IMPACT OF ISOFLAVONES AND *LACTOBACILLUS* ACIDOPHILUS ON THE FECAL MICROBIOLOGY STATUS IN HEALTHY FEMALE RATS

Iskandar Harahap[1], Maciej Kuligowski[2], Marcin Schmidt[3], Anna Brzozowska[4],
Joanna Suliburska[1✉]

[1]Department of Human Nutrition and Dietetics, Poznań University of Life Sciences
 Wojska Polskiego 31, 60-624 Poznań, **Poland**
[2]Department of Food Technology of Plant Origin, Poznań University of Life Sciences
 Wojska Polskiego 31, 60-624 Poznań, **Poland**
[3]Department of Biotechnology and Food Microbiology, Poznań University of Life Sciences
 Wojska Polskiego 48, 60-637 Poznań, **Poland**
[4]Department of Gastronomy Sciences and Functional Foods, Poznań University of Life Sciences
 Wojska Polskiego 31, 60-624 Poznań, **Poland**

ABSTRACT

Background. Isoflavones and probiotics are promising agents with preventive and therapeutic effects on bone health. However, their mechanism of action is not fully understood. The combined effect of probiotics and isoflavones on the composition of gut microbiota is yet to be investigated. Thus, this study aimed to determine whether the inclusion of isoflavones and probiotics in the diet had an effect on the content of *Lactobacillus* spp. in the feces of female rats.

Materials and methods. This study included 50 female Wistar rats aged 3 months old. The rats were randomly assigned to six groups. The control group (K) received a standard diet (AIN 93M), while the other five groups received a standard diet supplemented with the following: tempeh flour at an amount of 250 g/kg of diet (TP); soy flour at 250 g/kg (RS); daidzein and genistein at 10 and 100 mg/kg (DG), respectively; *Lactobacillus acidophilus* at 10^{10} CFU/day (LA); or daidzein, genistein, and *L. acidophilus* (DGLA). At the beginning of the experiment and after 8 weeks, fecal samples were collected from the examined rats and the content of *Lactobacillus* spp. in feces was determined.

Results. A comparison of *Lactobacillus* spp. content in fecal samples before and after the intervention showed a significant increase in bacteria in K, DG, and DGLA groups. A significant decrease in *Lactobacillus* spp. content was observed in the RS group compared to other groups after 8 weeks of intervention.

Conclusion. Isoflavones may improve the gut *Lactobacillus* status in healthy rats, while raw soybean decreases the content of these bacteria.

Keywords: isoflavones, probiotics, *Lactobacillus* spp., gut microbiota, fecal, rats

INTRODUCTION

Osteoporosis is characterized by a low bone mass, density, and abnormalities in bone microarchitecture, which result in weakened bones and an increased risk of fracture (McClung et al., 2014). It is an important public health issue, especially among women (Mahdavi-Roshan et al., 2015). During menopause, estrogen levels naturally decline in women, causing an increase in bone turnover, and subsequently loss of bone mass,

structural integrity, and strength (Rizzoli, 2014). Furthermore, the decrease in estrogen is accompanied by a decrease in the intestinal absorption and renal reabsorption of calcium, and an increase in the release of parathyroid hormone and bone resorption (Zhu and Prince, 2012). Thus, in order to increase bone mass and strength and improve bone microarchitecture, it is essential to stimulate bone development (McClung et al., 2014).

Recent research suggests that the gut microbiota could be a possible therapeutic target for treating osteoporosis after menopause (Xu et al., 2017). The gastrointestinal system can regulate the balance between osteoclast-mediated bone resorption and osteoblast-mediated bone formation (Harahap and Suliburska, 2021). It appears that the gut microbiome influences bone morphology, which in turn may have an impact on bone strength and fracture risk (Medina-Gomez, 2018). The gut microbiota-bone axis refers to the effect of microbial community or molecules produced by them on bone health (Villa et al., 2017).

Phytoestrogens, particularly isoflavones, have been the main ingredient of dietary alternatives to the Food and Drug Administration (FDA)-approved medications (Pawlowski et al., 2015). Isoflavones are abundant in soybeans, and fermented soybean products, such as tempeh, contain a higher concentration of these compounds than nonfermented soy products (Kuligowski et al., 2017). The therapeutic efficacy of soy isoflavones has been attributed to their biotransformation to equol, a potent estrogenic metabolite (Yuan et al., 2007).

On the other hand, preclinical studies based on animal models have shown that probiotics can improve bone mineralization and increase bone strength. A favorable effect on bone was observed in the *Lactobacillus* genus, and as a result, these organisms were widely studied in several clinical intervention trials (Villa et al., 2017). Moreover, *Lactobacillus acidophilus* has been characterized by the ability to increase bone microarchitecture, bone mineral density, and bone heterogeneity. The immunomodulatory effect of *L. acidophilus* administration on the host immune system is responsible for this favorable effect by inhibiting osteoclastogenic factors (IL-6, IL-17, TNF-α and RANKL) and improving anti-osteoclastogenic factors (IL-10, IFN-γ) (Dar et al., 2018).

The combined effect of isoflavones and probiotics on gut microbiota profile has not been explored so far. Therefore, this study aimed to determine the effect of isoflavones, particularly daidzein and genistein which can promote bone formation, combined with a probiotics strain, specifically *L. acidophilus*, on the content of *Lactobacillus* spp. in the fecal samples of healthy rats.

MATERIALS AND METHODS

Experimental design

The study was approved by a Local Ethical Committee in Poznań, Poland (no. 21/2021). A schematic of the study design is presented in Figure 1. Fifty 3-month-old healthy female Wistar rats were included in the study. The rats were acclimatized to the laboratory environment for the first 5 days and then housed separately in stainless steel cages coated with metal-free varnish and under 12-hour light / 12-hour dark cycles. The animals were randomly divided into six groups as follows: (1) The control group (K) received a standard diet (AIN 93M), while the remaining groups received a standard diet with the addition of (2) tempeh flour at an amount of 250 g/kg of diet (TP), (3) soy flour at 250 g/kg (RS); (4) daidzein and genistein at 10 and 100 mg/kg (DG), respectively; (5) *L. acidophilus* at 10^{10} CFU/day (LA); or (6) daidzein, genistein, and *L. acidophilus* (DGLA). Throughout the study, all groups of rats had *ad libitum* access to distilled water. The rats in each group were weighed weekly, and food consumption was recorded daily. Before and after the intervention, fecal samples were obtained from rats and stored at –80°C until the analysis. After 8 weeks, all animals were euthanized by decapitation following a 12-hour fast.

Diet preparation

The AIN 93M standard diet was purchased from the Zoolab company (Poland). Raw soy (*Glycine max*) of the Augusta variety was obtained from the Department of Genetics and Plant Breeding at Poznań University of Life Sciences (Poland). The soy was processed into a powder form by using a crusher machine. Pure isoflavones, namely daidzein and genistein, were procured from LC Laboratories (U.S.).

Tempeh was prepared following the method of Kuligowski et al. (2017) with some modifications. Briefly, the study used the same type and origin of raw soy
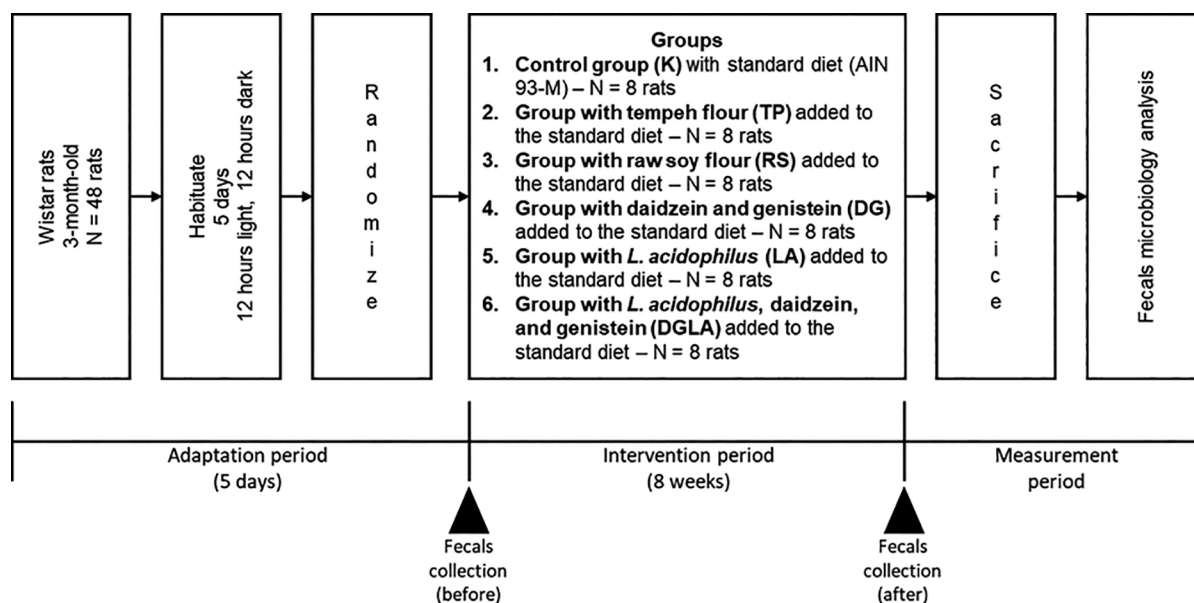
Fig. 1. Diagram of animal study design

in preparing tempeh. The hull of the soybeans was removed by decortication and soybeans were boiled for 40 minutes before cooling. Then, *Rhizopus oligosporus* NRRL 2710 obtained from Agricultural Research Service Culture Collection, Illinois, United States was inoculated in potato dextrose agar purchased from Merck KGaA, Darmstadt, Germany for 72 hours and subsequently mixed with soybeans and placed on disposable Petri plates. Fermentation was carried out by incubation for 24 hours at 30°C. Finally, the tempeh was defrosted, freeze-dried, and ground into a fine powder.

A probiotic strain of *L. acidophilus* DSM20079 was procured from the German Collection of Microorganisms and Cell Cultures, the Leibniz Institute DSMZ (Germany). To obtain single colonies, the De Man, Rogosa, and Sharpe (MRS) broth was first activated, and after 1 hour of activation, the freeze-dried stock of *L. acidophilus* DSM20079 was spread on the MRS agar and incubated at 37°C for 48 hours. An overnight culture of a single colony was used to inoculate 10 ml of MRS broth, which was then cultured for further 20 hours at 37°C before the culture volume was scaled up, and then new MRS broth was inoculated again with 2% of the overnight culture. The prepared culture was centrifuged for 10 minutes at 4°C, washed once in ice-cold sterilized saline, and then dissolved at 10^{11} CFU/ml in an ice-cold sterilized mix containing 10% milk powder and 20% maltodextrin. Then, the cells were eventually harvested and freeze-dried at room temperature with the condenser set to 55.5°C. The resulting mixture was transferred to sterile plates and frozen at –80°C. The viable count method was performed on powdered freeze-dried material after combining and random selection. Briefly, 1 g of freeze-dried product was dissolved in 9 ml of peptone water and vortexed. A series of dilutions were made in sterile peptone water. Then, pour-plated samples in sterile MRS agar were prepared at appropriate dilutions. After incubation at 37°C, the plates were incubated anaerobically in an inverted posture to allow the formation of colonies. The final formulation of the probiotic product contained 10^{10} CFU/g maize starch.

Composition of the diets

The diets used in the study consisted of proteins, lipids, ash, carbohydrates, and fiber. The protein content of the diets was determined using the Kjeldahl method (AOAC, 1995). The lipid content was determined using the Soxhlet method (PN-EN ISO 3947:2001; Soxtec System, Foss Tecator). The ash content was estimated after completely burning the sample in an

oven (AOAC, 2000). The carbohydrate content was calculated as 100% minus the percentage contributions of protein, fat, water, and ash. The fiber content was measured using the enzymatic-gravimetric (Asp, 2001). The energy value was calculated by Atwater factor [(9× fat mass) + (4× carbohydrate mass) + (4× protein mass)] kcal / 100 g (FAO, 2003).

The content of isoflavones in the diets was determined using a method proposed by Kuligowski and colleagues (Kuligowski et al., 2017). First, the isoflavones were extracted. Then, 1 g of soybean and tempeh powder were mixed in 80% acetonitrile, and the samples were homogenized vigorously at room temperature for 2 hours and subsequently centrifuged at 1310 g for 30 minutes. The supernatant was evaporated under vacuum (Buchi R-215), and the residue was diluted in methanol to a final volume of 2 ml. In the next step, the content of isoflavones was measured using a Waters 2695 HPLC system equipped with a Waters 2996 photodiode array detector (Waters Associated, Milford, MA) after separation on an Alltech Alltima C18 reversed-phase column (4.6 × 250 mm, 5 μm; Alltech Co., Deerfield, IL).

Body mass gain and food intake measurement
Body mass gain was estimated based on weekly body weight measurements, while diet intake was estimated based on daily food consumption measurements. Body mass and food weight were determined using a digital scale weight.

Fecal microbiological measurement
After five days of the adaptation period, fecal samples on 1st day of intervention (baseline) and the 56th day of intervention (endline) were collected from all study groups and subjected to microbiological analysis to determine the content of *Lactobacillus* spp. After measuring the fecal mass, the samples were soaked in 100 ml sterile buffered peptone water (Millipore, Poland) mixed with 0.1% Tween 80 (Millipore, Poland), and homogenized using a stomacher device. Then, the fecal suspension was serially diluted in buffered peptone water and plated in triplicate on MRS agar (Millipore, Poland). The plated agar media were incubated with anaerobic gas-generating sachets (AnaeroGen, Oxoid) in gas-tight boxes at 37°C for 48 hours (MRS agar plates). After incubation, the bacterial colonies

were counted, and the bacterial cell counts in feces were calculated based on the dilution factor and the mass of the starting material.

Statistical analysis
Statistically significant differences between the groups were determined by analysis of variance (ANOVA) using the statistical software Statistica by TIBCO for Windows. Tukey's honestly significant difference test was used when ANOVA indicated a significant difference among the means. The differences were considered statistically significant at a 5% level.

RESULTS

Diet composition
The proximate composition of each type of diet is presented in Table 1. The TP diet had the highest content of protein (23.56 ±0.83 g/100 g wet mass), fat (7.61 ±0.23 g/100 g wet mass), and soluble fiber (3.78 ±0.04 g/100 g wet mass), and the lowest content of carbohydrates (59.37 ±1.20 g/100 g wet mass). On the other hand, the RS diet had the highest content of insoluble fiber (14.88 ±0.66 g/100 g wet mass), total fiber (17.64 ±0.96 g/100 g wet mass), and ash (3.26 ±0.12 g/100 g wet mass). The composition of K, DG, LA, and DGLA diets was comparable.

The isoflavone content of raw soybeans and tempeh is shown in Table 2. The levels of both daidzein and genistein were significantly higher in tempeh than in soybean. Soybean contained 14.19 ±0.29 μg/g wet mass of daidzein, while tempeh contained 38.39 ±2.75 μg/g wet mass of daidzein. Similarly, the level of genistein in soybean was 15.9 ±0.65 μg/g wet mass, and that in tempeh was 424.99 ±36.24 μg/g wet mass. This clearly shows that fermentation significantly enhanced the isoflavone content of soybean.

Body mass and food intake
The results of daily weight gain and food consumption measurements are shown in Table 3. Body mass gain was found to be significantly lower in the RS group (51.00 ±6.32 g) than in TP (77.63 ±10.00 g), DG (95.25 ±21.61 g), LA (97.75 ±12.91 g), and DGLA groups (92.25 ±13.55 g). In addition, food intake was significantly lower in RS (18.12 ±0.39 g/day), TP (18.43 ±0.45 g/day), and K (18.93 ±0.29 g/day) groups

Table 1. Proximate composition of diets (by wet mass)

Group	Protein g/100 g	Fat g/100 g	Insoluble fibre g/100 g	Soluble fibre g/100 g	Fibre g/100 g	Carbohy-drates g/100 g	Ash g/100 g	Energy Kcal/100 g
K	14.95 ±0.64[a]	4.28 ±0.19[a]	5.92 ±0.45[a]	1.87 ±0.03[a]	7.78 ±0.42[a]	70.78 ±0.62[c]	2.68 ±0.07[ab]	346.45 ±1.83[c]
TP	23.56 ±0.83[c]	7.61 ±0.23[c]	11.61 ±0.75[b]	3.78 ±0.04[c]	15.38 ±0.78[b]	59.37 ±1.20[a]	2.92 ±0.09[b]	337.89 ±3.18[b]
RS	21.04 ±0.89[b]	5.14 ±0.02[b]	14.88 ±0.66[c]	2.76 ±0.31[b]	17.64 ±0.96[c]	63.13 ±0.97[b]	3.26 ±0.12[c]	311.49 ±3.37[a]
DG	14.11 ±0.14[a]	4.51 ±0.05[a]	5.67 ±0.01[a]	1.92 ±0.05[a]	7.58 ±0.04[a]	71.28 ±0.20[c]	2.45 ±0.08[a]	351.83 ±0.57[c]
LA	14.19 ±0.17[a]	4.46 ±0.17[a]	5.71 ±0.02[a]	1.83 ±0.03[a]	7.54 ±0.01[a]	71.34 ±0.61[c]	2.51 ±0.15[a]	352.14 ±0.72[c]
DGLA	14.16 ±0.34[a]	4.61 ±0.08[a]	5.98 ±0.13[a]	1.86 ±0.08[a]	7.83 ±0.07[a]	71.07 ±0.32[c]	2.47 ±0.04[a]	351.17 ±0.68[c]

Mean values with a different superscript letter ([a,b,c]) in each column are significantly different for $p < 0.05$.

Table 2. Isoflavones content of raw soybean and tempeh, µg/g wet mass

Sample	Daidzein	Genistein
Soybean	14.19 ±0.29[a]	15.9 ±0.65[a]
Tempeh	38.39 ±2.75[b]	424.99 ±36.24[b]

Mean values with a different superscript letter ([a,b]) in each column are significantly different for $p < 0.05$.

compared to DG (22.32 ±0.99 g/day), LA (21.82 ±0.88 g/day), and DGLA groups (21.68 ±0.79 g/day). Daidzein and genistein intake was significantly higher in the TP group and the group receiving pure isoflavones than in the RS group.

Comparison of *Lactobacillus* spp. content in rats' feces before and after the intervention

A comparison of the content of *Lactobacillus* spp. in the fecal samples of the studied groups before and after the intervention is presented in Figure 2. Only the RS diet caused reduction in the content of *Lactobacillus* spp. in fecal samples (from 9.04 ±0.4310 to 8.76 ±0.55 log10 CFU/g), while other diets caused an increase. The fecal samples from groups receiving the DG diet (from 8.88 ±0.18 to 9.60 ±0.18 log10 CFU/g), DGLA diet (from 9.15 ±0.21 to 9.63 ±0.17 log10 CFU/g), and standard diet (from 8.82 ±0.23 to 9.34 ±0.24 log10 CFU/g) had significantly higher content of *Lactobacillus* spp. TP diet increased the *Lactobacillus* spp. content in fecal samples from 9.04 ±0.33

Table 3. The results of body mass gain and food intake

Group	Body mass gain g	Food intake g/day	Daidzein intake µg/day	Genistein intake µg/day	Probiotic intake CFU/day
K	69.63 ±11.86[ab]	18.93 ±0.29[a]	0	0	0
TP	77.63 ±10.00[bc]	18.43 ±0.45[a]	223.04 ±5.42[b]	2469.10 ±59.96[c]	0
RS	51.00 ±6.32[a]	18.12 ±0.39[a]	84.64 ±1.84[a]	94.84 ±2.06[a]	0
DG	95.25 ±21.61[c]	22.32 ±0.99[b]	223.19 ±9.89[b]	2231.83 ±98.86[b]	0
LA	97.75 ±12.91[c]	21.82 ±0.88[b]	0	0	10^{10}
DGLA	92.25 ±13.55[c]	21.68 ±0.79[b]	216.81 ±7.87[b]	2168.10 ±78.73[b]	10^{10}

Mean values with a different superscript letter ([a,b,c]) in each column are significantly different for $p < 0.05$.
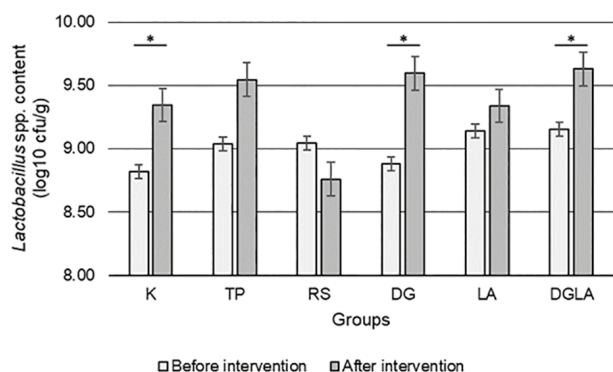
Fig. 2. The comparison *Lactobacillus* spp. content before and after intervention in healthy rats' fecals. *Significant differences with *p* < 0.05

to 9.54 ±0.16 log10 CFU/g; however, the difference was not statistically significant. Surprisingly, the LA diet did not significantly increase the fecal *Lactobacillus* spp. content (from 9.14 ±0.24 to 9.34 ±0.28 log10 CFU/g).

Lactobacillus spp. profile in rats' feces after the intervention

The content of *Lactobacillus* spp. determined in the fecal samples of the studied groups after the 8-week intervention is shown in Figure 3. The content of *Lactobacillus* spp. was significantly lower in the RS group
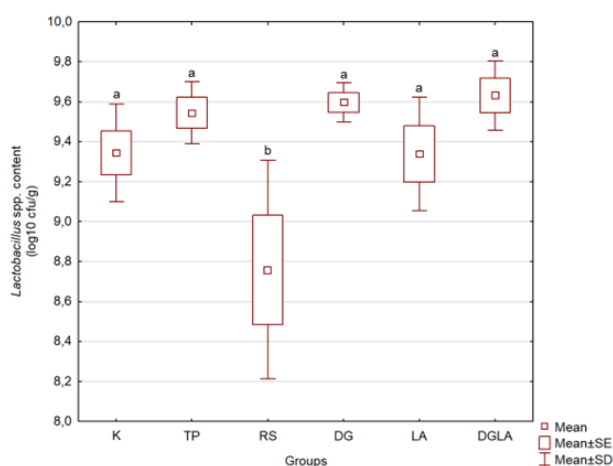

Fig. 3. Box plot result of *Lactobacillus* spp. profile after intervention in healthy rats' fecals. [a,b]Significant differences between groups

(8.76 ±0.55 log10 CFU/g) compared to other groups. Meanwhile, the content of *Lactobacillus* spp. was relatively the highest in DGLA diet (9.63 ±0.17 log10 CFU/g), followed by the DG diet (9.60 ±0.10 log10 CFU/g) and the TP diet (9.54 ±0.16 log10 CFU/g). Unexpectedly, the fecal samples from the LA group (9.34 ±0.28 log10 CFU/g) and the group receiving the standard diet (9.34 ±0.24 log10 CFU/g) had comparable amounts of *Lactobacillus* spp. at the end of the intervention period.

DISCUSSION

The most significant finding of this study was that raw soybean significantly decreased *Lactobacillus* spp. content in feces. On the other hand, pure isoflavones (daidzein and genistein) and products rich in isoflavones, such as tempeh, seem to improve fecal *Lactobacillus* spp. content. Unexpectedly, the fecal content of *Lactobacillus* spp. increased in the control group during the experiment. As a result, the level of *Lactobacillus* spp. was comparable in all groups except the group receiving raw soybean. It is difficult to explain the change observed in the control group in comparison to groups receiving diets supplemented with isoflavones and *L. acidophilus*. Nevertheless, besides macronutrients, some microorganisms, including *Lactobacillus* group, exhibit age-related associations (Salazar et al., 2019). Moreover, the abundance of bacterial species gradually decreased with age (Flemer et al., 2017).

In fact, isoflavones have the ability to increase intestinal flora, particularly lactobacilli (Tamura et al., 2002), and *L. acidophilus* has been shown to influence the intestinal microbiota, primarily intestinal lactobacilli (Lahtinen et al., 2012). However, the increase in fecal *Lactobacillus* spp. content found in the control group could be attributed to the presence of fiber, which has an impact on the intestinal *Lactobacillus* community (Pan et al., 2016). The obtained result of the highest level of insoluble fiber in the raw soy diet resulted in the low content of *Lactobacillus* spp. in feces. It shows that digestive microbes tend to be less able to break down the insoluble fibers (Holscher, 2017). The decrease in the fecal *Lactobacillus* population caused by raw soybean may be related to the presence of soy proteins. It is known that soy proteins

produce putrefactive compounds in the intestines such as ammonia, hydrogen sulfide, amines, phenols, and indoles, which are recognized as carcinogens and toxins (An et al., 2014).

The increase in the gut *Lactobacillus* population observed in TP, DG, and DGLA groups (Fig. 1) in this study can be linked with the presence of daidzein and genistein which were found in greater concentrations in tempeh than in raw soybean. Daidzein is metabolized to equol by the gut microflora. Dihydrodaidzein racemase is a key enzyme in *Lactococcus garvieae*, a lactic acid bacterium that generates equol from daidzein, and was found to increase equol biosynthesis by 5.86-fold (Shimada et al., 2012). Similarly, dihydrodaidzein racemase increased equol production from daidzein in *Limosilactobacillus fermentum* strains (Ruiz de la Bastida et al., 2021). Besides daidzein, genistein increased the count of *Lactobacillus* by up to 85% in a culture of mouse fecal bacteria (Huang et al., 2016). Similarly, genistein increased the number of *Lactobacillales* family by up to 20-fold in the gut of humanized mice (Paul et al., 2017).

Unexpectedly, *L. acidophilus*, a probiotic bacterium, had no significant effect on the *Lactobacillus* spp. content in the present study. The lack of agreement with the findings of other studies may be associated with the inability of *L. acidophilus* to colonize in the intestine. According to Sui et al. (2002) the probiotic *L. acidophilus* strain NCFM® can survive in the human digestive tract for 2 weeks after consumption, but it is incapable of colonizing. The viability of probiotics during gastrointestinal transit is affected by various factors, such as gastric acid, digestive enzymes, and bile acids in the upper gastrointestinal tract, as well as colonization resistance induced by commensal bacteria (Han et al., 2021).

Although the combination of daidzein, genistein, and *L. acidophilus* resulted in the highest fecal *Lactobacillus* spp. content in other intervention groups, such a result seems to be the effect of isoflavones, and not their combination with *Lactobacillus*.

Our hypothesis that oral supplementation of *L. acidophilus* can increase *Lactobacillus* content in feces was not confirmed. Although several other studies have reported beneficial results, it should be mentioned that different strains of bacteria were used by the authors. In one study, *Lactobacillus rhamnosus*-fermented

soymilk was used, which increased the content of *Bacteroides* and *Lactobacillus* bacteria in the feces of healthy male BALB/c mice (Dai et al., 2019). In another study, an aqueous soy extract fermented with *Enterococcus faecium* CRL 183 and *Lactobacillus helveticus* 416 was used, which caused a significant increase in fecal microbiota, including *Lactobacillus* spp. (Cavallini et al., 2011). Furthermore, in diabetes mellitus mice receiving tempeh cofermented with *L. plantarum* (40 mg/kg), the predominant bacteria in the fecal samples was found to be *Lactobacillus* (Huang et al., 2018). Similar findings were observed in a human clinical study. In a randomized, double-blind, placebo-controlled trial on fermented soymilk supplemented with *Lactobacillus casei* Shirota, the relative abundance levels of *Lactobacillaceae* were found to be significantly higher during the 8-week intervention compared to that at baseline (Nagino et al., 2018). However, this study did not confirm the synergistic effects between isoflavones and of *L. acidophilus* in improving the composition of the gut microbiota, particularly the content of *Lactobacillus*. The lack of expected results in the present study may be attributed to use of *L. acidophilus* in healthy rats. Moreover, this finding may be related to fecal microbiota content in the lower digestive tract differs significantly from microbiota content in feces (Tang et al., 2020).

The limitation of this study was that it did not analyze the diversity of fecal microbiota which is important to gain a detailed picture of how isoflavones and probiotics alter gut microbial communities. The use of culture-independent molecular approaches, such as real-time quantitative polymerase chain reaction, for the quantification of bacterial growth may allow yielding more accurate results than the culturing methodology applied in this study. Finally, the study used healthy female rats on a complete diet, so the obtained results indicate that the combination of isoflavones and *L. acidophilus* may be safe for microbiota composition in healthy conditions.

CONCLUSION

To summarize, the oral supplementation of combination between daidzein, genistein *L. acidophilus* DSM20079 may improve the fecal *Lactobacillus* spp. status in healthy female rats, while raw soybean

decreases the fecal content of these bacteria. The oral supplementation with *L. acidophilus* DSM20079 only seems to have no effect on fecal *Lactobacillus* spp. content in healthy female rats.

REFERENCES

An, C., Kuda, T., Yazaki, T., Takahashi, H., Kimura, B. (2014). Caecal fermentation, putrefaction and microbiotas in rats fed milk casein, soy protein or fish meal. Appl. Microbiol. Biotechnol., 98(6), 2779–2787. https://doi.org/10.1007/s00253-013-5271-5

AOAC (1995). Official methods of analysis of AOAC International. Washington, DC: Association of Official Analytical Chemists.

AOAC (2000). Official methods of analysis of AOAC International. Gaithersburg, Md.: Association of Official Analytical Chemists.

Asp, N. (2001). Development of dietary fibre methodology. In B. V. McCleary, L. Prosky (Eds.), Advanced dietary fibre technology (pp. 77–86). Oxford: Blackwell Science.

Cavallini, D. C., Suzuki, J. Y., Abdalla, D. S., Vendramini, R. C., Pauly-Silveira, N. D., Roselino, M. N., …, Rossi, E. A. (2011). Influence of a probiotic soy product on fecal microbiota and its association with cardiovascular risk factors in an animal model. Lipids Health Dis., 10(1), 126. https://doi.org/10.1186/1476-511X-10-126

Dai, S., Pan, M., El-Nezami, H. S., Wan, J. M. F., Wang, M. F., Habimana, O., …, Shah, N. P. (2019). Effects of lactic acid bacteria-fermented soymilk on isoflavone metabolites and short-chain fatty acids excretion and their modulating effects on gut microbiota. J. Food Sci., 84(7), 1854–1863. https://doi.org/10.1111/1750-3841.14661

Dar, H. Y., Shukla, P., Mishra, P. K., Anupam, R., Mondal, R. K., Tomar, G. B., …, Srivastava, R. K. (2018). *Lactobacillus acidophilus* inhibits bone loss and increases bone heterogeneity in osteoporotic mice via modulating Treg-Th17 cell balance. Bone Reports, 8(February), 46–56. https://doi.org/10.1016/j.bonr.2018.02.001

FAO (2003). FAO Food and Nutrition 77. Food energy – methods of anlysis and conversion factors. Rome, Italy: FAO Food and Agriculture Organization of the United Nations.

Flemer, B., Gaci, N., Borrel, G., Sanderson, I. R., Chaudhary, P. P., Tottey, W., …, Brugère, J. F. (2017). Fecal microbiota variation across the lifespan of the healthy laboratory rat. Gut Microb., 8(5), 428–439. https://doi.org/10.1080/19490976.2017.1334033

Han, S., Lu, Y., Xie, J., Fei, Y., Zheng, G., Wang, Z., …, Li, L. (2021). Probiotic gastrointestinal transit and colonization after oral administration: A long journey. Front. Cell. Inf. Microbiol., 11. https://doi.org/10.3389/fcimb.2021.609722

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. https://doi.org/10.3390/foods10112685

Holscher, H. D. (2017). Dietary fiber and prebiotics and the gastrointestinal microbiota. Gut Microb., 8, 2. https://doi.org/10.1080/19490976.2017.1290756

Huang, H., Roman Arocho, G. M., Davis, C., Yu, L., Wang, T. T. Y. (2016). Consumption of selected cruciferous vegetables and soy phytochemical dietary supplements can alter gut microbiome composition. FASEB J., 30(1), 1176.26–1176.26. https://doi.org/10.1096/fasebj.30.1_supplement.1176.26

Huang, Y. C., Wu, B. H., Chu, Y. L., Chang, W. C., Wu, M. C. (2018). Effects of tempeh fermentation with *Lactobacillus plantarum* and *Rhizopus oligosporus* on streptozotocin-induced type II diabetes mellitus in rats. Nutrients, 10(9). https://doi.org/10.3390/nu10091143

Kuligowski, M., Pawłowska, K., Jasińska-Kuligowska, I., Nowak, J. (2017). Composición de isoflavonas, contenido de polifenoles y actividad antioxidante de las semillas de soja durante fermentación de tempeh [Isoflavone composition, polyphenols content and antioxidative activity of soybean seeds during tempeh fermentation]. CyTA J. Food, 15(1), 27–33. https://doi.org/10.1080/19476337.2016.1197316

Lahtinen, S. J., Forssten, S., Aakko, J., Granlund, L., Rautonen, N., Salminen, S., …, Ouwehand, A. C. (2012). Probiotic cheese containing *Lactobacillus rhamnosus* HN001 and *Lactobacillus acidophilus* NCFM® modifies subpopulations of fecal lactobacilli and *Clostridium difficile* in the elderly. AGE, 34(1), 133–143. https://doi.org/10.1007/s11357-011-9208-6

Mahdavi-Roshan, M., Ebrahimi, M., Ebrahimi, A. (2015). Copper, magnesium, zinc and calcium status in osteopenic and osteoporotic post-menopausal women. Clin. Cases Min. Bone Metab., 12(1), 18–21. https://doi.org/10.11138/ccmbm/2015.12.1.018

McClung, M. R., Grauer, A., Boonen, S., Bolognese, M. A., Brown, J. P., Diez-Perez, A., …, Bone, H. G. (2014). Romosozumab in postmenopausal women with low bone mineral density. New Eng. J. Med., 370(5), 412–420. https://doi.org/10.1056/NEJMoa1305224

Medina-Gomez, C. (2018). Bone and the gut microbiome: a new dimension. J. Lab. Prec. Med., 3, 96–96. https://doi.org/10.21037/jlpm.2018.11.03

Nagino, T., Kaga, C., Kano, M., Masuoka, N., Anbe, M., Moriyama, K., …, Miyazaki, K. (2018). Effects of fermented soymilk with *Lactobacillus casei* Shirota on skin condition and the gut microbiota: A randomised clinical pilot trial. Ben. Microb., 9(2), 209–218. https://doi.org/10.3920/BM2017.0091

Pan, Y., Wang, F., Sun, D.-W., Li, Q. (2016). Intestinal *Lactobacillus* community structure and its correlation with diet of Southern Chinese elderly subjects. J. Microbiol., 54(9), 594–601. https://doi.org/10.1007/s12275-016-6131-7

Paul, B., Royston, K. J., Li, Y., Stoll, M. L., Skibola, C. F., Wilson, L. S., …, Tollefsbol, T. O. (2017). Impact of genistein on the gut microbiome of humanized mice and its role in breast tumor inhibition. PLOS ONE, 12(12), e0189756. https://doi.org/10.1371/JOURNAL.PONE.0189756

Pawlowski, J. W., Martin, B. R., McCabe, G. P., McCabe, L., Jackson, G. S., Peacock, M., …, Weaver, C. M. (2015). Impact of equol-producing capacity and soy-isoflavone profiles of supplements on bone calcium retention in postmenopausal women: a randomized crossover trial. Am. J. Clin. Nutr., 102(3), 695–703. https://doi.org/10.3945/ajcn.114.093906

Rizzoli, R. (2014). Nutritional aspects of bone health. Best Pract. Res. Clin. Endocrinol. Metab., 28(6), 795–808. https://doi.org/10.1016/j.beem.2014.08.003

Ruiz de la Bastida, A., Peirotén, Á., Langa, S., Arqués, J. L., Landete, J. M. (2021). Heterologous production of equol by lactic acid bacteria strains in culture medium and food. Int. J. Food Microbiol., 360, 109328. https://doi.org/10.1016/j.ijfoodmicro.2021.109328

Salazar, N., Arboleya, S., Fernández-Navarro, T., de los Reyes-Gavilán, C. G., Gonzalez, S., Gueimonde, M. (2019). Age-associated changes in gut microbiota and dietary components related with the immune system in adulthood and old age: A cross-sectional study. Nutrients, 11(8). https://doi.org/10.3390/nu11081765

Shimada, Y., Takahashi, M., Miyazawa, N., Abiru, Y., Uchiyama, S., Hishigaki, H. (2012). Identification of a novel dihydrodaidzein racemase essential for biosynthesis of equol from daidzein in *Lactococcus* sp. Strain 20-92. Appl. Environ. Microbiol., 78(14), 4902–4907. https://doi.org/10.1128/AEM.00410-12

Sui, J., Leighton, S., Busta, F., Brady, L. (2002). 16S ribosomal DNA analysis of the faecal lactobacilli composition of human subjects consuming a probiotic strain *Lactobacillus acidophilus* NCFMR. J. Appl. Microbiol., 93(5), 907–912. https://doi.org/10.1046/j.1365-2672.2002.01767.x

Tamura, M., Hirayama, K., Itoh, K., Suzuki, H., Shinohara, K. (2002). Effects of soy protein-isoflavone diet on plasma isoflavone and intestinal microflora in adult mice. Nutr. Res., 22(6), 705–713. https://doi.org/10.1016/S0271-5317(02)00378-0

Tang, Q., Jin, G., Wang, G., Liu, T., Liu, X., Wang, B., Cao, H. (2020). Current sampling methods for gut microbiota: A call for more precise devices. Front. Cell. Inf. Microbiol., 10(April). https://doi.org/10.3389/fcimb.2020.00151

Villa, C. R., Ward, W. E., Comelli, E. M. (2017). Gut microbiota-bone axis. Crit. Rev. Food Sci. Nutr., 57(8), 1664–1672. https://doi.org/10.1080/10408398.2015.1010034

Xu, X., Jia, X., Mo, L., Liu, C., Zheng, L., Yuan, Q., Zhou, X. (2017). Intestinal microbiota: A potential target for the treatment of postmenopausal osteoporosis. Bone Res., 5(1), 17046. https://doi.org/10.1038/boneres.2017.46

Yuan, J. P., Wang, J. H., Liu, X. (2007). Metabolism of dietary soy isoflavones to equol by human intestinal microflora – implications for health. Mol. Nutr. Food Res., 51, 7, 765–781. https://doi.org/10.1002/mnfr.200600262

Zhu, K., Prince, R. L. (2012). Calcium and bone. Clin. Biochem., 45(12), 936–942. https://doi.org/10.1016/j.clinbiochem.2012.05.006