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WYDZIAŁ BIOTECHNOLOGII I NAUK O ŻYWNOŚCI
KATEDRA ROZWOJU FUNKCJONALNYCH PRODUKTÓW ŻYWNOŚCIOWYCH

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Department of Functional Food Products Development

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Ph. D. Thesis Evaluation Report

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

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The PhD thesis evaluation report was prepared on the basis of the resolution of the Scientific Council of the Food and Nutrition Technology Discipline of the University of Life Sciences in Poznan on October 24, 2024 in connection with the procedure of the award of the degree of doctor of philosophy in agricultural science in the discipline Food technology and nutrition for MSc Iskandar Azmy Harahap.

The selection and meaning of the topic

The selection of the doctoral thesis's topic, focuses on calcium deficiency in postmenopausal women in relation to the incidence of osteoporosis, was based on the identified gap in the state of the art in this area of human nutrition and medicine. Calcium is an essential and the most abundant mineral in human body playing and assisting many functions in the proper functioning of the organism, just to mentioned bone and teeth structure, muscle contraction, as well as nervous and cardiovascular systems operations. Calcium is found in many popular food like dairy and fish (sardines) products, as well as in leafy vegetables including broccoli and kale, however its intake can be substantially reduced by limited bioavailability or special diets treatments (vegetarian and vegan), food preferences (children) and medications. Calcium deficiency can lead to various serious problems in the body a.o. distortions of muscles, heart and brain functions, but most of all calcium deficiency disease (hypocalcemia) including

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osteopenia y osteoporosis affecting the skeletal system and teeth. The demographic groups very susceptible to calcium deficiency are elderly people and a (post)menopausal women in which the decline in the estrogen secretion causes a woman's bones to thin faster reducing bone density and increase the risk of fracture. Due to poor calcium absorption related to aging, digestive issues, or vitamin D deficiency (limited exposure to sunlight), as well as adverse effects of bisphosphonates treatment, exploring other ways of calcium intake and its bioavailability, bioaccessability and status are crucial in nutrition and health sciences.

Formal aspects of the work

The Ph.D. thesis presented by MSc Iskandar Azmy Harahap entitled: "The study on the impact of isoflavones and probiotics on calcium bioaccessibility and calcium status – in vitro and in vivo studies" consists of 52 pages divided to the following parts: Summaries in Polish and English, Short description of the PhD candidate biography and achievements, List and a brief description of the published papers with short justification of the monothematic cycle, Introduction, Materials and Methods, The most crucial research findings, Conclusions, Novelty and Limitations of the conducted research, Future perspectives, Bibliography, List of 13 supporting publications co-authored by the doctoral candidate. Attached to the descriptive part of PhD dissertation reprints of the publications constituting the doctoral dissertation followed by the Statements of the co-authors of the publications of the doctoral dissertation are added. The whole dissertations consists of five scientific papers, one review and four original research papers, already published in MDPI and Frontiers journals. The whole cycle presents well set and carried out research focused on the main topic of calcium deficiency in postmenopausal women treated by tempeh, daidzein and *Lactobacillus acidophilus* divided into *in vitro*, *in vivo* and clinical studies. Total ranking of the cycle of the papers equals IF=23,2 that is an equivalent of 560 MEiN points. MSc. Harahap is the first author of all the presented publications.

The assessed dissertation meets the formal requirements for this type of work presented in the procedure for the doctoral degree, i.e. it is experimental and contains all the necessary chapters arranged in a typical sequence. The study is a coherent and it is correctly written in clear and understandable language.

Content of the work

Introduction – Introductory part brings crucial information about calcium, as an essential nutrient and physiological element, in human body. Author pointed out the proposed mechanism of calcium absorption and problems related to calcium deficiency, especially in postmenopausal women. M.Sc. Iskandar Harahap drew also some attention to already



established medical treatment in osteoporosis, like limitations and risks of bisphosphonates therapy. As an alternative PhD candidate, based on the literature data, proposed traditional Indonesian fermented soy product, tempeh, in the combination with probiotic strain of *Lactobacillus acidophilus* to overcome calcium deficiency issue in postmenopausal women. The part of the introduction was also related to information collected in **the first publication** (Probiotics and Isoflavones as a Promising Therapeutic for Calcium Status and Bone Health: A Narrative Review, *Foods*, 2021, 10, 2685), which is a coherent and informative review paper on probiotics and isoflavones potential in maintaining calcium status in the human body.

Purpose and objective of the research – The literature data led MSc. Iskandar Azmy Harahap to formulate three short, clear and informative research hypotheses as a starting point for drawing the one general and four specific objectives of the study. He claimed that isoflavones (tempeh, daidzein) and probiotics (*Lactobacillus acidophilus*) enhance calcium bioaccessibility and status in organism improving bone health in postmenopausal women, especially due to their simultaneous deposition and synergistic effect. The objectives are formulated properly and are possible to achieve by the correctly planned set of experiments.

Results and discussion – The scientific content of the four presented research papers forming the monothematic cycle are related to the calcium bioavailability and status in the selected tissues after treatment with tempeh, daidzein and *Lactobacillus* on three levels *in vitro*, *in vivo* and in a clinical study involving postmenopausal women. All publications were prepared based on the original research results collected by M.Sc. Iskandar Harahap during his doctoral school experimental works.

Publication 2 Effects of daidzein, tempeh, and a probiotic digested in an artificial gastrointestinal tract on calcium deposition in human osteoblast-like Saos-2 cells, published in International Journal of Molecular Sciences (25(2), 1008, IF 4.9; 140 pts.) presents the results of the first investigation on the effects of daidzein, tempeh, and *Lactobacillus acidophilus* on calcium deposition in human osteoblast-like Saos-2 cells by digestion in an artificial gastrointestinal model. Experimental work focused on bioaccessability of calcium from tested materials in the conditions of simulated digestion, followed by intestinal absorption in the intestinal epithelial Caco-2 cell model and finished on bioavailability and bone-related cellular processes in human osteoblast-like Saos-2 cells. The main discovery of the cellular studies was that there is no synergistic effect and substantial improvement in calcium deposition when daidzein, tempeh and *Lactobacillus acidophilus* were used. The authors suggested also the effects of tested isoflavones and probiotic bacteria on the osteogenic differentiation in Saos-2 cells, which can possibly preserve or improve bone health.

Questions: 1. On which base was a particular *Lactobacillus acidophilus* strain selected for the study?



2. What are the limitations of tempeh consumption and what is the recommended daily intake of the product?

3. In the presented study (P2), *in vitro*, tempeh, *Lactobacillus acidophilus* and daidzein had no effect on calcium deposition in Saos-2 cells. One of the possible reasons, mentioned by Mr Harahap, is inhibitory activity of substances present in tempeh. However, *in vivo* there was a very strong deposition of calcium in the femoral bones of the rats fed with tempeh. Does this mean that those substances present in tempeh did not express their inhibitory activity *in vivo*? Are there known in literature any other possible mechanisms for this phenomenon?

Publication 3 Effect of tempeh and daidzein on calcium status, calcium transporters, and bone metabolism biomarkers in ovariectomized rats. (Nutrients, 16, 651, IF 4.8; 140 pts.) presents the results of the ovariectomized rat model study in which animals were fed daily with tempeh and daidzein. Their calcium status, calcium transporters expression, and bone metabolism biomarkers were analyzed to simulate postmenopausal osteoporosis conditions. The main achievement of this experiment showed that, comparing to bisphosphonate drugs treatment, tempeh and daidzein improved calcium status, enhanced the expression of calcium transporters, and positively influenced rat bone metabolism biomarkers. The study suggested that selected isoflavones could be a good dietary source of calcium in the prevention and treatment of calcium deficiency.

The third experimental part of the study presented in the Ph.D. dissertation (**Publication 4**: Impact of *Lactobacillus acidophilus* and its combination with isoflavone products on calcium status, calcium transporters, and bone metabolism biomarkers in a postmenopausal osteoporotic rat model. Nutrients (16, 2524, IF 4.8; 140 pts.) focuses on the synergistic effect of *Lactobacillus acidophilus*, tempeh and daidzein on calcium status, calcium transporters, and bone metabolism biomarkers in an ovariectomized rat model. The collected results and observations of the study revealed that the daily consumption of probiotic *Lactobacillus acidophilus* and its combination with isoflavones enhanced femoral bone calcium levels, however this type of the diet reduced calcium concentration in blood serum. Bone metabolism biomarkers, hematological parameters and cholesterol and triglyceride levels were positively affected by the diet, however with simultaneous elevation of blood glucose levels.

Question: 1. In *in vivo* experiments on rats (P3 and P4), it is evident that daidzein, tempeh and *L. acidophilus* lead to a significant decrease in calcium concentration in the rat's blood. According to some literature data (doi:10.1538/expanim.61.399), normal calcium level in a serum of rats is between 1.32 and 3.24 mmol/L. In the carried out studies Mr Harahap showed that in the group of rats fed with tempeh calcemia dropped to the level of 1.4 mM (almost to the lower limit), while the same group had the highest calcium intake of all other groups. Does this mean that consuming daidzein, tempeh with or without additional *L. acidophilus*, over a



longer period of time and in larger quantities, can hypothetically lead to the effects of hypocalcemia and all the consequences, including the functioning of the nervous system and muscles?

2. In the study presented in P3 daidzein, and in P4 tempeh with presence of lactobacilli, seriously increased the level of AST (Aspartate Aminotransferase) in experimental rats. Does this indicate their possible hepatotoxicity if used for longer period of time and in larger quantities?

3. It is stated that daidzein (P3) and daidzein with lactobacilli and tempeh with lactobacilli (P4) significantly reduced the level of triglycerides in the serum of experimental rats (especially tempeh with lactobacillus). Would this mean that daidzein and tempeh can be hypothetically used to lower triglycerides in humans?

The last part of the thesis (**Publication 5** 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, IF 4.0; 70 pts.) relates to the human clinical study on the postmenopausal women supplemented their daily diet with *Lactobacillus acidophilus* on calcium levels, bone metabolism biomarkers, and bone mineral density profiles. This study is concluded by the statement that however the probiotic supplementation did not significantly influence bone mineral density, it helped to stabilize bone turnover. The study suggested also that this dietary intervention disturbed calcium and blood glucose levels in humans.

Questions: 1. What was the reason for not testing a probiotic supplementation of human diet with *Lactobacillus acidophilus* together with tempeh and daidzein, as in the previous experiments *in vitro* and *in vivo* on rats.

2. Are there any unfavorable effects of simultaneous (and long lasting) consumption of probiotics and isoflavones on human health?

The main findings of the work are summarized by four conclusions corresponding with the publications included in the PhD thesis. The overall conclusion summed up the whole series of experiments published in the papers. The good point of the conclusions part is a practical recommendation, which however is a bit too general. Novelty and future directions of research in the area of calcium deposition and mineral regulation mechanisms are also a good points of the dissertation. Identification of the presented study limitations is a very interesting point of the work suggesting other potential pathways and ways of understanding of metabolic processes and mechanisms.



Final conclusion

Summing up, I would like to state that, the evaluated PhD thesis has a high scientific potential with novel aspects and mechanisms of elimination of calcium deficiency, especially in case of dietary treatment with isoflavone rich products in combination with probiotic strains of bacteria in postmenopausal women. All experiments were carefully planned and carried out with properly selected and available methodology and equipment on *in vitro*, *in vivo* and in a clinical studies. The collected results were carefully collected, statistically analyzed, discussed and interpreted in a thorough manner, which enabled them to be published in the recognized scientific journals, which prove the novelty and high level of the performed research (Total IF 23.2 and 560 ministerial point). The content of the doctoral dissertation of MSc Iskandar Azmy Harahap entitled: "The study on the impact of isoflavones and probiotics on calcium bioaccessibility and calcium status – *in vitro* and *in vivo* studies" and the resulting valuable practical application possibilities give me the right to conclude that the evaluated PhD dissertation meets all the requirements specified. The doctoral dissertation meets the conditions specified in art. 187 paragraphs 1-4 of the Act of 20 July 2018 - The Law on Higher Education and Science (Journal of Laws of 2023, item 742, as amended). Therefore, with full conviction, I submit an application to the Council of the discipline Food Technology and Nutrition at the University of Life Sciences in Poznan for the admission of MSc Iskandar Azmy Harahap to the public defense of his PhD thesis.

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