'POLISH MOTHER'S MEMORIAL HOSPITAL' RESEARCH INSTITUTE

DEPARTMENT OF PERINATOLOGY, OBSTETRICS AND GYNECOLOGY

Head of Department: Mariusz Grzesiak MD PhD Associate Professor, Institute Professor

ul. Rzgowska 281/289, 93-338 Łódź,
tel. 042 / 271 10 61 e-mail: sek16@iczmp.edu.pl

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Review of the dissertation for the degree of Doctor of Agricultural Sciences, discipline of Food Technology and Nutrition

by Iskandar Azmy Harahap, MSc, titled **"Investigation of the effects of isoflavones and probiotics on calcium bioavailability and metabolism - in vitro and in vivo studies."**

Postmenopausal osteoporosis represents a significant medical problem with considerable socioeconomic implications, characterized by progressive bone tissue deterioration and disruption of its structural microarchitecture. The pathophysiology of this condition is closely linked to estrogen deficiency, which leads to accelerated bone resorption while simultaneously slowing bone formation processes, resulting in a negative bone balance. Calcium metabolism disorders and its limited bioavailability present significant challenges in the context of prevention and therapy of metabolic bone disorders. Intensified research on

natural bioactive compounds and probiotics opens new perspectives in optimizing calcium homeostasis.

Soy isoflavones, belonging to the group of plant polyphenols, demonstrate complex biological activity through modulation of calcium transport-related gene expression and regulation of proteins involved in its metabolism. Particular attention is given to potential molecular mechanisms through which these compounds may increase the expression of calcium transporters in the intestinal epithelium and influence osteoclast and osteoblast activity.

In parallel, increasing scientific evidence points to the fundamental role of gut microbiota in regulating calcium metabolism. Probiotic bacteria, through various biochemical and physiological mechanisms, can significantly modify calcium bioavailability. Key mechanisms include modulation of intestinal pH environment, production of short-chain fatty acids (SCFAs), and synthesis of specific metabolites affecting calcium transport-related gene expression.

The interaction between soy isoflavones and probiotics presents a particularly interesting research area, suggesting potential synergistic action. Probiotic bacteria, through enzymatic transformation of isoflavones into their more bioavailable forms, can enhance their biological effectiveness. Simultaneously, bacterial metabolites can modulate isoflavone receptor expression and influence their affinity for transport proteins.

The study of interaction mechanisms between soy isoflavones and probiotics in the context of calcium bioavailability requires the use of advanced analytical techniques and in vitro and in vivo models. Understanding these complex mechanisms of interaction between soy isoflavones and probiotics in calcium metabolism regulation may contribute to the development of new, effective therapeutic strategies for disorders related to calcium metabolism and bone tissue disorders.

In his doctoral dissertation, Iskandar Azmy Harahap, MSc, addresses these issues by presenting a thematically coherent cycle of five publications. In this review, I will address each of the attached manuscripts separately.

The first publication, **"Probiotics and Isoflavones as a Promising Therapeutic for Calcium Status and Bone Health: A Narrative Review" (Foods. 2021 Nov 3;10(11))** provides a comprehensive review of the current state of knowledge regarding the role of probiotics and isoflavones in calcium metabolism and bone health. It presents a broad context of the

problem, paying particular attention to the global nature of osteoporosis and its impact on patients' quality of life. It systematically discusses issues related to bone metabolism, the role of calcium, and the importance of gut microbiota in maintaining bone health. It presents current osteoporosis treatment methods along with their limitations, justifying the need to search for alternative therapeutic solutions.

The methodology was presented clearly and in detail. The use of recognized scientific databases and time limitation to the last ten years ensures the currency of presented results. The article selection process was illustrated using a clear PRISMA diagram, which significantly increases the transparency of the methodology.

In the results section, the Doctoral Candidate and co-authors systematically analyze scientific evidence from three types of studies: in vitro, animal models, and clinical trials with human participants. This comprehensive analysis allows for a deeper understanding of the mechanisms of action of probiotics and isoflavones at different levels of biological organization.

In the section on in vitro studies, the authors provide detailed descriptions of how different probiotic bacterial strains affect the expression of calcium metabolism-related genes and their role in regulating bone cell functions. Equally interesting are the findings regarding isoflavones and their metabolites, particularly in the context of their effects on osteoblasts and osteoclasts.

In their analysis of animal model studies, they provide convincing evidence for the effectiveness of probiotic and isoflavone combinations in preventing bone mass loss. They present various dosing regimens and their impact on bone parameters, which has significant implications for potential clinical applications.

In the section devoted to clinical studies, particularly valuable is the analysis of various patient populations and diverse supplementation protocols. The authors emphasize the importance of therapy individualization and the need to consider multiple factors affecting its efficacy.

In the discussion, the authors critically analyze the collected scientific evidence and indicate potential mechanisms of synergistic action between probiotics and isoflavones, while noting the need for further research in this area.

A certain limitation of the work is the lack of more detailed analysis of potential interactions with commonly used medications, which could have significant clinical implications.

Nevertheless, the article represents an extremely valuable source of knowledge for both scientists and clinicians. The authors not only summarize the current state of knowledge but also indicate future research directions and potential clinical applications. Particularly valuable are the recommendations regarding standardization of doses and administration protocols, as well as suggestions for the development of new supplementation forms.

The article **"Effects of Daidzein, Tempeh, and a Probiotic Digested in an Artificial Gastrointestinal Tract on Calcium Deposition in Human Osteoblast-like Saos-2 Cells" (Int J Mol Sci. 2024 Jan 13;25(2))** presents a comprehensive study evaluating the effects of tempeh, daidzein, and *Lactobacillus acidophilus* on calcium absorption and utilization in bone cells.

The PhD candidate and co-authors employed an innovative research approach, combining digestion process simulation with cellular assessment using two cell lines - Caco-2 (modeling intestinal epithelium) and Saos-2 (osteoblast-like cells). This combination enabled not only a comprehensive evaluation of calcium bioavailability but also its impact on bone cell metabolism.

In the methodological section, the authors provided detailed descriptions of the test substance preparation process. Particular attention should be paid to the careful selection of experimental conditions and quality control at each stage of the study. The simulated digestion process was conducted using digestive enzymes (pepsin and pancreatin), which allowed for the recreation of physiological conditions.

The study results yielded several significant observations. First, it was demonstrated that tempeh contains significantly higher calcium content compared to unprocessed soybeans, with calcium release being 2.5 times higher. This is an important discovery from the perspective of tempeh's potential use as a dietary calcium source.

An interesting aspect of the study was the assessment of the test substances' effects on Saos-2 cells. The PhD candidate demonstrated that tempeh, daidzein, and *L. acidophilus* did not significantly affect extracellular calcium deposition in cells cultured without mineralization stimulators. However, when osteogenesis was stimulated, an increase in intracellular calcium content was observed under the influence of calcium citrate, tempeh, and *L. acidophilus*.

Particularly valuable are the observations regarding Saos-2 cell proliferation. All tested substances at concentrations from 0.05 to 1 mg/ml showed stimulating effects on cell

multiplication without exhibiting cytotoxic effects. This is crucial information from the perspective of safety for potential applications of these substances.

The authors also noted the absence of synergistic effects between isoflavones and *L. acidophilus* in the context of calcium deposition and osteogenic differentiation. This is an important observation that may have implications for dietary supplement design.

The scientific value of the article is enhanced by a detailed discussion of results, in which the authors address the molecular mechanisms of observed phenomena. Attention was drawn to the role of soy's antinutritional components, which may affect calcium bioavailability, and the importance of fermentation in reducing these compounds.

The PhD candidate identified existing limitations of the conducted study resulting from the use of a simplified in vitro digestion model and the lack of assessment of other metabolites relevant to bone metabolism. These limitations, however, do not diminish the value of the conducted research and indicate directions for further research work.

In conclusion, it should be emphasized that the article represents a valuable contribution to understanding the role of products containing isoflavones and probiotics in calcium metabolism and bone cell function.

In the publication **"Effect of Tempeh and Daidzein on Calcium Status, Calcium Transporters, and Bone Metabolism Biomarkers in Ovariectomized Rats" (Nutrients 2024 Aug 2;16(15))**, the Doctoral Student and Co-Authors address the significance of natural soy compounds in the context of postmenopausal osteoporosis, whose prevalence is significantly increasing in aging societies.

The authors highlight the limitations of current therapies, particularly bisphosphonates, which convincingly justifies the need to search for alternative therapeutic solutions.

The research methodology was developed with great attention to detail. While the use of an ovariectomized rat model is a widely accepted approach in postmenopausal osteoporosis research, the authors introduced several innovative elements to their research protocol. Of particular note is the two-phase nature of the experiment, comprising a calcium deficiency induction period and the actual intervention phase. This carefully designed research protocol allowed for better replication of clinical conditions and more accurate assessment of the tested substances' effectiveness.

The selection of research groups was conducted with great care. The inclusion of a sham control group allowed for eliminating the impact of the surgical procedure itself on the studied parameters. The group sizes were determined based on previous studies and statistical power analysis, ensuring appropriate reliability of the obtained results.

A particularly valuable methodological aspect is the application of a broad spectrum of research techniques. The authors did not limit themselves to basic biochemical measurements but utilized advanced methods such as bone histopathological analysis, calcium transporter expression studies, and comprehensive assessment of bone metabolism markers. This multifaceted approach allowed for obtaining a more complete picture of the studied substances' effects.

The study results were presented in a clear and logical manner. The authors demonstrated that both daidzein and tempeh can positively affect bone calcium content and modify calcium transporter expression in the intestines. Moreover, these effects were comparable to bisphosphonates, suggesting potential therapeutic applications for the studied compounds.

The histopathological analysis of bones provided valuable information about structural changes occurring under the influence of the tested substances. Particularly significant are the observations regarding the reduction in adipocyte-occupied area in bone marrow in groups receiving daidzein and tempeh. These changes may have crucial importance for bone formation and strength.

An extremely important aspect of the study is the analysis of molecular mechanisms underlying the observed effects. The authors demonstrated that both daidzein and tempeh can influence the expression of key calcium transporters (TRPV5 and TRPV6) in the intestines. This is a particularly important discovery as it suggests that the studied substances may improve calcium absorption through modulation of its transport at the cellular level.

The bone metabolism markers portion of the study deserves special attention. The authors conducted a comprehensive analysis of both bone resorption markers (pyridinoline, deoxypyridinoline, type I collagen C-telopeptide) and bone formation markers (bone alkaline phosphatase, osteocalcin, type I procollagen N-terminal propeptide). They showed that tempeh can affect both processes, leading to a favorable balance in bone metabolism. This is a significant discovery as it suggests that tempeh may act through complex mechanisms, not limiting itself to inhibiting bone resorption as is the case with bisphosphonates.

The Doctoral Student thoroughly acknowledges the study's limitations, including the lack of vitamin D and K measurements, which play important roles in calcium and bone metabolism. They also note differences in experimental diet compositions that could have influenced the observed effects. This critical analysis of their own results demonstrates the high scientific quality of the conducted research.

The discussion of results is comprehensive and well-thought-out. Particularly valuable is the analysis of potential mechanisms of action of the studied substances in the context of signaling pathways related to bone metabolism.

The practical implications of the results are very promising. The study suggests that natural soy products, especially tempeh, may constitute a valuable alternative or complement to conventional osteoporosis therapies. This is particularly important in the context of searching for safe methods of long-term treatment for this disease.

The authors appropriately indicate the need for further research, particularly clinical studies, to confirm the observed effects in humans. They also propose specific directions for future work, including dosage optimization and assessment of long-term safety of the studied substances.

The article **"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"** published in "Nutrients" presents an in-depth study on the effects of the probiotic Lactobacillus acidophilus and its combinations with isoflavone-containing products on bone health in a rat model of postmenopausal osteoporosis.

The Doctoral Student and Collaborators designed and conducted a meticulous experimental study using an animal model. Of particular note is the inclusion of both a sham-operated control group and a bisphosphonate-treated group, which enabled valuable comparison of the studied interventions' effects with current pharmacological treatment. The two-phase structure of the experiment, comprising a calcium deficiency induction phase followed by a dietary intervention phase, allowed for precise evaluation of the tested substances' effects under conditions similar to actual postmenopausal osteoporosis.

The study yielded several interesting observations. The most significant results concerned the synergistic action of L. acidophilus in combination with isoflavone-containing products,

particularly tempeh. A significant increase in calcium levels in femoral bones was observed, accompanied by a decrease in serum calcium levels. Histopathological analysis showed marked improvement in bone structure, and bone metabolism markers indicated favorable changes in bone remodeling processes. Notably, these effects were comparable to bisphosphonates, suggesting therapeutic potential for the studied substances.

The innovation of this research lies in its comprehensive approach to postmenopausal osteoporosis. The combination of probiotics with natural sources of isoflavones represents a novel perspective on therapeutic possibilities, particularly in the context of seeking alternatives to conventional pharmacotherapy, which often involves unwanted side effects.

However, certain study limitations should be noted. The lack of parathyroid hormone measurements, which is a key regulator of calcium metabolism, and the absence of densitometric studies represent certain methodological shortcomings. Additionally, the increased blood glucose levels observed in some intervention groups require further investigation and explanation of the mechanisms behind this phenomenon.

The clinical implications of the presented results deserve emphasis. The study suggests that combining probiotics with isoflavone-rich foods may represent a promising nutritional strategy in the prevention and support of postmenopausal osteoporosis treatment. This is particularly significant in light of growing interest in natural methods for supporting bone health.

In the publication **"Effects of daily probiotic supplementation with Lactobacillus acidophilus on calcium status, bone metabolism biomarkers, and bone mineral density in postmenopausal women: a controlled and randomized clinical study" (Front Nutr. 2024 Jul 1;11)**, the Doctoral Student and Collaborators presented an exceptionally significant clinical study examining the complex relationship between Lactobacillus acidophilus supplementation and calcium metabolism, bone metabolism markers, and bone mineral density in postmenopausal women.

The authors hypothesized that probiotic use could beneficially influence calcium metabolism and bone metabolism. This innovative approach to addressing osteoporosis deserves particular attention as it attempts to utilize mechanisms related to the gut-bone axis in the prevention and treatment of bone disorders.

The study design was carefully conceived and executed according to the highest methodological standards. A randomized controlled trial model with double-blind methodology was implemented, significantly enhancing the reliability of the obtained results. The study recruited 55 postmenopausal women who met strictly defined inclusion and exclusion criteria.

The study population was divided into two subgroups: an experimental group of 30 participants receiving L. acidophilus probiotic and a control group of 25 participants receiving placebo.

The intervention period lasted 12 weeks, during which systematic and multidirectional measurements were conducted. The range of diagnostic tests performed is particularly impressive, including:

- Comprehensive body composition analysis using the InBody 270 device, enabling precise assessment of anthropometric parameters and detailed body composition analysis, including fat and muscle tissue distribution
- Detailed biochemical diagnostics covering not only standard biochemical parameters but also specialized bone metabolism markers
- Advanced densitometric examinations, utilizing DXA methodology as the gold standard in bone mineral density assessment

In the probiotic group, a statistically significant decrease in serum calcium levels was observed compared to baseline values. This unexpected result requires particular attention and further research, as it may have significant clinical implications. Interestingly, no analogous changes were observed in hair calcium levels, suggesting the complexity of mechanisms regulating calcium metabolism. One of the most important discoveries was the observation of probiotics' stabilizing effect on bone turnover markers. This is particularly significant in the context of osteoporosis prevention, where excessive resorptive activity can lead to bone mass loss. The results suggest that probiotics may play a role in regulating bone metabolism.

Another important observation was the significant reduction in both total body fat content and visceral fat in the probiotic group. This additional metabolic effect may have significant implications for the overall health status of postmenopausal women.

A concerning trend in glucose metabolism was also noted, showing increased glucose concentration in the probiotic group. This aspect requires particular attention and further

research, as it may have significant implications for the safety of probiotic use, especially in patients with carbohydrate metabolism disorders.

As a reviewer, I must point out certain limitations of the conducted study. The twelve-week observation period may be too short for a complete assessment of probiotics' effect on bone metabolism, particularly regarding changes in bone mineral density. Furthermore, the lack of gut microbiota analysis limits the possibility of fully understanding the mechanisms of probiotic action.

In my assessment, the conducted research has significant implications for clinical practice, providing new information about the potential use of probiotics in the prevention and treatment of metabolic disorders in postmenopausal women. Simultaneously, the Doctoral Student and Co-authors emphasize the necessity of monitoring metabolic parameters during supplementation. They also stress the need for further research evaluating the long-term effects of probiotic use, particularly regarding further analysis of probiotics' impact on bone metabolism and calcium homeostasis, as well as determining optimal dosage and supplementation duration for achieving the best therapeutic effects.

The presented publication constitutes a significant contribution to the development of knowledge regarding practical possibilities of using probiotics in the prevention and treatment of metabolic disorders in postmenopausal women. The study results open new perspectives in the application of probiotics in medicine while indicating the need for further, in-depth analyses in this area.

The research presented in five publications forming the core of the dissertation was planned, executed, and analyzed in a manner demonstrating the Doctoral Candidate's scientific maturity. The doctoral dissertation has significant cognitive value and can serve as a foundation for further research in this field. I would like to emphasize that the minor reservations or limitations regarding the conducted research that I have pointed out do not diminish the significant scientific and clinical value of this dissertation.

The doctoral dissertation submitted for my review meets the conditions specified in Article 187 of the Act of July 20, 2018, Law on Higher Education and Science (Journal of Laws of 2018, item 1688) regarding scientific degrees and academic titles, as well as degrees and titles in the field of art.

Therefore, I am addressing the esteemed Scientific Council of the Food Technology and Nutrition Discipline at the Poznań University of Life Sciences with a request to accept this dissertation and admit Iskandar Azmy Harahap, M.Sc., to further stages of the doctoral proceedings.

Mariusz Grzesiak MD PhD Associate Professor, Institute Professor
Specialist in obstetrics and gynecology
Specialist in perinatology

Head of Perinatology, Obstetrics and Gynecology Department
'Polish Mother's Memorial Hospital' Research Institute Lodz, Poland
Head of II Chair and Department of Gynecology and Obstetrics
Medical University of Lodz, Lodz Poland