavone products down-regulated TRPV5 expression in the duodenum and jejunum. This suggests a potential role of these interventions in modulating calcium absorption in post-menopausal osteoporotic rats. However, it is important to interpret these changes in mRNA expression cautiously, as they may not always correlate directly with protein activity [40].

One notable correlation we observed was a significant positive association between serum calcium levels and the expression of TRPV5 in the duodenum. This correlation aligns with our current findings: the intake of isoflavones and probiotic *L. acidophilus* simultaneously decreased calcium transporter expression and serum calcium levels. The decrease in serum calcium levels and the reduction in calcium transporter expression may result from complex regulatory mechanisms aimed at maintaining calcium homeostasis. This alteration to the calcium transport mechanisms might occur in response to changes in dietary intake, hormonal signals, or other factors to maintain overall calcium balance within cells and tissues. For instance, the parathyroid gland plays a crucial role in regulating calcium levels; primarily through parathyroid hormone, which stimulates bone resorption, enhances intestinal calcium absorption, and increases active renal calcium absorption [41]. Additionally, we observed a significant positive correlation between TRPV6 expression in the duodenum and DPD levels, suggesting that bone resorption may increase as calcium absorption decreases.

Conversely, the significant negative correlation between PINP and serum calcium levels, as well as TRPV5 expression in the duodenum, suggests an inverse relationship between calcium distribution and bone formation in ovariectomized rats. This finding aligns with our current study, where the intake of isoflavone products and probiotic *L. acidophilus* simultaneously decreased serum calcium levels and TRPV5 expression, followed

by an increase in PINP concentrations. This negative correlation may imply that increased TRPV5 expression and enhanced serum calcium levels are associated with impaired bone formation, resulting in lower levels of PINP.

In addition to these findings on calcium status and calcium transporter expression, the daily consumption of probiotic *L. acidophilus* and tempeh simultaneously resulted in significantly higher levels of hemoglobin, hematocrit, and glucose, along with significantly lower levels of cholesterol and triglycerides. Another notable finding in our study is the observed increase in leukocytes and lymphocytes among ovariectomized rats. This increase may be attributed to dysregulation of the immune system associated with estrogen deficiency resulting from ovariectomy. Estrogen plays a crucial role in modulating immune function, including regulating the production and activity of various immune cells [42]. Therefore, the removal of estrogen through ovariectomy may lead to an imbalance in immune cell populations, resulting in an increase in leukocytes and lymphocytes as part of the body's response to the hormonal changes associated with menopause.

In previous studies, probiotics such as *Lactobacillus* species have shown varying effects on hemoglobin and hematocrit levels. While our previous research demonstrated no significant changes with probiotic *L. acidophilus* alone or in combination with daidzein and genistein in healthy female rats [43], our current findings suggest that daily consumption of probiotic *L. acidophilus* and tempeh may enhance hemoglobin and hematocrit levels. This effect could be attributed to mechanisms such as improved iron bioavailability facilitated by *L. acidophilus* and the potential erythropoietic peptides generated during tempeh fermentation [44–46].

In addition, favorable outcomes were observed in terms of lipid metabolism, with a decrease in cholesterol and triglyceride levels following the daily intake of probiotic *L. acidophilus* and its combination with tempeh. These findings are consistent with previous research demonstrating the potential of dietary interventions—such as consuming tempeh and co-incubating probiotics during soy tempeh fermentation—to improve lipid profiles in diabetic rat models [47,48]. However, it is worth noting that an adverse effect on blood glucose levels was also observed, with increases observed in the OL and OTL groups. Despite this, previous studies have shown that daily consumption of tempeh led to improvements in blood glucose levels in diabetic rat models, possibly due to the presence of short-chain fatty acids, such as propionate, which enhance the uptake of glucose by skeletal muscles and regulate glucose levels through the release of GLP-1. Additionally, propionate inhibits liver gluconeogenesis and modulates lipid metabolism, thereby reducing blood glucose levels. Alterations in the gut microbiota composition—particularly, reductions in propionate producers—can lead to elevated glucose levels, highlighting the relationships between diet, the gut microbiota, and metabolic health [49]. Furthermore, the enhancement of isoflavone absorption mediated by lactic acid bacteria, such as *Lactobacillus*, suggests a potential mechanism for improving overall metabolic health [50]. Thus, while this study revealed some adverse effects on glucose levels, the observed benefits in lipid metabolism underscore the potential effects of dietary interventions involving probiotic *L. acidophilus* and its combination with tempeh.

Our findings suggest that *L. acidophilus* intake may contribute to reduced blood cholesterol and lipid levels through mechanisms involving the down-regulation of microsomal fatty acid elongation and glycerolipid metabolism pathways, as well as the inhibition of key cholesterol biosynthesis-related enzymes by soy isoflavones [51,52]. These systemic effects—although not the primary focus of our study—underscore the potential implications of our interventions beyond bone health. Future research is needed to elucidate the interconnected pathways linking glucose–lipid metabolism and bone health.

Limitations and Future Perspective of Study

In our study, several strategies were implemented to enhance the robustness and reliability of the findings. First, the inclusion of a sham group in the study design allowed for the control of potential confounding factors associated with the surgical procedure,

thus ensuring that observed effects were specifically attributable to the interventions under investigation. Additionally, the incorporation of a group treated with the drug bisphosphonate provided a valuable reference point for comparing the efficacy of the interventions against a conventional pharmacological approach commonly used in osteoporosis management. This comparative analysis with a conventional osteoporosis management drug offers valuable context for the research findings, offering insights into the potential benefits of utilizing *L. acidophilus* and its combination with isoflavone products in post-menopausal osteoporotic rats.

While our findings revealed promising trends in calcium metabolism and bone biomarkers, several limitations should be acknowledged. Our study did not include measurements of parathyroid hormone (PTH)—a crucial parameter in calcium metabolism—which limits our ability to comprehensively evaluate calcium homeostasis. Furthermore, although our research suggested that the combination of *L. acidophilus* with isoflavone products (e.g., tempeh and daidzein) might offer synergistic effects on calcium status and bone health, the role of taurine in calcium signaling and bone metabolism was not investigated. Future studies exploring taurine levels and its interactions with other biomolecules could provide valuable insights into new therapeutic approaches for osteoporosis.

Considering the growing significance of environmentally friendly approaches for handling bone health in the elderly demographic, our discoveries encourage nutritional support in the context of pharmacological treatment. Future research endeavors should explore optimal dosages, durations, and modes of administration for isoflavones and probiotics in order to maximize their therapeutic effects. Additionally, investigating potential synergistic effects with lifestyle modifications such as exercise could enhance bone health outcomes. Ultimately, the translation of our findings to clinical practice warrants human clinical studies to validate the efficacy and safety of the interventions in this population. To further enhance the depth of our research findings, future studies could incorporate the methodology to measure BMD and BMC using DEXA, in addition to analyzing bone metabolism biomarkers. This approach would allow for a comprehensive assessment of changes in bone architecture over time. Through including DEXA measurements, future research endeavors could identify any gradual changes in bone structure, providing valuable insights into the efficacy of interventions studied.

5. Conclusions

In conclusion, the daily consumption of probiotic *L. acidophilus* and its combination with isoflavone products may enhance femoral bone calcium levels while concurrently reducing serum calcium levels in ovariectomized rats. Additionally, this intervention also led to promising improvements in bone metabolism biomarkers, with comparable effects to bisphosphonate on bone histopathology. Furthermore, consumption of probiotic *L. acidophilus* and tempeh simultaneously appeared to positively influence hematological parameters and reduce cholesterol and triglyceride levels. In addition, the daily intake of probiotic *L. acidophilus* alone or in combination with tempeh resulted in elevated blood glucose levels. However, it is worth noting that the daily intake of probiotic *L. acidophilus* alone did not significantly affect bone resorption biomarkers, calcium transporter expression, or various blood parameters in ovariectomized rats, highlighting the need for further research to elucidate the potential interactions between probiotics and other nutrients in modulating bone health.

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Informed Consent Statement: Not applicable.

Data Availability Statement: All data generated or analyzed during this study are available from the corresponding author on reasonable request.

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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

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Background: Menopause poses significant health risks for women, particularly an increased vulnerability to fractures associated with osteoporosis. Dietary interventions have emerged as promising strategies, focusing on mitigating the risk of osteoporosis rather than solely addressing the established disease. This 12-week randomized controlled trial aimed to analyze the effects of consuming *Lactobacillus acidophilus* probiotics on calcium levels, biomarkers of bone metabolism, and bone mineral density (BMD) profiles in postmenopausal women.

Methods: Fifty-five participants were randomly assigned to receive either a placebo (n = 25) or the probiotic *L. acidophilus* UALa-01^{TM} (n = 30) daily via oral intervention. Throughout the study, evaluations included body composition, blood biochemical parameters, serum calcium levels, and biomarkers of bone metabolism. Additionally, Dual-energy X-ray absorptiometry was used to measure BMD profiles.

Results: The findings delineated that the probiotic group experienced a decrease in serum calcium levels compared to their initial levels. However, hair calcium levels and biomarkers related to bone metabolism showed no notable changes within this group. Consumption of probiotic *L. acidophilus* also seemed to prevent fluctuations in bone turnover markers. Moreover, there were no significant alterations in BMD levels at the lumbar spine, left femur, and total body in the probiotic group. Additionally, probiotic intake led to favorable outcomes by significantly reducing both body fat and visceral fat during the intervention period. Conversely, an adverse effect of consuming probiotic *L. acidophilus* was observed with a significant increase in glucose concentration.

Conclusion: In conclusion, the consumption of *L. acidophilus* probiotics daily for 12 weeks among postmenopausal women does not affect the profile of BMD, but it may help in stabilizing bone turnover. It is important to note that most measured parameters were within the normal range for this population. However, it is worth noting that 3 months of probiotic supplementation could potentially disrupt calcium and glucose status in postmenopausal women.

KEYWORDS

probiotic, calcium status, bone health, DXA (dual-energy X-ray absorptiometry), menopause

1 Introduction

Menopause, a natural biological transition, marks the end of a woman's reproductive years and is typically characterized by hormonal changes, particularly a decline in estrogen levels. This phase, occurring between the ages of 40 and 60 years, signifies a crucial period in a woman's life, carrying significant implications for her health and overall wellbeing. Women experiencing menopause face various health challenges due to hormonal fluctuations and metabolic changes, making them particularly vulnerable (1). Among these challenges, osteoporosis emerges as a major concern. Osteoporosis is a skeletal condition marked by reduced bone mineral density (BMD) and increased susceptibility to fractures, posing substantial health risks and complications for postmenopausal women (2, 3). According to WHO guidelines, the primary sites for measuring BMD are the proximal femur and the lumbar spine. Typically, BMD assessments are conducted on the L1–L4 section of the lumbar spine (4). Although both femurs can be assessed, the left femur is commonly chosen for diagnostic purposes as per standard practice (5).

Apart from osteoporosis, menopause is linked to a range of metabolic risks, including cardiovascular disease, obesity, and metabolic syndrome. These risks stem from changes in hormonal balance and metabolic function during menopause (6). Fragility fractures, particularly those affecting the spine and hip, contribute significantly to greater morbidity and mortality. The costs associated with fractures increased from €29.6 billion in 2010 to €37.5 billion in 2017, with fragility fractures causing 2.6 million disability-adjusted life years in European countries in 2016 (7). Moreover, menopause profoundly affects calcium status, a vital mineral crucial for bone health and overall metabolic function (8). Calcium is essential for bone formation, muscle contraction, nerve transmission, and blood clotting, playing a pivotal role in maintaining optimal health (9, 10). However, disruptions in calcium metabolism often occur during menopause, leading to imbalances in calcium levels and potentially increasing the risk of osteoporosis and other metabolic disorders (11). Understanding the interaction between menopause, calcium status, and metabolic health is crucial for developing effective strategies to mitigate the adverse health outcomes associated with menopause.

Treating menopausal symptoms presents significant challenges, particularly due to the limitations and adverse effects of hormone therapy. Although hormone therapy has traditionally been crucial for managing menopausal symptoms and reducing the risk of osteoporosis-related fractures, its use comes with notable adverse effects and safety concerns. One notable concern is the increased risk of cancer associated with prolonged hormone therapy, particularly breast cancer and endometrial cancer. These risks have led to hesitancy among healthcare providers and patients in considering hormone therapy as a long-term solution for menopausal osteoporosis (12). Moreover, existing pharmacological treatments for osteoporosis, such as bisphosphonates and SERMs (selective estrogen receptor modulators), have limitations that hinder their sustained use. While bisphosphonates effectively lower fracture risk, they are associated with gastrointestinal side effects such as esophageal irritation and ulceration, as well as rare but severe adverse events like osteonecrosis of the jaw and atypical femoral fractures (13). Similarly, although SERMs help maintain bone density and reduce fracture risk, they are associated with an increased risk of venous thromboembolism and hot flashes (14). Given the limitations and safety concerns associated with existing treatments, there is an urgent need to explore alternative therapeutic approaches that offer both effectiveness and long-term safety for menopausal women. Consequently, there is growing interest in identifying new preventive and treatment strategies that can ensure safe and effective long-term management of osteoporosis in menopausal women.

During menopause, alterations in the composition of gut microbiota, termed dysbiosis, have been observed, potentially impacting various physiological processes, including bone health (15). This phenomenon underscores the significance of the gut-bone axis, a two-way communication pathway between gut microbiota and the skeletal system (16).

Probiotics, recognized for their ability to modulate gut microbiota composition and function, have garnered attention for their potential role in regulating calcium homeostasis and bone metabolism. Specifically, probiotics have been shown to influence calcium absorption in the intestine, thereby affecting overall calcium status and bone mineralization (17). Moreover, probiotics have been implicated in regulating key biomarkers associated with

Abbreviations: BAI, body adiposity index; BMD, bone mineral density; BMI, body mass index; BSAP, bone-specific alkaline phosphatase; CONSORT, consolidated standards of reporting trials; CTX, C-terminal telopeptide of type I collagen; DXA, dual-energy X-ray absorptiometry; PINP, N-terminal propeptide of type I procollagen; SD, standard deviation; SERMs, selective estrogen receptor modulators; TRAP-5b, tartrate-resistant acid phosphatase isoform-5b; WHO, World Health Organization.

bone turnover, such as C-terminal telopeptide of type I collagen (CTX) and tartrate-resistant acid phosphatase isoform-5b (TRAP-5b), which indicate bone resorption rates (18). In addition to bone resorption markers, probiotics have also been found to impact biomarkers indicative of bone formation, including bone-specific alkaline phosphatase (BSAP) and N-terminal propeptide of type I procollagen (PINP) (19). Probiotics offer a promising approach to enhance bone health, with emerging evidence suggesting their potential to reduce risks associated with osteoporosis (20). Numerous studies have demonstrated the beneficial effects of probiotics on bone health, ranging from cellular assays to animal models and clinical trials involving menopausal conditions (21–23). Among the various probiotic strains studied, *Lactobacillus acidophilus* emerges as a particularly promising candidate. This specific strain has garnered significant attention due to its proven effectiveness in promoting bone health and preventing the progression of osteoporosis (24). *L. acidophilus* may aid in the process of osteogenic differentiation during bone mineralization, as evidenced by studies conducted on human osteosarcoma Saos-2 cells (25). In our previous research, we extensively investigated the effects of *L. acidophilus* supplementation using *in vitro* digestion models and healthy female rats (26–30), shedding light on its potential implications for calcium status and bone health.

The decision to conduct a human trial with *L. acidophilus* supplementation was rooted in several considerations. While our previous studies involving healthy rats did not yield statistically significant effects on calcium transport and bone metabolism biomarkers (27), our study involving healthy rats revealed noteworthy trends, such as a significant increase in calcium content in the femur of female rats following *L. acidophilus* DSM079 supplementation (31). It revealed promising trends suggesting a potential impact of *L. acidophilus* on calcium metabolism and bone health. Additionally, our *in vitro* cell study provided valuable insights by demonstrating that *L. acidophilus* may enhance osteogenic differentiation in Saos-2 cells (25), indicating its potential mechanisms of action on bone health. These findings underscored the need for further exploration in a human study. Despite the absence of a preclinical study specifically on a model of postmenopausal osteoporosis, existing literature has documented significant effects of *L. acidophilus* on bones and calcium metabolism in other experimental contexts (20). Therefore, based on these collective findings of *L. acidophilus* intake, we deemed it pertinent to investigate its potential benefits in postmenopausal women.

However, despite promising findings from both preclinical and clinical studies, there are still gaps in our understanding of how probiotics precisely influence the gut-bone axis and bone health. While dual-energy X-ray absorptiometry (DXA) serves as the gold standard for evaluating BMD (32), there remains a notable research gap in understanding the potential impact of probiotic supplementation on bone health in postmenopausal women. Despite extensive research on the effectiveness of probiotics in various health conditions, including gastrointestinal disorders (32) and immune function (33), few studies have explored their effects on bone metabolism and BMD profiles in this population. This dearth of research hinders our ability to fully comprehend the therapeutic potential of probiotics as a novel intervention for managing osteoporosis in postmenopausal women. Therefore, the present study aimed to investigate the effects of 12 weeks of oral and daily consumption of probiotic *L. acidophilus* UALa-01^{TM} on selected parameters of calcium status, bone metabolism biomarkers, and BMD profiles in postmenopausal women. It is crucial to emphasize that our hypothesis suggests a positive impact of probiotic supplementation with *L. acidophilus* on calcium status, bone metabolism biomarkers, and BMD profiles in postmenopausal women. This investigation seeks to address a notable research gap by shedding light on the potential benefits of probiotics in improving calcium status and bone health, as well as potentially mitigating the risk of osteoporotic fractures among this demographic. By exploring this area, we aim to provide novel insights that could have significant implications for public health strategies targeting postmenopausal women.

2 Materials and methods

2.1 Ethics of human clinical study

The clinical trial protocol for this study was approved by the Ethics Committee of Poznań University of Medical Sciences, Poland (approval no. 668/21 issued on 23 September 2021). Furthermore, the trial is registered with ClinicalTrials.gov under the identifier NCT05332626.[1] This investigation rigorously adheres to a comprehensive set of ethical and regulatory standards, as outlined in various legislations and regulations. Additionally, it complies with the CONSORT guidelines, ensuring transparent reporting of the study methodology and results. The study also aligns with the Declaration of Helsinki of the World Medical Association, which incorporates ethical principles for medical research involving humans and adheres to the guidelines set forth by the International Conference on Harmonization of Good Clinical Practice. This meticulous adherence to ethical and regulatory frameworks ensures the protection of participants' rights, safety, and wellbeing throughout the entirety of the clinical trial.

2.2 Study design: enrolment, allocation, and randomization

Initial assessments of postmenopausal patients were conducted at the Department of Treatment of Obesity, Metabolic Disorders, and Clinical Dietetics, Poznań University of Medical Sciences, Poland. To qualify for participation, individuals had to meet specific inclusion criteria, including providing written informed consent, being aged between 45 and 70 years, with a body mass index (BMI) between 27.0 and 34.9 kg/m^2, and confirming postmenopausal status by experiencing spontaneous amenorrhea for 12 months or longer. Exclusion criteria included a diagnosis of diabetes, secondary obesity, gastrointestinal diseases, recent use of dietary supplements, or pharmacotherapy for lipid disorders or hypertension within the 3 months before enrollment, presence of active clinically significant inflammatory processes, recent antibiotic intake within the month before enrollment, participation

1 https://clinicaltrials.gov/ct2/show/NCT05332626

in a body mass management study, or use of drugs known to affect body mass. Additionally, individuals who were current smokers or exhibited abuse of alcohol or drugs were also excluded from participation. Those meeting any exclusion criteria were not included in the study and were required to withdraw immediately if any exclusion criteria were identified during the course of the study. The study enrolled 64 subjects who met all inclusion criteria and provided written informed consent, undergoing the randomization procedure.

The study included 64 participants who were randomly assigned to two groups: the placebo group (n = 32) and the probiotic group (n = 32). Patient allocation was conducted in a blinded manner, ensuring both subjects and investigators remained unaware of the distribution. To uphold confidentiality, each participant received a unique identifier code from the study personnel. The randomization process was computer-generated, preventing any adjustments by the researcher directly involved with the patients. Despite stringent randomization procedures, baseline discrepancies were observed between the probiotic and placebo groups, particularly in body composition parameters and serum calcium levels. These differences may be attributed to the inherent challenges in achieving perfect homogeneity in participant characteristics, given the specific inclusion and exclusion criteria applied. While efforts were made to minimize these variations, such as rigorous screening protocols, it is acknowledged that residual confounding factors may have influenced the study outcomes. Out of the initial participants, 55 women completed the intervention and underwent subsequent statistical analysis, with 25 from the placebo group and 30 from the probiotic group. Figure 1 illustrates the study's progression through a flowchart, showing the number of patients lost to follow-up and explaining the reasons for their exclusion. Moreover, the analyses involved daily records, body composition assessment using the InBody device, serum bone metabolism biomarkers using ELISA, hair and serum calcium analysis using AAS, and bone density profiles using the DXA scanner. The study intervention and measurements were conducted between January 2022 and December 2023.

The probiotic groups were given a daily oral dose of 1×10^9 colony-forming units of *L. acidophilus* UALa-01^{TM}, with the excipient containing microcrystalline cellulose, silica, magnesium stearate, and a gelatin natural capsule. The probiotics were administered after meals in the morning time at 08:00 a.m ($\pm$ 1 h). In contrast, the placebo group received only the excipient orally, designed to be indistinguishable in taste, smell, and appearance from the probiotic mixture. The placebo also consisted of microcrystalline cellulose, silica, magnesium stearate, and a gelatin natural capsule. The study intervention lasted for 12 weeks, during which participants were explicitly instructed not to modify their regular physical activity. Figure 2, a research flowchart, visually outlines the research process and provides an overview of the assessment of collected samples.

2.3 Assessment of nutritional values of daily diet

The nutritional content of participants' daily diets was assessed using a standardized methodology involving a 3-day food recall procedure. This dietary assessment method utilized the 6.0 Diet Program (National Food and Nutrition Institute, Warsaw, Poland), known for its precision and reliability in capturing dietary intake data. Participants were given a questionnaire to record their dietary intake for 3 days before the start of the intervention and for 3 days preceding its conclusion. The 3-day food recall method allowed for the collection of detailed information on participants' dietary intake, including the types and quantities of foods consumed, meal timing, and cooking methods. Trained research personnel supervised the dietary recalls to ensure accuracy and consistency in data collection. Participants were guided through recalling their dietary intake for the specified periods, with a focus on recording all foods and beverages consumed, along with portion sizes and preparation methods. After completing the dietary recall questionnaires, the collected data were entered into the 6.0 Diet Program for analysis. This software facilitated the calculation of nutrient intakes based on reported food consumption, enabling the assessment of energy, macronutrient, and micronutrient intake levels. Specifically, nutrient compositions such as energy (Kcal), protein (gram), fat (gram), carbohydrates (gram), fiber (gram), calcium (gram), phosphorus (gram), calcium/phosphorus ratio, and vitamin D (gram) were analyzed. The analysis provided insights into the overall nutritional adequacy of participants' diets and allowed for comparisons between baseline and intervention phases to evaluate the impact of the dietary intervention on dietary quality and nutrient intake patterns.

2.4 Body composition and anthropometric measurements

Baseline and posttrial assessments included anthropometric measurements and body composition analyses. In a dedicated metabolic laboratory, all anthropometric measurements were meticulously conducted with participants wearing light clothing and no shoes, following an overnight fast and a period of rest. Body mass (kg) was measured using electronic scales with precision to the nearest 0.1 kg. BMI was calculated by dividing the mass by the square of the height (kg/m^2). Waist circumference (cm) was measured to the nearest 0.5 cm at the end of normal expiration between the iliac crest and the lower rib, providing additional anthropometric insights. Hip circumference (cm) was measured at the widest part of the buttocks, also to the nearest 0.5 cm. The waist-hip ratio was calculated by dividing the waist circumference by the hip circumference. Body composition assessment utilized the InBody 270 system (Cerritos, CA, USA), ensuring a comprehensive analysis of body composition parameters. The InBody 270 device employs a multifrequency 8-point tetrapolar touch electrode system to accurately measure body composition. By applying a range of frequencies to assess impedance across various body segments, the system allows for precise analysis of body adiposity index (BAI) (%), minerals (kg), and lean body mass (kg). Measurements were taken by placing electrodes on the hands and feet, with the device applying a low-level electrical current to measure impedance. Proprietary algorithms were used to analyze impedance data and generate body composition results within seconds. To ensure accuracy and reliability, participants stood barefoot on the device's foot electrodes

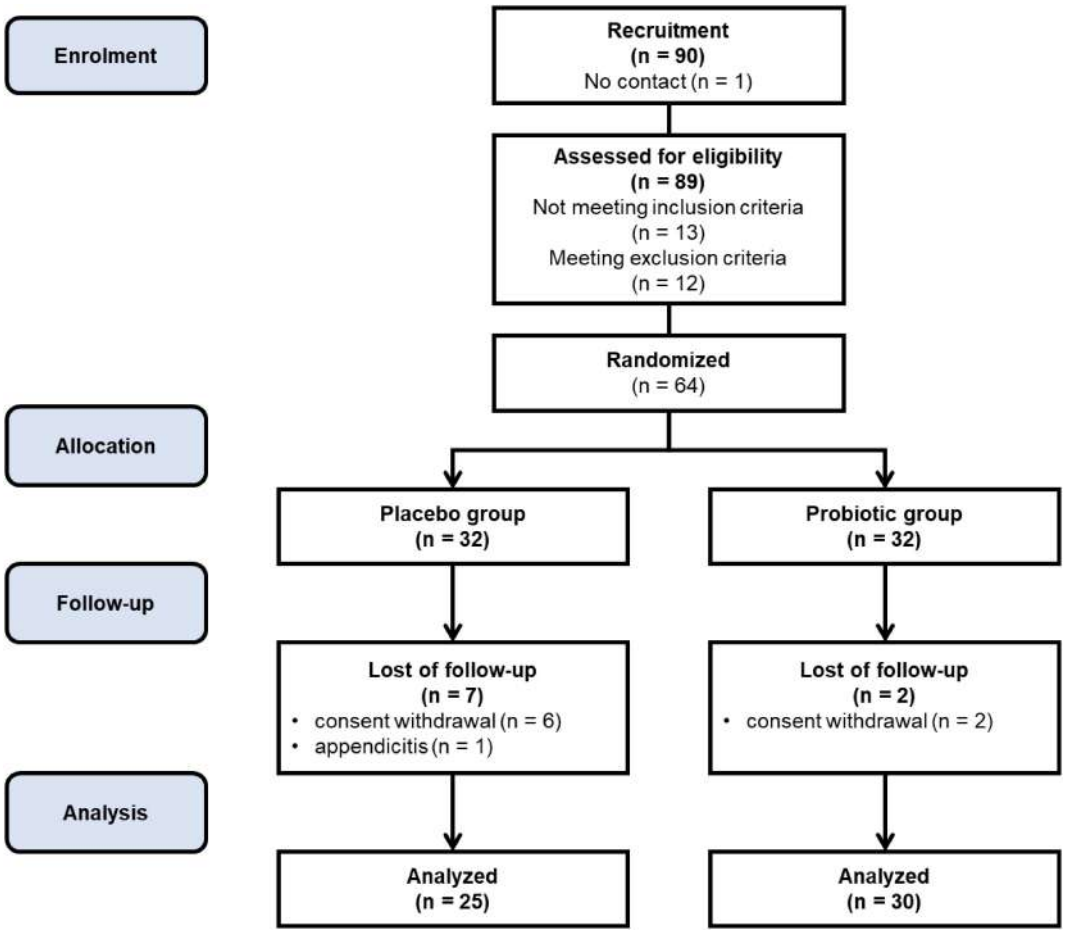

FIGURE 1
Flowchart of enrollment, allocation, and randomization of participants.

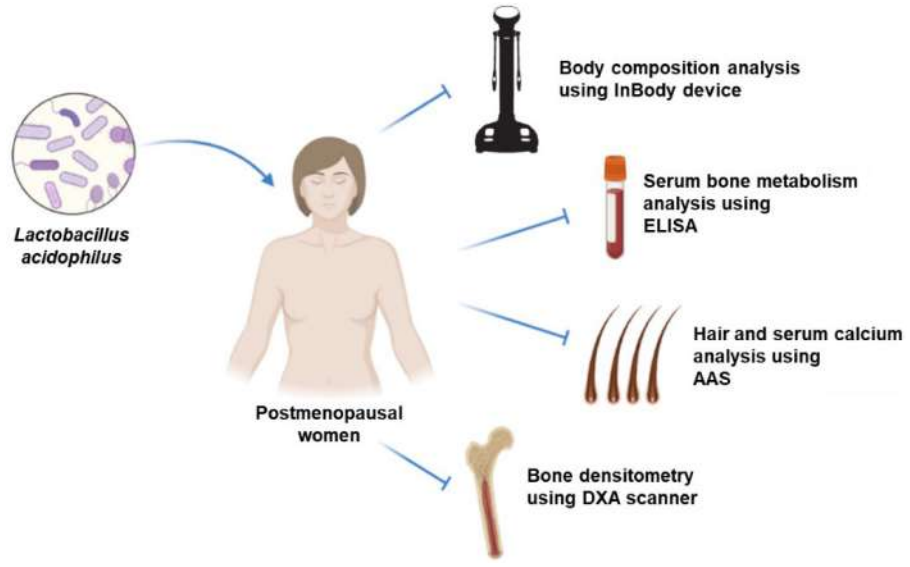

FIGURE 2
Experimental design of intervention and assessment; ELISA, enzyme-linked immunosorbent assay; AAS, atomic absorption spectrometry; DXA, dual-energy X-ray absorptiometry.

while holding onto the hand electrodes during measurements. The InBody 270 then generated a comprehensive body composition report, including values for skeletal muscle mass (kg), body fat percentage (%), and visceral fat level (point). Quality control measures were implemented to maintain consistency and precision throughout the study period. In addition, we acknowledge that certain factors related to participants' routines or health conditions could potentially affect the accuracy of the results. These factors may include hydration status, meal timing, exercise regimen, and specific health conditions affecting body composition (34). The InBody 270 was equipped with user-friendly software for easy data management and interpretation. Participant data were securely stored within the device's database, and results were automatically generated and printed for review by the research team. Before data collection, all study personnel underwent training on the proper use of the InBody 270 to minimize measurement error and ensure standardized procedures across all participants.

2.5 Serum and hair sample collection

Blood samples were obtained from a forearm vein after an overnight fast, both at baseline and upon completion of the trial. These samples were collected into serum-separating tubes for subsequent analysis of biochemical markers and minerals. Whole-blood morphological and biochemical parameters at baseline and endline were measured in a certified commercial laboratory (Alab Laboratories, Poznań, Poland) immediately after collection. The serum samples were promptly stored at −80°C to maintain their integrity until analysis. Concurrently, hair samples were gathered at the commencement and conclusion of the trial. Specifically, a hair sample was obtained from the occipital region, and when weighed as a whole, the average weight of the sample was found to be 0.5 grams. The samples were securely stored in individually labeled paper bags. Dyed and permed hair was not collected. Patients were explicitly instructed on the importance of adhering to this collection procedure for obtaining reliable results. Throughout the study, participants were instructed not to use hair spray or hair dye to ensure the purity of the collected samples. Additionally, the mass of each hair sample was meticulously measured. Hair samples were washed in acetone and deionized water, and then dried at 105°C.

2.6 Calcium content measurement

Calcium contents in serum and hair samples were analyzed using flame atomic absorption spectrometry (AAS-3, Carl Zeiss, Jena, Germany) following appropriate dilution with deionized water and the addition of 0.5% Lanthanum (III) chloride (Merck KGaA, Darmstadt, Germany). The quantification of calcium content was carried out at a specific wavelength of 422.7 nm. To assess the precision and reliability of this analytical method, certified reference materials, including human serum (Hum Asy Control 2, Sero, Billingstad, Norway) and human hair (NCS DC73347a LGC, Teddington, UK), were used. Results obtained from analyzing these reference materials demonstrated a notably high level of method accuracy, achieving a calculated accuracy rate of 91–93% for calcium quantification.

2.7 Bone biomarkers measurement

Commercial enzyme-linked immunosorbent assay (ELISA) kits procured from Qayee Bio-Technology Co., Ltd., Shanghai, China, were used along with absorption spectrophotometry (LEDetect96, Labexim, Lengau, Austria) to measure serum levels of markers associated with bone metabolism. Specifically, CTX and TRAP-5b, indicating bone resorption, were assessed, along with BSAP and PINP, biomarkers reflecting bone formation.

2.8 DXA bone mineral density assessment

All participants, both before and after the intervention, underwent a DXA scan at the Department of Human Nutrition and Dietetics, Poznań University of Life Sciences, Poland, administered by the same researcher using a GE Lunar Prodigy® machine (General Electric Healthcare, Madison, WI, USA). During the procedure, participants were asked to remove all metal components from their clothing and accessories to ensure accurate measurements. The BMD (g/cm^2) of the L1–L4 lumbar spines, left femur, and total body was assessed using the DXA software (enCORE by General Electric Healthcare, Madison, WI, US). Daily rigorous calibration and quality control of the DXA equipment were carried out to maintain the stability and reliability of the system. This meticulous approach to DXA scanning enhances the precision and credibility of the BMD assessments in our study.

2.9 Statistical analysis

IBM® SPSS® Statistics version 22 for Windows (Chicago, IL, US) was used for statistical analysis and figure generation. Measurements were presented as mean and median values, accompanied by their corresponding standard deviations with interquartile range. This approach provides a comprehensive depiction of the data distribution and variability, ensuring a thorough understanding of the central tendency and spread within the dataset. In addition to the analysis conducted, the normality of the data distribution was assessed using the Shapiro–Wilk test to ensure adherence to statistical assumptions crucial for subsequent analyses. Statistical analysis was performed to ascertain the significance of observed differences. For comparisons within the same group before and after the intervention (dependent groups), the Wilcoxon matched pairs test was used. For comparisons between the placebo and probiotic groups (independent groups), the Mann-Whitney U test was employed. The predetermined significance threshold for all observed differences was set at a 5% probability level. To investigate the relationships among serum calcium levels, bone biomarkers, and BMD profiles, Spearman's correlation analysis was employed. Furthermore, careful consideration was given to determining an appropriate sample size, with a minimum requirement of 25 subjects in each group established. This study followed the methodology outlined in the study by Soleimani et al. (35). A power calculation was performed considering a type I error (α) of 0.05 and a type II error (β) of 0.20, resulting in a power of 80%. Based on these parameters, it was determined that a minimum of 25 subjects in each group

would be necessary to detect statistically significant differences. This calculation accounted for the primary outcome measures, including changes in serum and hair calcium levels, as well as BMD profiles. This meticulous approach to assessing data distribution and determining sample size contributes to the methodological rigor of the study, enhancing its ability to produce robust and reliable findings within the specified statistical framework.

3 Results

3.1 Result of nutritional values of food recall

Table 1 depicts the dietary patterns of the study participants at baseline and endline. Before the intervention, no significant differences were observed in dietary intake variables between the placebo and probiotic groups. However, after the 12-week intervention, the probiotic group displayed a noticeable increase in energy, protein, carbohydrates, calcium, and phosphorus intake compared to the placebo group. Moreover, the placebo group exhibited a significant decrease in energy intake from preintervention to postintervention. Conversely, the probiotic group demonstrated a significant increase in dietary calcium intake at endline across all observed variables compared to the baseline period.

3.2 Result of body composition and anthropometric profiles

Table 2 displays the body composition and anthropometric profiles of study participants at baseline and endline. Before the intervention, significant differences were observed between the placebo and probiotic groups, including body mass, BMI, waist circumference, hip circumference, and BAI, with the probiotic group demonstrating lower values in these variables compared to the placebo group. Following the 12-week intervention, the probiotic group exhibited a noteworthy reduction in the percentage of body fat and visceral fat between preintervention and postintervention assessments. However, it is important to note that these changes were not significantly different when compared to the placebo group.

3.3 Result of blood morphology and biochemical parameter profiles

Table 3 illustrates the blood morphology profiles of study participants before and after a 12-week intervention. Before the intervention, significant differences were observed between the placebo and probiotic groups in hemoglobin, hematocrit, and triglyceride levels, with the probiotic group exhibiting significantly lower levels of these variables than the placebo group. Following the intervention period, the placebo group demonstrated significant decreases in hemoglobin and hematocrit values compared to the preintervention period. It is noted that the probiotic group exhibited significantly lower levels of hemoglobin, hematocrit, and triglycerides compared to placebo group at the preintervention period. Conversely, the placebo group demonstrated a significant decrease in hemoglobin and hematocrit levels following the intervention period. However, it is important to note that despite these changes, all values remained within the normal physiological range for postmenopausal women, and there were no significant differences observed between the probiotic and placebo groups. In contrast, the probiotic group exhibited a significant increase in glucose concentration from the preintervention to the postintervention period. However, it is noteworthy that no significant difference in glucose concentration was observed between the probiotic group and placebo group at the postintervention period.

3.4 Results of calcium concentration in serum, hair, and bone metabolism biomarkers

Table 4 presents the results of calcium concentration in serum, hair, and bone metabolism biomarkers of study participants before and after a 12-week intervention. Before the intervention, the placebo group exhibited significantly lower calcium concentration in serum compared to the probiotic group, although no significant differences were observed in calcium concentration in hair and bone metabolism biomarkers between the two groups at baseline.

At the endline period, no significant differences were found between the placebo and probiotic groups. Additionally, the placebo group exhibited a significant increase in serum calcium levels compared to the baseline period. Furthermore, after the 12-week intervention, the placebo group showed a significant decrease in CTX levels and a significant increase in TRAP-5b levels compared to the baseline period. In contrast, the probiotic group demonstrated a significant decrease in serum calcium levels compared to the baseline period. No significant differences were noted in hair calcium, bone resorption, and bone formation biomarkers within the probiotic group between the preintervention and postintervention periods.

3.5 Result of DXA assessment

Table 5 displays the BMD profiles of the lumbar spine, left femur, and total body before and after a 12-week intervention period. No significant changes in BMD levels were observed in either the placebo or probiotic groups from baseline to endline. However, it is worth noting that during the baseline period, the probiotic group exhibited a significantly lower BMD level at the left femur compared to the placebo group.

Table 6 presents a comparison of differences in BMD profiles assessed by DXA between the placebo and probiotic groups at baseline and endline. Although significant differences in the comparison between preintervention and postintervention periods were not observed in either the placebo or probiotic groups, it is noteworthy that the probiotic group, consumed by postmenopausal women for 12 weeks, demonstrated slightly larger differences in BMD across the spine, left femur, and total body, ranging from

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TABLE 1 Characteristics of dietary pattern from participating subjects at baseline and endline.

Variable	Group	Baseline		Endline		*P*-value	Sig.
		Mean ± SD	Median (IQR)	Mean ± SD	Median (IQR)		
Energy (Kcal)	Placebo	1,792.96 ± 502.88	1,776.50 (1,545.60–2,135.45)	1,576.95 ± 373.62	1,573.20 (1,312.45–1,799.35)	0.01	Sig.
	Probiotic	1,827.56 ± 577.35	1,883.70 (1,442.55–2,262.55)	1,922.93 ± 650.91	1,831.80 (1,423.50–2,414.75)	0.47	NS
	P-value	0.97		0.00			
	Sig.	NS		Sig.			
Protein (gram)	Placebo	77.20 ± 27.46	77.90 (66.00 –90.80)	73.62 ± 19.98	73.70 (61.60–82.15)	0.42	NS
	Probiotic	77.86 ± 22.18	80.70 (60.40–92.40)	87.50 ± 28.18	87.90 (62.45–107.55)	0.05	NS
	P-value	0.83		0.01			
	Sig.	NS		Sig.			
Fat (gram)	Placebo	70.90 ± 29.80	64.80 (51.55–94.10)	58.08 ± 22.86	54.40 (39.55–74.30)	0.05	NS
	Probiotic	68.52 ± 29.79	65.70 (44.85–82.10)	73.34 ± 32.78	72.70 (50.00–91.05)	0.73	NS
	P-value	0.51		0.06			
	Sig.	NS		NS			
Carbohydrates (gram)	Placebo	195.16 ± 63.36	196.60 (152.40–238.60)	177.04 ± 48.98	169.50 (141.15–209.40)	0.12	NS
	Probiotic	205.21 ± 74.61	204.20 (148.30–259.10)	210.04 ± 80.63	190.30 (147.45–280.60)	0.56	NS
	P-value	0.87		0.03			
	Sig.	NS		Sig.			
Fiber (gram)	Placebo	24.12 ± 7.91	25.10 (17.05–29.55)	24.34 ± 8.77	24.50 (17.60–28.75)	0.87	NS
	Probiotic	26.37 ± 10.53	26.10 (20.10–32.25)	27.09 ± 10.77	26.90 (18.75–33.60)	0.84	NS
	P-value	0.70		0.22			
	Sig.	NS		NS			
Calcium (gram)	Placebo	927.06 ± 354.63	903.50 (659.20–1,177.30)	894.72 ± 381.84	795.70 (627.65–1,068.60)	0.53	NS
	Probiotic	895.82 ± 357.92	816.40 (661.10–1,090.45)	1,115.81 ± 529.76	947.50 (716.30–1,426.00)	0.03	Sig.
	P-value	0.38		0.03			
	Sig.	NS		Sig.			
Phosphorus (gram)	Placebo	1,354.52 ± 428.68	1,409.70 (1,114.65–1,596.55)	1,314.72 ± 374.96	1,260.60 (1,050.20–1,472.35)	0.58	NS
	Probiotic	1,467.68 ± 776.82	1,391.50 (1,038.75–1,630.90)	1,563.40 ± 566.34	1,534.00 (1,179.30–1,963.45)	0.12	NS
	P-value	0.91		0.01			
	Sig.	NS		Sig.			
Calcium/phosphorus (gram)	Placebo	0.69 ± 0.17	0.71 (0.54–0.81)	0.67 ± 0.17	0.70 (0.57–0.81)	0.37	NS
	Probiotic	0.64 ± 0.18	0.66 (0.52–0.79)	0.72 ± 0.23	0.67 (0.54–0.87)	0.18	NS
	P-value	0.22		0.50			
	Sig.	NS		NS			
Vitamin D (gram)	Placebo	3.77 ± 9.31	1.70 (0.75–3.65)	5.17 ± 8.26	1.80 (1.05–4.90)	0.25	NS
	Probiotic	2.41 ± 3.10	2.00 (1.00–2.70)	2.80 ± 2.99	1.80 (0.90–3.15)	0.46	NS
	P-value	0.51		0.25			
	Sig.	NS		NS			

SD, standard deviation; IQR, interquartile range. Statistical analysis was conducted using a Wilcoxon two-related samples test with a significance threshold set at $p = 0.05$. Sig., Significance; NS, not significance.

TABLE 2 Characteristics of body composition and anthropometric profiles from participating subjects at baseline and endline.

Variable	Group	Baseline		Endline		*P*-value	Sig.
		Mean ± SD	Median (IQR)	Mean ± SD	Median (IQR)		
Body mass (kg)	Placebo	78.30 ± 14.63	75.50 (66.50–88.80)	73.88 ± 13.45	68.90 (65.60–83.30)	0.14	NS
	Probiotic	68.56 ± 13.43	65.30 (58.80–71.70)	72.22 ± 15.21	69.10 (59.60–81.40)	0.40	NS
	P-value	0.01		0.65			
	Sig.	Sig.		NS			
Body mass index (kg/m^2)	Placebo	28.58 ± 4.35	28.10 (24.70–30.90)	27.66 ± 4.41	27.60 (23.90–30.50)	0.35	NS
	Probiotic	25.29 ± 4.76	23.80 (21.90–28.60)	26.35 ± 5.00	24.00 (22.30–29.50)	0.57	NS
	P-value	0.01		0.46			
	Sig.	Sig.		NS			
Waist circumference (cm)	Placebo	96.47 ± 12.40	98.00 (86.50–105.00)	93.72 ± 12.04	93.00 (84.50–104.00)	0.39	NS
	Probiotic	86.24 ± 12.13	85.75 (76.50–90.00)	88.04 ± 13.34	89.25 (77.25–96.00)	0.62	NS
	P-value	0.01		0.28			
	Sig.	Sig.		NS			
Hip circumference (cm)	Placebo	110.78 ± 8.76	110.00 (103.75–116.50)	107.57 ± 10.51	106.00 (100.75–111.50)	0.15	NS
	Probiotic	102.48 ± 9.54	99.50 (95.00–107.75)	104.19 ± 10.71	103.00 (95.50–111.50)	0.57	NS
	P-value	0.01		0.20			
	Sig.	Sig.		NS			
Waist-Hip ratio	Placebo	0.87 ± 0.07	0.89 (0.84–0.91)	0.87 ± 0.09	0.87 (0.82–0.90)	0.48	NS
	Probiotic	0.84 ± 0.07	0.82 (0.79–0.90)	0.84 ± 0.07	0.84 (0.78–0.89)	0.99	NS
	P-value	0.14		0.25			
	Sig.	NS		NS			
Body adiposity index (%)	Placebo	34.29 ± 4.16	34.14 (31.50–37.34)	32.86 ± 5.79	33.07 (28.61–36.52)	0.14	NS
	Probiotic	30.91 ± 4.84	28.94 (27.12–35.89)	31.77 ± 5.96	30.48 (27.11–35.73)	0.60	NS
	P-value	0.02		0.41			
	Sig.	Sig.		NS			
Minerals (kg)	Placebo	3.15 ± 0.47	3.22 (2.90–3.48)	3.11 ± 0.45	3.03 (2.78–3.40)	0.82	NS
	Probiotic	3.29 ± 0.44	3.26 (3.01–3.53)	3.33 ± 0.42	3.25 (3.01–3.56)	0.23	NS
	P-value	0.28		0.06			
	Sig.	NS		NS			
Lean body mass (kg)	Placebo	44.58 ± 7.01	43.30 (41.80–48.30)	45.29 ± 5.61	44.10 (41.10–50.00)	0.44	NS
	Probiotic	46.54 ± 6.09	45.70 (42.10–52.50)	46.79 ± 5.98	46.00 (42.20–52.00)	0.47	NS
	P-value	0.47		0.48			
	Sig.	NS		NS			
Skeletal muscle mass (kg)	Placebo	26.21 ± 5.95	24.20 (23.00–28.40)	27.19 ± 7.61	24.60 (22.30–29.90)	0.34	NS
	Probiotic	25.55 ± 3.65	24.80 (22.90–29.20)	25.67 ± 3.63	25.30 (22.90–28.90)	0.54	NS
	P-value	0.94		0.98			
	Sig.	NS		NS			
Body fat (%)	Placebo	36.96 ± 7.35	37.40 (32.50–42.30)	37.29 ± 6.80	39.90 (31.60–42.10)	0.84	NS
	Probiotic	34.49 ± 8.28	34.60 (27.60–40.80)	33.91 ± 8.12	34.30 (28.30–39.90)	0.04	Sig.
	P-value	0.44		0.25			
	Sig.	NS		NS			
Visceral fat (point)	Placebo	12.96 ± 4.63	11.00 (9.00–18.00)	13.20 ± 4.45	12.00 (10.00–17.00)	0.45	NS
	Probiotic	12.00 ± 5.02	11.00 (8.00–16.00)	11.33 ± 5.27	11.00 (7.00–15.00)	0.00	Sig.
	P-value	0.48		0.29			
	Sig.	NS		NS			

SD, standard deviation; IQR, Interquartile Range. Statistical analysis was conducted using a Wilcoxon two-related samples test with a significance threshold set at $p = 0.05$. Sig., significance; NS, not significance.

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TABLE 3 Blood morphology and biochemical parameters in subjects at baseline and endline.

Variable	Group	Baseline		Endline		*P*-value	Sig.
		Mean ± SD	Median (IQR)	Mean ± SD	Median (IQR)		
WBC	Placebo	5.66 ± 0.96	5.37 (4.80–6.30)	6.03 ± 0.89	5.80 (5.59–7.00)	0.06	NS
	Probiotic	5.94 ± 1.08	5.18 (5.60–6.88)	5.32 ± 0.90	5.30 (4.65–5.90)	0.05	NS
	P-value	0.65		0.19			
	Sig.	NS		NS			
RBC	Placebo	4.59 ± 0.28	4.54 (4.35–4.82)	4.43 ± 0.35	4.49 (4.29–4.58)	0.09	NS
	Probiotic	4.41 ± 0.36	4.41 (4.24–4.59)	4.35 ± 0.29	4.35 (4.17–4.59)	0.74	NS
	P-value	0.08		0.68			
	Sig.	NS		NS			
Hemoglobin (g/dL)	Placebo	14.26 ± 0.87	14.30 (13.80–14.70)	13.55 ± 0.99	14.00 (12.90–14.20)	0.01	Sig.
	Probiotic	13.58 ± 0.93	13.80 (13.05–14.30)	13.71 ± 0.83	13.65 (13.38–14.33)	0.82	NS
	P-value	0.02		0.91			
	Sig.	Sig.		NS			
Haematocrit (%)	Placebo	41.72 ± 2.49	42.00 (40.20–43.00)	39.87 ± 2.72	41.00 (37.50–41.80)	0.01	Sig.
	Probiotic	38.79 ± 6.25	40.20 (37.40–41.63)	39.00 ± 6.28	39.80 (38.60–41.63)	0.92	NS
	P-value	0.02		0.81			
	Sig.	Sig.		NS			
Platelet	Placebo	259.42 ± 61.53	259.00 (225.00–304.00)	265.16 ± 63.17	273.00 (232.00–302.00)	0.75	NS
	Probiotic	268.84 ± 49.33	263.00 (230.00–306.50)	251.56 ± 48.95	251.00 (215.50–277.00)	0.28	NS
	P-value	0.72		0.62			
	Sig.	NS		NS			
TC (mg/dl)	Placebo	234.67 ± 44.53	231.00 (206.00–270.00)	214.22 ± 42.72	208.00 (187.00–240.00)	0.06	NS
	Probiotic	224.35 ± 49.42	226.00 (195.50–241.75)	227.58 ± 42.86	226.50 (187.75–261.00)	0.76	NS
	P-value	0.32		0.36			
	Sig.	NS		NS			
HDL (mmol/l)	Placebo	1.59 ± 0.40	1.65 (1.28–1.75)	1.57 ± 0.39	1.53 (1.32–1.74)	0.18	NS
	Probiotic	1.67 ± 0.28	1.68 (1.50–1.79)	1.74 ± 0.65	1.66 (1.48–1.95)	0.44	NS
	P-value	0.38		0.38			
	Sig.	NS		NS			
Triglycerides (mmol/l)	Placebo	1.31 ± 0.41	1.32 (0.95–1.63)	1.24 ± 0.50	1.13 (0.86–1.45)	0.41	NS
	Probiotic	1.09 ± 0.33	1.17 (0.80–1.32)	1.12 ± 0.38	1.06 (0.83–1.39)	0.79	NS
	P-value	0.02		0.24			
	Sig.	Sig.		NS			
LDL (mg/dl)	Placebo	150.44 ± 43.50	146.00 (112.00–193.00)	131.85 ± 38.19	124.00 (104.00–163.00)	0.07	NS
	Probiotic	139.81 ± 43.47	148.00 (108.75–158.50)	145.00 ± 41.27	148.00 (111.00–180.25)	0.70	NS
	P-value	0.41		0.21			
	Sig.	NS		NS			
Glucose (mg/dl)	Placebo	90.93 ± 10.31	89.00 (86.00–92.00)	89.04 ± 8.33	87.00 (83.00–95.00)	0.43	NS
	Probiotic	87.50 ± 8.76	86.00 (82.00–91.25)	96.42 ± 15.29	93.50 (89.00–97.25)	0.01	Sig.

(Continued)

TABLE 3 (Continued)

Variable	Group	Baseline		Endline		*P*-value	Sig.
		Mean ± SD	Median (IQR)	Mean ± SD	Median (IQR)		
	P-value	0.15		0.08			
	Sig.	NS		NS			
Insulin (μU/dl)	Placebo	6.63 ± 3.44	5.20 (4.00–9.00)	6.30 ± 3.18	5.40 (3.30–8.80)	0.75	NS
	Probiotic	6.07 ± 3.44	5.30 (3.23–8.40)	7.11 ± 3.89	6.75 (3.95–10.08)	0.52	NS
	P-value	0.71		0.47			
	Sig.	NS		NS			
HOMA-IR	Placebo	1.56 ± 1.07	1.14 (0.87–2.02)	1.42 ± 0.79	1.16 (0.73–2.11)	0.85	NS
	Probiotic	1.36 ± 0.86	1.02 (0.78–1.95)	1.81 ± 1.35	1.50 (0.91–2.48)	0.41	NS
	P-value	0.84		0.31			
	Sig.	NS		NS			
hs-CRP	Placebo	0.25 ± 0.20	0.18 (0.09–0.41)	0.58 ± 1.39	0.17 (0.10–0.49)	0.54	NS
	Probiotic	0.34 ± 0.48	0.10 (0.07–0.29)	0.17 ± 0.16	0.13 (0.04–0.21)	0.45	NS
	P-value	0.45		0.09			
	Sig.	NS		NS			

WBC, white blood cells; RBC, red blood cells; TC, total cholesterol; HDL, high-density lipoproteins; LDL, low-density lipoproteins; HOMA-IR, Homeostatic Model Assessment for Insulin Resistance; hs-CRP, high-sensitivity C-reactive Protein. SD, standard deviation; IQR, interquartile range. Statistical analysis was conducted using a Wilcoxon two-related samples test with a significance threshold set at $p = 0.05$. Sig., significance; NS, not significance. Hemoglobin, hematocrit, and triglyceride levels are reported, and all values fall within the normal physiological range for postmenopausal women.

-0.01 to 0.00 g/cm^2, compared to the placebo group, which showed no change.

4 Discussion

Exploring dietary interventions may reveal new therapeutic targets for managing metabolic changes and bone health during menopause. It is important to note that although we hypothesized that probiotic supplementation with *L. acidophilus* would positively impact calcium status, bone metabolism biomarkers, and BMD profiles in postmenopausal women, our results did not fully support this hypothesis. Notably, we observed a concerning decrease in serum calcium levels in the probiotic group within this population. Our current study's primary findings indicate that a 12-week daily probiotic *L. acidophilus* supplementation in postmenopausal women did not significantly influence the BMD profile. Additionally, our current findings highlighted that the observed values for various parameters, including hemoglobin, hematocrit, triglycerides, and glucose levels, remain within the normal physiological range for postmenopausal women. This suggests that while there may be some changes detected, major deviations from normal values are not to be expected.

Our main investigation extends to mineral status, with a particular focus on calcium and bone metabolism biomarkers. Menopause has been associated with changes in calcium balance and alterations in markers indicative of bone turnover (36). This study demonstrates a significant reduction in serum calcium concentrations among the probiotic group during the endline phase compared to the baseline phase, as depicted in Table 4. This result implies that consuming probiotic *L. acidophilus* may affect calcium balance in postmenopausal women. The observed decrease in serum calcium levels is consistent with findings from other studies, indicating the reliability of our results. Asemi and Esmaillzadeh (37), as well as Cheung et al. (38), have reported similar reductions in serum calcium and calcium absorption with probiotic interventions, suggesting a consistent trend in the impact of probiotics on calcium metabolism.

Moreover, in comparison to the placebo group, the probiotic group exhibited significantly increased energy, protein, and carbohydrate intake at the endline period, along with noticeable calcium intake before and after the intervention (Table 1). Surprisingly, despite significantly lower serum calcium levels in the probiotic group compared to the baseline period, this did not affect bone metabolism biomarkers (Table 3).

The mechanism underlying the decrease in serum calcium levels despite the high intake of carbohydrates, protein, phosphorus, and calcium involves several interconnected pathways. Firstly, phosphorus can form insoluble complexes with calcium in the intestines, thereby inhibiting calcium absorption. This reduced absorption contributes to lower circulating calcium levels. A reduced phosphorus intake triggers heightened synthesis of $1,25(OH)_2D_3$, leading to enhanced phosphorus absorption in the intestines. Furthermore, the heightened levels of $1,25(OH)_2D_3$ stimulate calcium absorption and raise serum calcium levels, subsequently reducing PTH levels and decreasing renal phosphorus excretion (39). Additionally, a high protein intake can elevate the excretion of calcium through the kidneys. This excessive calcium loss in urine diminishes the amount available in the bloodstream, further lowering serum calcium levels (40). Furthermore, increased protein intake can induce elevated acid production in the body, resulting in a state of metabolic acidosis (41, 42). These combined factors underscore the intricate balance among dietary composition, renal function, acid-base regulation, and bone metabolism in determining serum calcium levels.

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TABLE 4 Calcium concentration in serum and hair and bone metabolism biomarkers from participating subjects at baseline and endline.

Variable	Group	Baseline		Endline		*P*-value	Sig.
		Mean ± SD	Median (IQR)	Mean ± SD	Median (IQR)		
Calcium in serum (mmol/L)	Placebo	1.90 ± 0.23	1.93 (1.87–2.02)	2.04 ± 0.09	2.03 (1.96–2.11)	0.00	Sig.
	Probiotic	2.10 ± 0.33	2.17 (2.05–2.26)	1.88 ± 0.44	1.98 (1.82–2.16)	0.03	Sig.
	P-value	0.02		0.64			
	Sig.	Sig.		NS			
Calcium in hairs (mg/g dry mass)	Placebo	2.52 ± 1.33	2.26 (1.32–3.70)	2.42 ± 1.50	2.44 (0.94–3.23)	0.43	NS
	Probiotic	2.42 ± 1.49	2.30 (1.27–3.42)	2.15 ± 1.36	2.21 (0.85–3.17)	0.19	NS
	P-value	0.76		0.45			
	Sig.	NS		NS			
BSAP (ng/ml)	Placebo	11.49 ± 2.91	11.16 (9.04–13.64)	11.38 ± 1.96	11.90 (9.62–12.69)	0.77	NS
	Probiotic	12.24 ± 2.92	12.19 (8.99–15.01)	10.90 ± 1.56	11.26 (9.04–12.33)	0.24	NS
	P-value	0.65		0.18			
	Sig.	NS		NS			
PINP (ng/ml)	Placebo	2.73 ± 0.45	2.58 (2.37–3.29)	2.66 ± 0.52	2.36 (2.26–3.18)	0.28	NS
	Probiotic	2.42 ± 0.12	2.39 (2.32–2.58)	2.64 ± 0.52	2.46 (2.39–2.59)	0.31	NS
	P-value	0.72		0.47			
	Sig.	NS		NS			
CTX (pg/ml)	Placebo	6.24 ± 0.94	6.05 (5.61–7.22)	5.11 ± 0.52	5.29 (4.70–5.49)	0.01	Sig.
	Probiotic	5.66 ± 0.15	5.63 (5.55–5.79)	5.67 ± 0.55	5.39 (5.31–5.87)	0.61	NS
	P-value	0.14		0.14			
	Sig.	NS		NS			
TRAP-5b (ng/ml)	Placebo	18.00 ± 6.93	17.36 (13.18–24.45)	21.13 ± 6.33	18.87 (15.80–27.32)	0.04	Sig.
	Probiotic	23.43 ± 9.19	21.26 (15.71–31.06)	26.09 ± 9.31	21.69 (18.12–35.82)	0.37	NS
	P-value	0.14		0.07			
	Sig.	NS		NS			

BSAP, bone-specific alkaline phosphatase; PINP, N-terminal propeptide of type I procollagen; CTX, C-terminal telopeptide of type I collagen; TRAP-5b, Tartrate-resistant acid phosphatase isoform-5b; SD, standard deviation; IQR, interquartile range. Statistical analysis was conducted using a Wilcoxon two-related samples test with a significance threshold set at $p = 0.05$. Sig., significance; NS, not significance.

In our previous study, we investigated the effects of probiotic *L. acidophilus* DSM079 supplementation in healthy female rats, which showed that this strain did not significantly impact serum calcium levels, calcium transport, and bone metabolism biomarker values (27). Although the exact mechanism behind the decrease in serum calcium levels due to probiotic supplementation was not directly addressed in the rat study, several factors could potentially contribute to this observation. One possible explanation could be the interaction between probiotics and gut microbiota composition, which might influence calcium absorption in the intestines. Probiotics have been demonstrated to alter nutrient absorption by influencing gut microbial ecology, and alterations in microbial populations could impact calcium uptake and metabolism. Additionally, probiotics might indirectly affect calcium homeostasis by influencing factors such as intestinal pH, nutrient transporters, or regulatory pathways involved in calcium absorption and utilization (43). Further investigation into these mechanisms is necessary to fully understand the relationship between probiotic supplementation and serum calcium levels in both animal models and human subjects.

In our current study, we observed significant fluctuations in bone resorption markers, particularly CTX and TRAP-5b, within the placebo group. These findings align with prior research, such as the study by Park et al. (44) which delineated the alterations of CTX levels across various menopausal stages. Their findings revealed an increase in CTX levels with progressing menopause duration, followed by a subsequent decrease in women experiencing menopause for over 10 years (44). Additionally, Gurban et al. (45) demonstrated a notable connection between menopausal duration and bone turnover markers, particularly TRAP-5b, in osteoporotic women (45). The intricate relationship between menopause and bone turnover markers involves hormonal shifts, notably the decline in estrogen levels, which diminishes the regulatory influence on osteoclast activity and bone resorption (46, 47). CTX, reflecting collagen degradation during bone resorption, and TRAP-5b, an enzyme released by osteoclasts, act as indicators of increased bone turnover and potential bone loss

TABLE 5 Bone mineral density (BMD) profiles in dual-energy X-ray absorptiometry (DXA) assessment from participating subjects at baseline and endline.

Variable	Group	Baseline		Endline		*P*-value	Sig.
		Mean ± SD	Median (IQR)	Mean ± SD	Median (IQR)		
BMD Spine L1-L4 (g/cm^2)	Placebo	1.20 ± 0.20	1.14 (1.01–1.38)	1.18 ± 0.21	1.11 (1.03–1.38)	0.35	NS
	Probiotic	1.14 ± 0.17	1.10 (0.99–1.26)	1.14 ± 0.17	1.08 (1.01–1.30)	0.20	NS
	P-value	0.22		0.30			
	Sig.	NS		NS			
BMD Left Femur (g/cm^2)	Placebo	1.05 ± 0.13	1.03 (0.98–1.15)	1.05 ± 0.14	1.04 (0.97–1.14)	0.97	NS
	Probiotic	0.98 ± 0.12	0.97 (0.89–1.06)	0.98 ± 0.12	0.97 (0.88–1.05)	0.86	NS
	P-value	0.04		0.07			
	Sig.	Sig.		NS			
BMD Total body (g/cm^2)	Placebo	1.16 ± 0.12	1.14 (1.04–1.25)	1.15 ± 0.11	1.17 (1.07–1.23)	0.67	NS
	Probiotic	1.13 ± 0.12	1.12 (1.02–1.22)	1.12 ± 0.12	1.12 (1.04–1.21)	0.24	NS
	P-value	0.40		0.39			
	Sig.	NS		NS			

L1–L4: the first to the fourth vertebra of the lumbar spine. BMD, bone mineral density, the amount of minerals measured by DXA. SD, standard deviation; IQR, interquartile range. Statistical analysis was conducted using a Wilcoxon two-related samples test with a significance threshold set at $p = 0.05$. Sig., significance; NS, not significance.

TABLE 6 Comparison of differences in bone mineral density (BMD) profiles in DXA assessment between placebo and probiotic groups at baseline and endline.

Position	Variable	Placebo group	Probiotic group	*P*-value	Sig.
		Median (IQR)	Median (IQR)		
Spine L1-L4	Δ BMD (g/cm^2)	0.00 (−0.03 to 0.01)	0.00 (−0.02 to 0.03)	0.33	NS
Left femur	Δ BMD (g/cm^2)	0.00 (−0.02 to 0.01)	0.00 (−0.02 to 0.01)	0.67	NS
Total body	Δ BMD (g/cm^2)	0.00 (−0.03 to 0.02)	−0.01 (−0.04 to 0.01)	0.76	NS

L1–L4: the first to the fourth vertebra of the lumbar spine. BMD, bone mineral density, the amount of minerals measured by DXA. Δ BMD, a difference value between before and after intervention. SD, standard deviation; IQR, interquartile range. Statistical analysis was conducted using a Wilcoxon two-related samples test with a significance threshold set at $p = 0.05$. Sig., significance; NS, not significance.

in postmenopausal women (48, 49). These studies highlight the significance of our findings and contribute to understanding the dynamics of bone metabolism in menopausal women.

This observation suggests that probiotic consumption might contribute to stabilizing the fluctuation of these bone turnover markers over the study duration, potentially offering benefits for maintaining bone health. Fluctuations in bone turnover markers, particularly significant increases in bone resorption markers or decreases in bone formation markers, often indicate adverse effects on bone density and integrity, increasing the risk of conditions such as osteoporosis (50). By observing these fluctuations, it is suggested that consuming probiotics like *L. acidophilus* for 3 months may help mitigate the negative impacts of menopause, as evidenced by the higher concentrations of bone resorption in the placebo group.

The underlying mechanisms responsible for the observed stabilization of bone turnover markers by probiotic *L. acidophilus* are still not fully understood. Previous studies have reported potential mechanisms through which *L. acidophilus* could impact markers of bone turnover. These mechanisms often involve the modulation of gut microbiota (51–54), enhancement of mineral absorption (55), and production of bioactive compounds (56, 57) that affect bone health. The specific mechanisms may vary depending on factors such as the composition of the gut microbiota (58), individual differences (59), and the duration of intervention (60). It is worth noting that the 12-week duration of our intervention may be relatively short. In addition, our study cohort consisted of postmenopausal women without diagnosed osteoporosis, which could have influenced the observed outcomes.

In another finding from our current investigation, the consumption of probiotic *L. acidophilus* for 12 weeks did not have a significant impact on BMD levels at the lumbar spine, left femur, and total body between the preintervention and postintervention periods (Table 5). Despite demonstrating significant calcium intake before and after the intervention (Table 1), these variations in nutrient intake did not seem to affect BMD in postmenopausal women. However, it is worth noting that significant changes were observed in body fat composition. Typically, the loss of body fat is linked to improved bone health (61). However, the relatively short duration of 12 weeks in our study may have limited our ability to confirm such an association.

Our current study revealed a significant reduction in the percentage of body fat and visceral fat in the probiotic group between preintervention and postintervention assessments, as shown in Table 2. Insights from Kang et al. (62) shed light on the

potential impact of *L. acidophilus* on body weight and fat mass. In their study, mice fed a high-fat diet experienced a decrease in body weight and fat mass with *L. acidophilus* consumption. This effect coincided with the activation of brown adipose tissue, suggesting a role for *L. acidophilus* in modulating adiposity and potentially influencing metabolic processes. This reduction underscores the potential impact of probiotic *L. acidophilus* consumption on body composition and distribution of adipose tissue in postmenopausal women, suggesting avenues for future research.

Additionally, it is essential to consider the potential link between the observed changes in body composition and glucose levels. Typically, reductions in fat mass are associated with lower glucose levels (63). However, our current study revealed a significant increase in blood glucose concentration within the probiotic group from preintervention to postintervention, as demonstrated in Table 3. In addition, the probiotic group exhibited significantly higher energy, protein, and carbohydrate intakes compared to the placebo group (Table 1). Increased dietary energy and carbohydrate intake can raise blood glucose levels by stimulating insulin secretion and promoting glucose production through glycogenolysis and gluconeogenesis (64). Similarly, higher protein intake may contribute to elevated blood glucose through the gluconeogenic pathway, as amino acids can be converted into glucose in the liver (65). The findings of our current study align with previous research, indicating similar effects of probiotic consumption on glucose metabolism. For instance, the inclusion of probiotic yogurt containing *L. acidophilus* La5 and *B. animalis* subsp *lactis* Bb12 led to a significantly higher fasting glucose level in overweight men and women (66). These outcomes are consistent with a meta-analysis reviewing the effects of oral probiotic supplementation in postmenopausal women, which reported a nonsignificant reduction in glucose (67).

The observed increase in glucose levels in the probiotic group could potentially stem from several factors. Firstly, probiotics might affect glucose metabolism through interactions with the gut microbiota. Alterations in gut microbiota composition can influence carbohydrate fermentation and the production of short-chain fatty acids, which play a role in glucose homeostasis (68). Additionally, the decrease in serum calcium levels observed in the probiotic group may indirectly impact glucose metabolism. Calcium is involved in insulin secretion and sensitivity, and alterations in calcium levels can affect glucose uptake and utilization by pancreatic β-cells (69). Therefore, the decrease in serum calcium levels might have contributed to the observed increase in glucose concentration. Moreover, the slightly increased HOMA-IR index in the probiotic group suggests alterations in insulin resistance, which could further contribute to higher glucose levels. Overall, these findings suggest a complex interplay between probiotic supplementation, calcium metabolism, and glucose homeostasis, underscoring the need for further research to elucidate the underlying mechanisms. Variations in factors such as the type of probiotic administered (e.g., yogurt or capsules), the duration of the intervention, and the diversity of probiotic strains utilized could help clarify the disparities observed in research findings (67). Nevertheless, it is essential to acknowledge that participants in our study might have adjusted their dietary habits, a common occurrence during consultations with healthcare professionals. We did not monitor their dietary intake throughout the entire 12-week period. While the exact degree of importance attributed to these adjustments remains unclear, future research should aim to explore their potential impact on the outcomes of interest in similar studies. This acknowledgment underscores the need for further investigation into the relationship between dietary adjustments and study outcomes, providing valuable insights for the interpretation and generalization of study findings.

4.1 Study limitations and future perspectives

The strengths of our study are rooted in its rigorous methodology, which includes a controlled and randomized clinical design, meticulous measurement of key parameters, and comprehensive analysis of outcomes. Additionally, the use of advanced devices like the InBody for body composition assessment and DXA as the gold standard for assessing BMD enhances the reliability and precision of our findings. These robust methodological approaches bolster the reliability and validity of our findings, providing valuable insights into the potential clinical implications of probiotic supplementation in this demographic.

While our study represents a significant step forward, it is crucial to acknowledge certain limitations. These include the omission of measurements for inflammatory cytokine markers (70) and short-chain fatty acids (56), which are essential for a comprehensive understanding of the gut microbiota–bone interaction. Additionally, a detailed exploration of gut microbiota composition (51), intervention duration (59), and calcium transporter markers (71) was beyond the scope of our study, allowing for further investigation in subsequent research endeavors.

Furthermore, the short-term intervention period of 3 months in our study might not entirely capture the long-term benefits of probiotic consumption for menopausal women. Prior studies have indicated the beneficial effects of probiotics on bone health following longer intervention durations, spanning from 6 months (21, 70) to 12 months (72–74). Future studies with extended intervention periods could offer valuable insights into the sustained impact of probiotic supplementation on bone health outcomes in postmenopausal women.

Therefore, while our study provides valuable insights into the effects of probiotic supplementation on calcium status, bone metabolism biomarkers, and bone mineral density profiles in postmenopausal women, several limitations must be addressed. Firstly, we acknowledge that not screening for osteoporosis at baseline could impact the interpretation of our results, given that menopause is a known risk factor for osteoporosis. Secondly, although randomization was employed to minimize confounders, the lack of homogeneity in the study group remains a concern, potentially introducing variability among participants. Additionally, we recognize the importance of analyzing the composition of the gut microbiome at baseline and post-intervention to assess the effects of administering *L. acidophilus*, a consideration for future research. Furthermore, while our study was conducted over a 12-week period, future studies with longer durations at least a year are warranted to evaluate the sustained benefits of probiotic administration on overall bone

health. Lastly, self-reported data may be prone to recall bias and might not accurately capture the intricacies of participants' dietary habits. Moreover, relying solely on questionnaires lacks real-time or objective measures of dietary intake, potentially introducing inaccuracies into the analysis. Additionally, our study did not include continuous monitoring of dietary intake throughout the entire 12-week intervention period, representing another limitation. Continuous monitoring could offer valuable insights into changes in dietary patterns over time and their impact on study outcomes. By delving into these unexplored facets, researchers can contribute to the development of more effective nutritional interventions aimed at improving bone health in postmenopausal women.

Our study carries significant clinical implications for the bone health of postmenopausal women. The findings highlight the potential advantages of probiotic supplementation in managing bone health parameters. Understanding the effects of probiotics on calcium status, bone metabolism biomarkers, and BMD profiles offers valuable insights for healthcare providers. Integrating probiotic supplementation into tailored nutritional interventions for postmenopausal women could potentially reduce the risk of osteoporotic fractures and improve overall bone health. This study adds to the increasing body of evidence advocating for the incorporation of probiotics into clinical practice to optimize bone health outcomes in this population.

5 Conclusion

In conclusion, daily consumption of oral probiotic *L. acidophilus* UALa-01^{TM} for 12 weeks in postmenopausal women does not affect the BMD profile, but it may prevent fluctuations in bone turnover. However, such supplementation can disturb calcium and glucose status in postmenopausal women.

Further research is necessary to explore the long-term implications of probiotic consumption on calcium metabolism for postmenopausal women, to fully understand the potential benefits of probiotic interventions in this population.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The studies involving humans were approved by the Ethics Committee of Poznań University of Medical Sciences—Poland (approval no. 668/21 issued on 23 September 2021). The studies were conducted in accordance with the local legislation and institutional requirements. Written informed consent for participation in this study was provided by the participants' legal guardians/next of kin. Written informed consent was obtained from the individual(s) for the publication of any potentially identifiable images or data included in this article.

Author contribution

IH: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Validation, Visualization, Writing – original draft, Writing – review & editing. MM: Formal analysis, Investigation, Methodology, Writing – review & editing. MC-M: Formal analysis, Investigation, Methodology, Writing – review & editing. KS: Investigation, Methodology, Project administration, Writing – review & editing. PB: Methodology, Supervision, Writing – review & editing. JS: Conceptualization, Investigation, Methodology, Supervision, Validation, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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Declarations in the dissertation

Poznań, 05.08.2024

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Opinia Promotora pracy doktorskiej mgr Iskandara Azmy Harahap

Tematem pracy doktorskiej realizowanej przez mgr Iskandara Azmy Harahap jest „**Badanie wpływu izoflawonów i probiotyków na biodostępność i gospodarkę wapnia-badania in vitro i in vivo**".

Badania były realizowane w ramach dwóch projektów, których kierownikiem był mgr Harahap:
1/ Preludium, projekt finansowany przez NCN pt.: "DOBRY - [D]aidzein and pr[OB]iotic fo[R] health[Y]" A Combination of Daidzein and Lactobacillus acidophilus To Improve Calcium Status and Bone Health", nr 2021/41/N/NZ9/00838;
2/ grant promotorski finansowany przez Fundację Nutricia pt.: „Can *Lactobacillus acidophilus* decrease the risk of postmenopausal osteoporosis in women?", nr RG 3/2021.

Celem badan było określenie wpływu izoflawonów sojowych i probiotyków na biodostępność i gospodarkę wapnia oraz zdrowie kości w warunkach osteoporozy pomenopauzalnej. Cel badań został osiągnięty poprzez wykonanie badań in vitro, badań eksperymentalnych na szczurach oraz badań klinicznych z udziałem kobiet po menopauzie.
Podkreślić należy szerokie potraktowanie problemu przez Autora, który wykorzystał w badaniach zarówno źródło izoflawonów sojowych, czyli tempeh, jak i czystą daidzeinę oraz *Lactobacillus acidofilus* oraz połączenie tych czynników. Doktorant wykonał rzetelną analizę wpływu badanych czynników na odkładanie wapnia w komórkach osteoblastów ludzkich Saos-2 w badaniach in vitro, następnie sprawdził jak badane czynniki działają na biodostępność wapnia i parametry metabolizmu kości u szczurów po owakriektomii, a na koniec sprawdził działanie *L. acidophilus* w organizmie kobiet po menopauzie.
W wyniku przeprowadzonych badań Autor wykazał, że tempeh i daidzeina podawane osobno jak i w połączeniu z *L. acidophilus* zwiększają zawartość wapnia w kościach i korzystnie wpływają na metabolizm kości u szczurów. U kobiet po manopauzie probiotyki mogą stabilizować obrót kostny jednak zaburzają stężenie wapnia i glukozy w krwi. Doktorant rzetelnie opisał wyniki swoich badań, przedstawił również ich ograniczenia. Zamieszczona w dysertacji dyskusja świadczy o dojrzałości Autora, jego szerokiej wiedzy i umiejętności interpretowania wyników.

Uzyskane przez mgr Iskandara Harahap wyniki stanowią znaczący wkład w rozwój dyscypliny technologia żywności i żywienia. Mają one wartość zarówno poznawczą jak i aplikacyjną.

Moim zdaniem badania przeprowadzone przez mgr Iskandara Azmy Harahap oraz przygotowana dysertacja mają dużą wartość naukową i zasługują na wyróżnienie. Poza tym bardzo dobrze oceniam całokształt pracy naukowej mgr Harahap. Doktorant wyróżnia się dobrą organizacją pracy i sumiennością. Podczas realizacji pracy doktorskiej mgr Harahap dobrze opanował metody badawcze dotyczące przede wszystkim planowania i przeprowadzania badań eksperymentalnych na zwierzętach, analizy trawienia enzymatycznego in vitro, analizy pierwiastkowej materiału biologicznego oraz metodę testów immunoenzymatycznych ELISA. Magister Harahap jest również przygotowany do samodzielnego rozwiązywania problemów badawczych oraz pisania projektów i publikacji naukowych.
Mając na uwadze wiedzę Doktoranta, jak również doświadczenie i osiągnięcia zdobyte podczas studiów w Szkole Doktorskiej uważam, że jest wyróżniającym się młodym badaczem.

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I hereby declare that this doctoral dissertation entitled **"The study on the impact of isoflavones and probiotic on calcium bioaccessibility and calcium status – *in vitro* and *in vivo* studies"** has been prepared under my supervision and I hereby state that it meets the requirements for its submission in the degree conferral procedure.

Date 05 August 2024

Signature of the scientific supervisor of the doctoral dissertation

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Declaration of the author of the doctoral dissertation

I hereby declare that the submitted Doctoral dissertation entitled **"The study on the impact of isoflavones and probiotic on calcium bioaccessibility and calcium status – *in vitro* and *in vivo* studies"** has been written by me personally, i.e.

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I hereby declare that in the work *Harahap, I. A., Moszak, M., Czlapka-Matyasik, M., Skrypnik, K., Bogdański, P., & Sulibursk, J. (2024). 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. Frontiers in Nutrition, 11, 1401920. https://doi.org/10.3389/fnut.2024.1401920,* my individual contribution to its preparation consisted in Project Administration; Investigation; Methodology.

Date 27 th Jun

K. Skrypnik

signature

* enter as appropriate: authorship of the concept, preparation of a research project, elaboration of a critical literature review, elaboration of assumptions and methods, execution of research, including collection of data, analysis of results, interpretation of results and formulation of conclusions, statistical calculations, preparation of figures, preparation of the manuscript, others (indicate as appropriate)

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I hereby declare that in the work *Harahap, I. A., Moszak, M., Czlapka-Matyasik, M., Skrypnik, K., Bogdański, P., & Suliburska, J. (2024). 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. Frontiers in Nutrition, 11, 1401920. https://doi.org/10.3389/fnut.2024.1401920,* my individual contribution to its preparation consisted in Methodology; Supervision.

Date 21.06.2024 ..... Paw Bogdański .....

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Declaration on co-authorship

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Date 05 August 2024 .....

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Ethical Approval for Animal and Human Studies

UCHWAŁA NR **21/2021**

z dnia 21.05.2021 r.

Lokalnej Komisji Etycznej do spraw doświadczeń na zwierzętach w Poznaniu

§ 1

Na podstawie art. 48 ust. 1 pkt. 1 ustawy z dnia 15 stycznia 2015 r. o ochronie zwierząt wykorzystywanych do celów naukowych lub edukacyjnych (Dz. U. poz. 266), zwanej dalej „ustawą" po rozpatrzeniu wniosku pt.: ***„Określenie wpływu suplementacji Lactobacillus acidophilus ATCC 4356 i izoflawonów sojowych na gospodarkę wapnia u szczurów po owariektomii"*** z dnia **12.04.2021 r.**, złożonego przez **Uniwersytet Przyrodniczy w Poznaniu, Wydział Nauk o Żywności i Żywieniu**, adres **ul. Wojska Polskiego 31; 60-624 Poznań** zaplanowanego przez **prof. UPP dr hab. Joannę Sulibursk**ą

Lokalna Komisja Etyczna:

WYRAŻA ZGODĘ

Na przeprowadzenie doświadczeń na zwierzętach w zakresie wniosku.

§ 2

W wyniku rozpatrzenia wniosku o którym mowa w §1 , Lokalna Komisja Etyczna ustaliła, że:

1. Wniosek należy przypisać do kategorii: **PB10 (badania podstawowe); układ wewnątrzwydzielniczy lub metabolizm**
2. Najwyższy stopień dotkliwości proponowanych procedur to: **umiarkowany**
3. Doświadczenia będą przeprowadzane na gatunkach lub grupach gatunków: **Szczur wędrowny *(Rattus norvegicus)*, stado niekrewniacze Wistar, wiek 12 tygodni 64 samice**
4. Doświadczenia będą przeprowadzane przez: **prof. UPP dr hab. Joannę Sulibursk**ą**, mgr inż. Małgorzatę Tubacką, dr Katarzynę Skrypnik, mgr Iskandar Azmy Harahap**
5. Doświadczenie będzie przeprowadzane w terminie **od 01.06.2021 r. do 28.12.2024 r.**
6. Doświadczenie będzie przeprowadzone w ośrodku: **nie dotyczy**
7. Doświadczenie będzie przeprowadzone poza ośrodkiem **nie dotyczy**
8. Użyte do procedur zwierzęta dzikie zostaną odłowione przez ....., w sposób: **nie dotyczy**
9. Doświadczenie **nie zostanie** poddane ocenie retrospektywnej w terminie do ... miesięcy od dnia przekazania przez użytkownika dokumentacji, mającej stanowić podstawę dokonania oceny retrospektywnej. Użytkownik jest zobowiązany do przekazania ww. dokumentacji niezwłocznie, tj. w terminie, o którym mowa w art. 52 ust. 2 ustawy.

§ 3

Uzasadnienie:

Członkowie Lokalnej Komisji Etycznej do spraw doświadczeń na zwierzętach w Poznaniu (dalej: LKE w Poznaniu) po przeanalizowaniu wniosku 21/2021 pt. *„Określenie wpływu suplementacji Lactobacillus acidophilus ATCC 4356 i izoflawonów sojowych na gospodarkę wapnia u szczurów po owariektomii"* i akt sprawy dotyczących powyższego wniosku, poprzez jawne głosowanie na posiedzeniu w dniu 21 maja 2021 r., wyrazili zgodę na przeprowadzenie procedur w nim zawartych. Tym samym LKE w Poznaniu nie przychyliła się do wniosku Fundacji Międzynarodowy Ruch na Rzecz Zwierząt – Viva! (dalej: Fundacja), z dnia 25.03.2021 r., w którym to piśmie wnosiła o wydanie decyzji odmownej w powyższej sprawie. Jednocześnie LKE w Poznaniu, pragnie zauważyć, że Fundacja w piśmie z dnia 4 maja 2021 r. na odpowiedź Wnioskodawcy z dnia 9 kwietnia 2021 r., a dotyczącym wniosku 21/2021, w którym Fundacja podtrzymuje swoje stanowisko z dnia 23 marca 2021 r., wnioskuje o wydanie decyzji odmownej w sprawie innego wniosku o numerze 8/2021 pt. *„Ocena możliwości zastosowania Lactobacillus Plantarum oraz wzbogaconego w wapń miąższu dyni (Cucurbita L.) w prewencji i wspomaganiu leczenia osteoporozy pomenopauzalnej."*

LKE w Poznaniu stoi na stanowisku, że Wnioskodawca projektując doświadczenia, miał na uwadze to, aby schemat doświadczenia był adekwatny do procedur. Dlatego też procedury ściśle odnoszą się do schematu doświadczenia przedstawionego we wniosku. Ponadto Wnioskodawca w piśmie z dnia 9 kwietnia 2021 r. wskazuje, że Fundacja zarzuca mu źle rozplanowanie procedur i czynności, nie wskazując innego sposobu i nie argumentując tej uwagi. Dopiero w piśmie z dnia 4 maja 2021r. pojawia się propozycja wprowadzenia zmian w tym zakresie. Wnioskodawca jednak potrzymał swoje poprzednie stanowisko, uznając że układ procedur jest dobrze zaplanowany. Członkowie LKE w Poznaniu na posiedzeniu w dniu 21 maja 2021 r. nie wnieśli, żadnych uwag tym zakresie, w związku z tym, wniosek nie został skierowany do poprawy.

Czas trwania poszczególnych procedur jest opisany w punkcie 5 wniosku. Ponadto Fundacja zarzuca Wnioskodawcy, że czas trwania procedur nie jest adekwatny do planowanego terminu realizacji. Wydaje się, że słowem kluczowym jest tutaj określenie „planowanie". Wnioskodawca planuje ramy czasowe, w których wykona dane doświadczenia. Wydaje się, że tak przestawiony termin świadczy o dojrzałości eksperymentatora, który zadaje sobie sprawę, z możliwości wystąpienia trudnych do przewidzenia okoliczności, które mogą spowodować opóźnienie wykonania

doświadczenia. Wnioskodawca w swojej odpowiedzi wskazał chociażby czas pandemii, który pokrzyżował wielu Użytkownikom przeprowadzenie doświadczeń, a także toczące się postępowanie w sprawie tegoż wniosku. Ponadto zgodnie z art. 49 pkt. 3 Ustawy z dnia 15 stycznia 2015 r. o ochronie zwierząt wykorzystywanych do celów naukowych lub edukacyjnych (dalej: Ustawa) zgoda na przeprowadzenie doświadczenia jest udzielana na czas określony, nie dłuższy niż 5 lat, Wnioskodawca go nie przekroczył.

Dalej Wnioskodawca, wskazuje, że przytaczane przez Fundację nie dotyczą bezpośrednio celu, jaki jest stawiany w projekcie opisanym we wniosku 21/2021. Cytowane prace potwierdzają, jedynie to, że w projekcie został zastosowany właściwy model osteoporozy pomenopauzalnej. Ponadto w treści dokumentu Fundacja kilka razy podkreśla rolę żywienia w prewencji i rozwoju otyłości, zatem potwierdza ważność przedstawionego projektu, który skupia się właśnie na tym problemie. Wnioskodawca wraz z zespołem zajmuje się problemem osteoporozy pomenopauzalnej, która jest szczególnie nasilona u kobiet otyłych. Przeprowadzając badania podstawowe sprawdza możliwość zastosowania kombinacji różnych składników żywności, których spożywanie może zahamować rozwój osteoporozy lub też umożliwić zmniejszenie dawek leków stosowanych w tej chorobie. Szczególnie, że obecne metody leczenia osteoporozy, takie jak bisfosfoniany, nie są zalecane do długotrwałego stosowania.

W piśmie z dnia 25 marca 2021 r. Fundacja proponuje jako alternatywę doświadczenia na zwierzętach doświadczenie z udziałem ludzi. Zakres oznaczeń zaplanowanych *post mortem* w planowanym doświadczeniu wychodzi daleko poza oznaczenie tylko parametrów surowicy krwi ludzkiej. W kolejnym zdaniu Fundacja wskazuje, że ocenę zmian histopatologicznych u ludzi można wykonać dzięki trepanobiopsji tzw. wycinającej, która odbywa się przy użyciu specjalnego wiertełka zwanego trepanem (...). Można także przeprowadzić badanie za pomocą biopsji gruboigłowej pobierając fragment kości (...)" Poza tym Fundacja zachęca to wykorzystania metod z użyciem pierwiastków izotopowych. Zarówno członkom LKE jak i Wnioskodawcy trudno było zrozumieć jak można wskazać tak inwazyjny eksperyment medyczny z udziałem ludzi i wskazać go jako alternatywę dla badań żywieniowych na zwierzętach. Propozycja Fundacji odnośnie zaplanowania eksperymentu żywieniowego na ludziach, w którym miałyby być stosowane metody inwazyjne takie jak biopsja czy trepanobiopsja wydaje się absolutnym brakiem wyważenia korzyści i strat płynących z tego typu zabiegu. Ten punkt opinii i takie postawienie sprawy przez Fundację w ocenie komisji dyskwalifikuje jej stanowisko w sprawie niniejszego wniosku. Wydaje się, ze Fundacja chcąc złagodzić swoje stanowiska z marca, w następnym pisze, „że jedynie przedstawia dostępne badania, które wykonuje się na ludziach" jednocześnie powołując się na to, że przytacza też mało inwazyjne badania, tylko nie zważa na to, że nie dadzą one Wnioskodawcy wystarczającej odpowiedzi na

problem badawczy. Oczywistym, jest to, że lekarze wykonują wiele badań, czasami bardzo inwazyjnych, nowatorskich itp. ale dostosowują je do rodzaju i stopnia zaawansowania choroby, czyli inaczej mówiąc lekarz musi rozważyć, czy potencjalne korzyści z takiego działania nie przewyższą strat, kierując się zasadą *„Po pierwsze nie szkodzić"*. Ponadto wskazuje również na Kodeks Etyki Lekarza, który odnosi się do wykonywania badań naukowych z udziałem ludzi. W rozdziale II „Badania naukowe i eksperymenty biomedyczne" Kodeksu w Art. 42a. 1. czytamy „Lekarz przeprowadzając eksperyment leczniczy nie może narażać pacjenta na ryzyko w istotnym stopniu większe niż to, które grozi osobie nie poddanej temu eksperymentowi." i dalej w Art. 45.1 „Lekarz uczestniczący w eksperymentach medycznych musi je przeprowadzać zgodnie z zasadami badań naukowych. Eksperymenty z udziałem człowieka powinny być poprzedzone badaniami *in vitro* oraz *in vivo* na zwierzętach. Zwierzęta poddawane eksperymentom należy odpowiednio traktować i w miarę możliwości chronić przed cierpieniem."

LKE w Poznaniu już w tym miejscu pragnie podkreślić, że zaplanowane procedury we wniosku 21/2021 są zgodne z art. 5 ust.1 punkt 1., ponieważ projekt zakłada ocenę wpływu stosowanej interwencji na biodostępność wapnia, jego wykorzystanie w organizmie oraz metabolizm kości. Oznaczenie zawartość tego pierwiastka w narządach wewnętrznych oraz zbadanie struktury kości są możliwe do wykonania tylko na modelu zwierzęcym.

Fundacja zwraca uwagę na zaplanowany w projekcie lek - alendronian. W doświadczeniu zaplanowano zostanie alendronian w dawce 30mg/kg diety (a nie jak podaje Fundacja 30 mg /kg masy ciała) wraz z dietą z odpowiednią zawartością wapnia. Lek jest dobrze tolerowanym lekiem stosowanym w profilaktyce i leczeniu osteoporozy po menopauzie oraz z powodzeniem stosowany w badaniach modelowych osteoporozy pomenopauzalnej (Mohamed et al., Biomed Pharma 2019;89:1115-1124; Piao et al. Biomed Eng Biomed Tech 2019, doi:10.1515/bmt-2018-0087; Jiang et al., J. Oral. Pathol. Med. 2016, doi:10.1111/jop.12499). W doświadczeniu zastosowany będzie alendronian sodu. Ponadto, Fundacja dokładnie opisuje budowę chemiczną, stosowanie leku u ludzi /etc/, co Wnioskodawcy jest doskonale znane z racji posiadanych kwalifikacji przedstawionych w punkcie 2 wniosku. Wnioskodawcy, który posiada stopień doktora nauk farmceutycznych budowa jak i działanie tego leku jest znana i nie musi posiłkować się krótkimi, wybranymi przez Fundację fragmentami z wielostronicowej charakterystyki produktu leczniczego. Biodostępność alendronianu jest niższa, gdy jest przyjmowany z pokarmem, dlatego u ludzi zaleca się przyjmowanie go na czczo. Jednak w projekcie, aby uniknąć podawania leku sondą dożołądkową i związanych z tym dodatkowych cierpień i stresu dla szczurów oraz uniknięcia uszkodzenia przewodu pokarmowego i pojawienia się ewentualnych stanów zapalnych z tym związanych, zaplanowano podawanie leków z pokarmem. Dawka jest odpowiednio dostosowana i obliczona na podstawie danych literaturowych.

W stanowisku Fundacji czytamy „w dostępnej literaturze są już opublikowane badania wpływu alendronianu i/lub simwastatyny na szczury po owariaktomii będące na diecie wysokotłuszczowej". Jak podkreśla Wnioskodawca, zgadza się z tym stwierdzeniem i Fundacja podaje doskonały przykład artykułu, w którym wykazano przeciwosteoporotyczne działanie alendronianu. Jednocześnie zaznacza, że w zaplanowanym projekcie grupa z alendronianem jest grupą porównawczą, do której właśnie będzie porównane działanie daidzeiny, genisteiny, tempehu i *Lactobacillus acidophilus* na metabolizm kości.

W stanowisku Fundacji, można przeczytać, że izoflawony sojowe, bakterie Lactobacillus oraz tempeh były badane i mają wpływ na organizm, w tym na układ kostny. W tekście cytowane są szczególnie artykuły, gdzie badano wpływ tych czynników żywieniowych na parametry związane z chorobami sercowo-naczyniowymi, chorobami tarczycy, cukrzycą i otyłością. Poza tym, przywołane są również prace, gdzie wykazano, że wpływ tych czynników na metabolizm kości nie jest jednoznaczny (np. pozycja 16). Wnioskodawca stwierdza, że cytowane badania nie mają bezpośredniego związku z celem projektu. W punkcie 5 wniosku wyraźnie zostało zaznaczone, że poszczególne czynniki żywieniowe, które planuje się wykorzystać w badaniu, mają działanie osteoprotekcyjne i zostały poparte cytowaniami. Planowana kompozycja składników jest autorska, stworzona przez osobę planującą doświadczenia i została wybrana właśnie z uwagi na ich możliwe synergistyczne i pozytywne działanie na metabolizm kostny i na estrogeny. Dlatego, Fundacja nie może zarzucić Wnioskodawcy, że takie badania zostały już wykonane. Fundacja skupiła się na tym, aby wyszukać badania wpisując osobno poszczególne słowa kluczowe w bazę danych i osobno opisać znalezione doświadczenia, o czy świadczą poszczególne akapity stanowiska, nie zważając na to, że problem badawczy jest zupełnie inny. Ponadto Wnioskodawca składając wniosek w punkcie 9 opisuje w jaki sposób zaplanował projekt badawczy. Badanie pozwoli sprawdzić korzystne działanie połączenia tempehu lub izoflawonów sojowych z *Lactobacillus acidophilum* na metabolizm kostny oraz ocenić ewentualnie niekorzystny wpływ tego połączenia na organizm szczurów z osteoporozą. To niekorzystne działanie może wynikać z nieznanych interakcji między izoflawonami sojowymi, składnikami tempehu a *Lactobacillus acidophilus* jak i z niekorzystnego wpływu tych składników na zmiany fizjologiczne i metaboliczne w organizmie. Połączenie nawet nieszkodliwych substancji o znanym działaniu może bowiem wywołać nieprzewidziane skutki uboczne. Takie kompleksowe zbadanie wpływu planowanej interwencji na organizm umożliwiają badania na modelu zwierzęcym. Fundacja zarzuca Wnioskodawcy, że nie podaje w opisie projektu ilości tempehu jaką mają spożywać szczury. Ilość tempehu została jednak podana w punkcie 5 wniosku, jak i w opisie procedur.

Fundacja wskazuje, że Wnioskodawca nie odniósł się odpowiednio do zasady udoskonalenia i zastosował tylko szklane miseczki jako materiał wzbogacający. Takie postępowanie jest uzasadnione tym, że w badaniach żywieniowych każdy czynnik, który może być spożyty przez zwierzę, może mieć istotny wpływ na wynik badania, unika się zatem wprowadzania innych materiałów do klatki, które są przez zwierzęta zjadane. Ponadto na posiedzeniu w 14 lutego 2020 r. był , rozpatrywany wniosek nr 57/2019 tego samego Wnioskodawcy, również był to projekt żywieniowy, w którym wpisał jako urozmaicenia szklane miseczki. Pełnomocnik Fundacji, która równocześnie jest członkiem LKE w Poznaniu, zgłosiła zapytanie dotyczące, braku ściółki i typowych dla gryzoni urozmaiceń, proponując w zamian szklane domki lub kule. Na komisji wyjaśniono powody ograniczenia ściółki, a funkcję szklanych domków i kul, których trudno szukać na rynku jako urozmaiceń dla szczurów, dlatego właśnie z powodzeniem spełniają te funkcje szklane miseczki. Wspomniany wniosek otrzymał zgodę komisji. Rok później Wnioskodawca złożył kolejny wniosek żywieniowy 8/2021, zresztą dobrze znany Fundacji, z racji jej udziału w postępowaniu na prawach strony, który pierwszy raz był rozpatrywany 22 stycznia 2021 r., (LKE w Poznaniu głosowała nad dopuszczeniem Fundacji w postępowaniu administracyjnym w dniu 26.02.2021 r.) i wtedy w uwagach dr Arlety Sierakowskiej nie było już zarzutu o brak urozmaiceń. Dlatego dużym zaskoczeniem jest to, że ten temat powrócił w stanowisku Fundacji. Wskazane przez Fundację podłoża i urozmaicenia, nie są możliwe w stosowaniu doświadczeniach żywieniowych. Co więcej niektórzy eksperymentatorzy, nie tylko prowadzący badania żywieniowe, rezygnują również z plastikowych urozmaiceń np. tuneli, ponieważ są one właśnie skutecznie obgryzane przez gryzonie, co w dobie odchodzenia od plastiku, właśnie ze względu na szkodliwość dla organizmu nie trzeba tłumaczyć. Jak widać nie zawsze założenia teoretyczne, mają przełożenie w praktyce. Zwierzęta będą miały codzienny kontakt z człowiekiem (z jego dotykiem, głosem i zapachem), bowiem codziennie będzie sprzątana klatka, będzie podawane jedzenie i woda. Klatki ze zwierzętami ustawione są szeregowo, mają otwory, ściany są przezroczyste szczury zatem czują swój zapach, słyszą się nawzajem i widzą się. Warunki życia szczurów są zgodne z Dyrektywą 2010/63/UE z dnia 22 września 2010 r. w sprawie ochrony zwierząt wykorzystywanych do celów naukowych.

Kolejne zagadnienie poruszane przez Fundację, to brak lekarza weterynarii w punkcie 8 wniosku. Obowiązkiem Użytkownika jest zawarcie umowy z lekarzem weterynarii, o czym mówi art. 23 Ustawy, na co zresztą Wnioskodawczyni wskazała w odpowiedziach. W związku z tym lekarz weterynarii nie musi być wpisany w punkcie 8 wniosku, ponieważ świadczy on w ośrodku usługi weterynaryjne z racji zatrudnienia wynikającego z wymogów Ustawy. Swoją wiedzą i doświadczeniem zawodowym obejmie nie tylko zwierzęta z tego wniosku, ale z każdego innego, który będzie realizowany w ośrodku. Dlatego też podczas całego doświadczenia, również w czasie

operacji zwierzęta będą pod nadzorem weterynarza - lek. wet. Piotra Kempskiego, który sprawuje opiekę nad zwierzętarnią Wydziału Nauk o Żywności i Żywieniu Uniwersytetu Przyrodniczego w Poznaniu w Poznaniu, gdzie doświadczenie będzie przeprowadzone.

Zgodnie z sugestią Fundacji zmieniona została dawka ketaminy i cepetora na odpowiednio: 50-75 mg/kg (K) i 1 mg/kg (Cept), Dodatkowo zastosowany będzie lek przeciwbólowy meloksykam (dootrzewnowo) w dawce 1-2 mg co 24 h przez pierwsze 3 dni po operacji. Podczas operacji będzie zastosowana maść chroniąca oczy przed wysychaniem rogówki. Wszystkie zmiany zostały uwzględnione w poprawionej wersji wniosku. Ponadto Wnioskodawca wskazuje, że nacięcie skóry i usunięcie jajników zostanie wykonane w ten sposób, że rana znajdować się będzie na grzbiecie zwierząt, co uniemożliwi wylizywanie i wygryzanie rany. Trudno określić procent spadku masy ciała kwalifikujący do wczesnej śmierci lub też podać przedziały czasowe, kiedy te oznaki wystąpią, wszystko bowiem zależy od innych czynników np. od nasilenia oznak świadczących o cierpieniu zwierzęcia. Zespół wykonujący doświadczenie zapewni właściwą opiekę pooperacyjną, jednak nawet bardzo dobra opieka medyczna (zarówno u zwierząt jak i u ludzi) nie zawsze pozwala na uniknięcie pojedynczych przypadków powikłań w postaci stanów zapalnych. Ogólne ramy czasowe oznak wskazujących na wczesne humanitarne uśmiercenie, mogą przynieś więcej szkody i cierpienia dla zwierząt, ponieważ kierując się ściśle teoretycznymi wytycznymi, a może przesłonić faktyczny stan zwierzęcia.

Ponadto Wnioskodawca i LKE w Poznaniu nie wie na jakiej podstawie Fundacja kieruje swoje merytoryczne zarzuty do wniosku a tym samym uwagi do pracy naukowej Wnioskodawcy. Trudno odpowiadać na zarzuty i dyskutować z osobami, które sformułowały uwagi, jeśli nie zna się ich wykształcenia, specjalności i doświadczenia w tym zakresie. Wyszukanie w bazach publikacji związanych z poszczególnymi związkami, bądź podanie charakterystyki produktu leczniczego oraz zacytowanie kilku fragmentów uchwał nie można nazwać merytoryczną dyskusją. Ponadto wskazanie alternatywy, jaką według Fundacji są badania na człowieku, której część z nich jest nieetyczna, a zastosowanie innych nie da odpowiednich danych na rozwiązanie problemu badawczego postawionego przez Wnioskodawcę. Dlatego też propozycje Fundacji są niemożliwe do zrealizowania i dodatkowo podważają wysokie kompetencje Wnioskodawcy, który od lat zajmuje się wpływem czynników żywieniowych, farmakologicznych i środowiskowych na gospodarkę mineralną. Świadczy o tym chociażby index h=18, liczba cytowań 1187, oraz to, że w 2020 r. Wnioskodawczyni znalazła się gronie najbardziej wpływowych 2 proc. naukowców pod względem cytowalności ich publikacji na świecie. Wnioski zawierają autorskie pomysły na przeprowadzenie badań, które zgodnie z Ustawą z dnia 4 lutego 1994 r. o prawie autorskim i prawach pokrewnych objęte są prawami autorskimi.

Mając na względzie powyższe argumenty należy stwierdzić, iż Komisja wydając zgodę na przeprowadzenie doświadczenia pt. *„Określenie wpływu suplementacji Lactobacillus acidophilus ATCC 4356 i izoflawonów sojowych na gospodarkę wapnia u szczurów po owariektomii"* działa zgodnie z art. 5 ust.1 pkt 1 i pkt 3 oraz z art. 47 ust. 1 pkt 1, pkt 2, i pkt 5 Ustawy z dnia 15 stycznia 2015 r. o ochronie zwierząt wykorzystywanych do celów naukowych lub edukacyjnych.

§ 4

Integralną część niniejszej uchwały stanowi kopia wniosku, o którym mowa w § 1.

(Pieczęć lokalnej komisji etycznej)

LOKALNA KOMISJA ETYCZNA
do Spraw Doświadczeń na Zwierzętach
Uniwersytet Przyrodniczy w Poznaniu
60-637 Poznań, ul. Wołyńska 35
tel. 61 8487198, tel. 61 8466085

Podpis przewodniczącego komisji

PRZEWODNICZĄCY
Lokalnej Komisji Etycznej
do Spraw Doświadczeń na Zwierzętach
dr Paweł Kołodziejski

Pouczenie:
Zgodnie z art. 33 ust. 3 i art. 40 ustawy w zw. z art. 127 § 1 i 2 oraz 129 § 2 ustawy z dnia z dnia 14 czerwca 1960 r. Kodeks postępowania administracyjnego (Dz. U. 2017, poz. 1257 – t.j.; dalej KPA) od uchwały Lokalnej Komisji Etycznej strona może wnieść, za jej pośrednictwem, odwołanie do Krajowej Komisji Etycznej do Spraw Doświadczeń na Zwierzętach w terminie 14 od dnia doręczenia uchwały.
Na podstawie art. 127a KPA w trakcie biegu terminu do wniesienia odwołania strona może zrzec się prawa do jego wniesienia, co należy uczynić wobec Lokalnej Komisji Etycznej, która wydała uchwałę. Z dniem doręczenia Lokalnej Komisji Etycznej oświadczenia o zrzeczeniu się prawa do wniesienia odwołania przez ostatnią ze stron postępowania, decyzja staje się ostateczna i prawomocna.
Otrzymuje:
1) Użytkownik,
2) a/a
Użytkownik kopie przekazuje:
- Osoba planująca doświadczenie
- Zespół ds. dobrostanu

UNIWERSYTET MEDYCZNY IM. KAROLA MARCINKOWSKIEGO W POZNANIU

KOMISJA BIOETYCZNA PRZY UNIWERSYTECIE MEDYCZNYM
IM. KAROLA MARCINKOWSKIEGO W POZNANIU

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Uchwała nr 668/21

Na podstawie przepisów Ustawy z dnia 5 grudnia 1996 r. o zawodach lekarza i lekarza dentysty (tj. z dnia 28 lutego 2020 r., Dz.U. z 2020 r. poz. 514)z późn. zm.); Rozporządzenia Ministra Zdrowia i Opieki Społecznej z dnia 11 maja 1999 r. w sprawie szczegółowych zasad powoływania i finansowania oraz trybu działania komisji bioetycznych (Dz. U. z 1999 r., Nr 47, poz. 480); Rozporządzenia Ministra Finansów, Funduszy i Polityki Regionalnej z dnia 30.12.2020r. w sprawie obowiązkowego ubezpieczenia odpowiedzialności cywilnej podmiotu przeprowadzającego eksperyment medyczny (Dz.U. z 2020r. poz.2412); Ustawy z dnia 6 września 2001 r. Prawo farmaceutyczne (tj. z dnia 15 maja 2020 r. (Dz.U. z 2020 r. poz. 944)z późn. zm.); Rozporządzenia Ministra Finansów z dnia 30 kwietnia 2004 r. w sprawie obowiązkowego ubezpieczenia odpowiedzialności cywilnej badacza i sponsora (Dz. U. z 2004 Nr 101, poz. 1034 z późn. zm.); Rozporządzenia Ministra Zdrowia z dnia 30 kwietnia 2004 r. w sprawie sposobu prowadzenia badań klinicznych z udziałem małoletnich (Dz. U. z 2004 r. Nr 104, poz. 1108); Rozporządzenia Ministra Zdrowia z dnia 30 kwietnia 2004 r. w sprawie zgłaszania niespodziewanego ciężkiego niepożądanego działania produktu leczniczego (Dz. U. z 2004 r. Nr 104, poz. 1107); Rozporządzenia Ministra Zdrowia z dnia 17 lutego 2016 r. w sprawie wzorów wniosków związanych z badaniem klinicznym wyrobu medycznego lub aktywnego wyrobu medycznego do implantacji oraz wysokości opłat za złożenie tych wniosków (Dz. U. z 2016 r., poz. 208); Ustawy z dnia 20 maja 2010 r. o wyrobach medycznych (t.j. z dnia 13 grudnia 2019 r., Dz.U. z 2020 r. poz. 186), z późn. zm.); Rozporządzenie Ministra Finansów z dnia 6 października 2010 r. w sprawie obowiązkowego ubezpieczenia odpowiedzialności cywilnej sponsora i badacza klinicznego w związku z prowadzeniem badania klinicznego wyrobów (Dz. U. z 2010 r. Nr 194, poz. 1290); Ustawy z dnia 18 marca 2011 r. o Urzędzie Rejestracji Produktów Leczniczych, Wyrobów Medycznych i Produktów Biobójczych (t.j. tj. z dnia 4 maja 2020 r., Dz.U. z 2020 r. poz. 836) z późn. zm.); Rozporządzenia Ministra Zdrowia z dnia 2 maja 2012 r. w sprawie Dobrej Praktyki Klinicznej (Dz. U. z 2012 r., poz. 489); Rozporządzenia Ministra Zdrowia z dnia 12 października 2018 r. w sprawie wzorów dokumentów przedkładanych w związku z badaniem klinicznym produktu leczniczego oraz opłat za złożenie wniosku o rozpoczęcie badania klinicznego (Dz. U. z 2018 r., poz. 1994); w oparciu o Deklarację Helsińską Światowego Stowarzyszenia Lekarzy (WMA - World Medical Association) - Etyczne zasady prowadzenia badań medycznych Z udziałem ludzi oraz przepisy ICH GCP.

Komisja Bioetyczna, na posiedzeniu w dniu 23 września 2021 r.

rozpatrzyła wniosek dotyczący prowadzenia eksperymentu badawczego.

Kierownicy projektu: **prof. dr hab. Paweł Bogdański**
dr hab. n. o zdr. Joanna Sulibursk a, prof. UPP

Miejsce prowadzenia badań:
Katedra i Zakład Leczenia Otyłości, Zaburzeń Metabolicznych i Dietetyki Klinicznej Uniwersytetu Medycznego w Poznaniu

Główny badacz: **mgr Iskandar Harahap**

Członkowie zespołu badawczego: **prof. dr hab. Paweł Bogdański**
dr hab. n. o zdr. Joanna Sulibursk a, prof. UPP
mgr Iskandar Azmy Harahap

Temat badań:
„Czy lactobacillus acidophilus obniża ryzyko rozwoju osteoporozy u kobiet po menopauzie?”.

Okres prowadzenia badań: **wrzesień 2021 r. – wrzesień 2024 r.**

Komisja wydała uchwałę o pozytywnym zaopiniowaniu tego wniosku

Przewodniczący Komisji
M. Krawczyński
prof. dr hab. Maciej Krawczyński

Podpisy członków Komisji Bioetycznej podejmujących Uchwałę nr 668/21 ***z dnia 23.09.2021r.***

Lp.	Imię i Nazwisko	Specjalność	Miejsce Pracy	Podpis
1.	Przewodniczący Komisji **prof. dr hab. Maciej Krawczyński**	genetyka kliniczna, okulistyka	Katedra i Zakład Genetyki Medycznej UMP, ul. Rokietnicka 8, 60-806 Poznań.	
2.	Z- ca Przewodniczącego Komisji **prof. dr hab. Janusz Wiśniewski**	filozof	Wydział Nauk Politycznych i Dziennikarstwa UAM, ul. Uniwersytetu Poznańskiego 5, 61-614 Poznań.	
3.	dr Krystyna Babiak	prawnik	Kancelaria Radcy Prawnego, dr Krystyna Babiak, ul. Czartoria 1/2, 61-102 Poznań.	
4.	dr n. med. Magdalena Badura-Stronka	genetyka kliniczna, neurologia	Katedra i Zakład Genetyki Medycznej UMP, ul. Rokietnicka 8, 60-806 Poznań.	
5.	ks. prof. UAM dr hab. Andrzej Bohdanowicz	teologia	Wydział Teologiczny Uniwersytetu Adama Mickiewicza w Poznaniu, ul Wieżowa 2/4, Poznań.	----------
6.	prof. dr hab. Katarzyna Derwich	pediatria, onkologia, hematologia, transplantologia kliniczna	Klinika Onkologii, Hematologii i Transplantologii Pediatrycznej UMP, ul. Szpitalna 27/33, 60-572 Poznań.	
7.	prof. dr hab. Wojciech Dyszkiewicz	chirurgia, torakochirurgia, transplantologia kliniczna	Klinika i Oddział Torakochirurgii Wielkopolskiego Centrum Pulmonologii i Torakochirurgii im. Eugenii i Janusza Zeylandów, ul. Szamarzewskiego 62, 60-569 Poznań.	
8.	prof. dr hab. Przemysław Guzik	kardiologia, choroby wewnętrzne	Klinika Intensywnej Terapii Kardiologicznej i Chorób Wewnętrznych UMP, ul. Przybyszewskiego 49, 60-355 Poznań.	
9.	mgr Jolanta Łojko-Kołodziejczak	pielęgniarka	Pielęgniarka Oddziałowa Izby Przyjęć Pediatrii Szpitala Klinicznego im. Karola Jonschera UMP, ul. Szpitalna 27/33, 60-572 Poznań.	
10.	mgr Krystyna Malinger	farmaceuta	emerytowany kierownik Apteki Szpitalnej	
11.	dr hab. n. med. Anna Mania	pediatria, choroby zakaźne	Klinika Chorób Zakaźnych i Neurologii Dziecięcej UMP, ul. Szpitalna 27/33, 60-572 Poznań.	
12.	prof. dr hab. Andrzej Marszałek	patomorfologia	Katedra i Zakład Patologii i Profilaktyki Nowotworów UMP, ul. Garbary 15, 61-866 Poznań.	----------
13.	prof. dr hab. Maciej Owecki	choroby wewnętrzne, endokrynologia	Katedra Medycyny Społecznej UMP, ul. Rokietnicka 4, 60-806 Poznań.	
14.	Przedstawicielka OIL **prof. dr hab. Agnieszka Słopień**	psychiatria, psychiatria dzieci i młodzieży, psychoterapia dzieci i młodzieży	Klinika Psychiatrii Dzieci i Młodzieży UMP, ul. Szpitalna 27/33, 60-572 Poznań.	
15.	prof. dr hab. Robert Spaczyński	ginekologia i położnictwo endokrynologia, endokrynologia ginekologiczna i rozrodczości	Klinika Niepłodności i Endokrynologii Rozrodu UMP, ul. Polna 33, 60-535 Poznań.	