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Research Article

# The Health Benefits of Tofu, an Unfermented Soybean Food that Regulates the Intestinal Environment

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## Abstract

Products based on soybean isoflavones are gaining attention globally for their health benefiting properties in multiple systems including the intestinal microbiota. Tofu is a healthy, unfermented soybean food which is widely available and commonly consumed, especially in Japan. However, few studies have focused on the beneficial effects of Tofu on the intestinal environment, particularly constipation, and the dependency of health benefits on the bioavailability of isoflavones. In this study, the effects of Tofu and isoflavone bioavailability on digestive health were investigated. Seventy-nine Japanese participants consumed either Tofu containing isoflavones (Tofu group) or placebo-Tofu containing no isoflavones (p-Tofu group) for four weeks. Urinary isoflavones (daidzein, genistein, and equol) were measured to evaluate isoflavone bioavailability. The results showed that the constipation improvement rate was higher in Tofu group than in the p- Tofu group (p=0.05). Specifically, the improvement rate in females in the Tofu group was significantly higher than females in the p-Tofu group (p<0.05). At the end of the intervention, urinary isoflavones tended to be higher and lower than at baseline in the Tofu group and the p-Tofu group, respectively. Although not statistically significant, this effect was much stronger in females than males in the p-Tofu group. When only participants with improvements in constipation were analyzed, daidzein levels were significantly higher post-intervention than pre-intervention in the Tofu group (p<0.05). Furthermore, when only participants without improvements in constipation were analyzed, equol-producers in the p-Tofu group tended to have a lower equol level and had a significantly lower equol conversion efficiency from daidzein post-intervention than pre-intervention (p<0.05). In conclusion, our results suggest that isoflavone-containing Tofu may be a functional food that regulates the intestinal environment, relieving constipation and maintaining isoflavone levels.

**Keywords:** Bioavailability; Constipation; Equol; Intestinal environment; Isoflavones; Tofu

## Introduction

In recent years, an increasing number of foods have been recognized and studied for their beneficial health effects. The health effects of soybean isoflavones are well known, specifically their anti-inflammatory and anti-oxidative effects, as well as their disease specific effects, including preventing cardiovascular and hormonal disorders and maintaining bone metabolism [1,2]. There are also few studies on the beneficial effects of soy isoflavones on gut microbiota. These studies demonstrate that soy isoflavones can modulate the gut microbiota and suppress the growth of harmful bacteria, suggesting that isoflavones influence the regulation of the intestinal environment [3]. A clinical study using soymilk, a fermented soybean product, suggests that the beneficial effects of soy isoflavones and aglycones occur through their fermentation, digestion, and absorption and modulation of the intestinal microbiota [4].

The relationship between isoflavone intake and absorption of functional aglycones mainly depends on the individual intestinal environment, including the presence of specific digestion/metabolization bacteria and β-glucosidase activity. Aglycones absorbed from the intestine then circulate and act throughout the body [5], meaning the pharmacokinetics/dynamics of isoflavones vary greatly depending on the individual. Therefore, reports related to isoflavones must be understood on this basis. For example, among clinical studies on breast cancer, consumption of isoflavones is not always associated with beneficial effects [6,7]; however, aglycones measured in the blood are associated with the prevention of breast cancer [8]. Thus, to determine the health benefits of isoflavones, it is important that not only the intake/consumption of isoflavones and/or aglycones, but also absorption of aglycones, is examined by using clinical samples such as blood and urine.

There are two types of soybean products: fermented products (miso, soy sauce, soymilk, etc.) and unfermented products (tofu, etc.). While fermented products include isoflavones and aglycones, the unfermented products have only isoflavones, which can be metabolized to aglycones by intestinal microbiota. Tofu is prepared by coagulating soymilk and natural mineral ingredients obtained from surface seawater such as calcium sulfate, magnesium chloride, calcium chloride, and others, known as nigari (bittern) in Japan [9]. Nigari is a concentrated solution of salts left over after the crystallization of seawater,

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which possesses many minerals. However, because of cost implications, most companies use the chemical components to produce tofu instead of natural nigari.

Although the beneficial effects of tofu on various aspects of human health are well established [10], clinical reports focused on the effects of tofu on human intestinal health are still insufficient. Here, we investigated the effects of tofu intake on human health, particularly intestinal environment (constipation), in a randomized double-blind control study using a self-administrated questionnaire and urinary isoflavone aglycone measurements. We prepared tofu with natural nigari obtained from deep seawater, which possesses more minerals at higher concentrations compared to surface seawater [11]. Drinking water generated from deep seawater has been reported in clinical research using urinary isoflavones analysis as safe and beneficial to human health [12,13].

## Materials and Methods

Preparation for Tofu and intake schedule: Tofu produced using original natural ingredients (nigari) from deep seawater collected in the offshore of Muroto, Kochi, Japan, (patent No. 6486529; Muroto-kaiyoushinsousui Co., Ltd., Kochi, Japan), and a commercially available placebo-tofu without soy isoflavones were used in this study (Tofu and p-Tofu groups, respectively). The participants consumed 80g/day of tofu or 100g/day of p-tofu for 4 weeks. Eighty grams of tofu contains 26.4mg of isoflavones, while 100g of p-tofu does not contain any isoflavones.

### Study Design

This randomized double-blind controlled study was performed to compare the intestinal environment of individuals in response to consumption of tofu containing soy isoflavones and p-tofu containing no soy isoflavones using a self-administered questionnaire and urine sample analysis. The study was conducted in Muroto, Kochi, Japan, 2023. The study protocol, although severely restricted in terms of time and budget, was ethically approved, and was conducted in accordance with the ethical standards described in the 1964 Declaration of Helsinki and its later amendments.

### Subjects

Seventy-nine of the 82 healthy adults enrolled participated in this study. We excluded participants with any illnesses and any prescription or commercial drug use. Pregnant females and isoflavone-related supplement users were also excluded. After obtaining written informed consent, 79 participants were randomly divided into two groups: a group consuming tofu (n=41; mean age 40 years; range: 19-74 years; 14 males and 27 females) and a group consuming p-tofu (n=38; mean age 43 years; range 18-75 years; 17 males and 21 females). The characteristics of participants are shown in table 1. During the study period, all participants continued with their normal daily routines without any special restrictions, except for tofu consumption.

### Measurement for Urinary Isoflavones

We measured urinary isoflavones by HPLC according to previous reports [12,13]. Briefly, 800μL of urine was mixed with 80μL of 1M sodium acetate (pH 4.5) and 8μL of β-glucuronidase/sulfatase solution (Sigma- Aldrich, Tokyo, Japan) and incubated at 37°C for 4h to hydrolyze conjugated isoflavones. Next, 80μL of propyl 4-hydroxybenzoate (100μg/mL) was added to the mixture as an internal standard. Samples were then extracted with 1.5mL of dichloromethane and evaporated at 50°C. Twenty microliters of residue dissolved in 400μL methanol and injected into the HPLC system (Shimadzu Co., Ltd., Koto, Japan). The detection limits were 100ng/mL for genistein and daidzein, and 200ng/mL for equol. The amounts of the three isoflavones were corrected for the presence of creatinine, expressed as mg/g Cre. The conversion efficiency of daidzein to equol was calculated as equol/equol + daidzein (g/g E+D).

### Measurement of Dietary Intake and Health

Using a self-administered questionnaire, we evaluated general health, intestinal environment (constipation), consumption of unusual drinks/foods, and the use of supplements and drugs during the study.

### Statistical Analyses

Baseline characteristics, including differences in urinary isoflavones such as daidzein, genistein, and equol between the two groups, were analyzed using the Mann–Whitney U test. Differences in corrected urinary isoflavone data pre- and post-intervention were analyzed using the Wilcoxon signed-rank non-parametric test. Differences in questionnaire responses pre- and post-intervention were analyzed using the chi-square test. Data were analyzed using Bell-Curve for Excel ver. 3.20 (Social Survey Research Information Co., Ltd., Tokyo, Japan). $p<0.05$ was considered statistically significant.

## Results

Baseline characteristics are shown in table 1; there were no statistically significant differences in any parameters between the Tofu and p-Tofu group. There were no adverse effects of tofu or p-tofu consumption on any participants in the study.

### Self-Administered Questionnaire

The self-administered questionnaire revealed that the constipation improvement rate was 17/41 (41.5%) and 8/38 (21%) in individuals in the Tofu and p-Tofu groups, respectively (Figure 1). Overall, the improvement rate tended to be higher in the Tofu group than the p-Tofu group ($p=0.05$). In females, the improvement rate in the Tofu group (48%) was significantly higher than in the p-Tofu group (19%; $p<0.05$).

Next, we analyzed the difference in constipation improvement rate between equol-producers and non-equol-producers in the two groups (Figure 2). Equol-producers were defined as individuals with ≥200ng/mL urinary equol. Overall, the improvement rate was higher in the Tofu group than in the p-Tofu group in both equol-producers and non-equol-producers. Especially, the largest difference between the Tofu and p-Tofu groups was observed in female equol-producers; no difference was observed in male equol-producers.

### Urinary Isoflavones Analysis

Urinary isoflavones such as daidzein and genistein were detected in all 79 participants. Daidzein and genistein tended to increase from pre-intervention to post-intervention in the Tofu group and tended to decrease in the p-Tofu group (all $p>0.05$; Table 2); this effect was more pronounced in females than males in the p-Tofu group, although it remained non-significant ($p<0.1$; Table 2).

| | Tofu | | | | | | Placebo-Tofu | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **All Subjects** | **Total (n=41)** | | **Male (n=14)** | | **Female (n=27)** | | **Total (n=38)** | | **Male (n=22)** | | **Female (n=16)** | |
| Age (year) | 40 | (33-53) | 38 | (33-50) | 45 | (33-53) | 43 | (33-52) | 39 | (33-52) | 46 | (36-53) |
| Daidzein (mg/g-Cre) | 1.00 | (0.32-2.37) | 0.59 | (0.30-2.17) | 1.03 | (0.34-2.24) | 1.49 | (0.54-2.35) | 1.17 | (0.35-2.20) | 1.66 | (0.68-2.87) |
| Genistein (mg/g-Cre) | 0.97 | (0.46-2.43) | 1.24 | (0.68-2.38) | 0.84 | (0.36-2.48) | 1.12 | (0.39-2.93) | 0.89 | (0.39-2.84) | 2.09 | (0.66-2.84) |
| **Equol-Prodecers** | **Total (n=13)** | | **Male (n=5)** | | **Female (n=8)** | | **Total (n=9)** | | **Male (n=5)** | | **Female (n=4)** | |
| Age (year) | 52 | (34-61) | 39 | (33-72) | 53 | (47-57) | 52 | (37-54) | 52 | (38-64) | 45 | (34-53) |
| Daidzein (mg/g-Cre) | 1.73 | (0.48-2.46) | 1.18 | (0.39-2.46) | 1.92 | (0.87-2.45) | 1.83 | (1.46-3.59) | 1.83 | (1.46-3.77) | 2.46 | (1.36-3.25) |
| Genistein (mg/g-Cre) | 1.79 | (0.58-2.79) | 2.43 | (2.23-2.79) | 0.88 | (0.52-2.65) | 0.93 | (0.36-3.52) | 1.31 | (0.93-3.62) | 0.53 | (0.23-1.50) |
| Equol (mg/g-Cre) | 1.03 | (0.57-2.46) | 0.82 | (0.59-2.90) | 1.08 | (0.50-1.48) | 1.66 | (0.92-2.19) | 1.78 | (0.94-2.19) | 1.03 | (0.31-2.66) |
| Equol (g/g-E+D) | 0.47 | (0.32-0.54) | 0.54 | (0.33-0.68) | 0.40 | (0.28-0.52) | 0.32 | (0.24-0.64) | 0.60 | (0.24-0.68) | 0.31 | (0.28-0.40) |

**Table 1:** Preintervention characteristics of the participants from the two groups.

Note: The data was shown as median and IQR in parentheses; non-parametric analysis (Mann-Whitney U test).

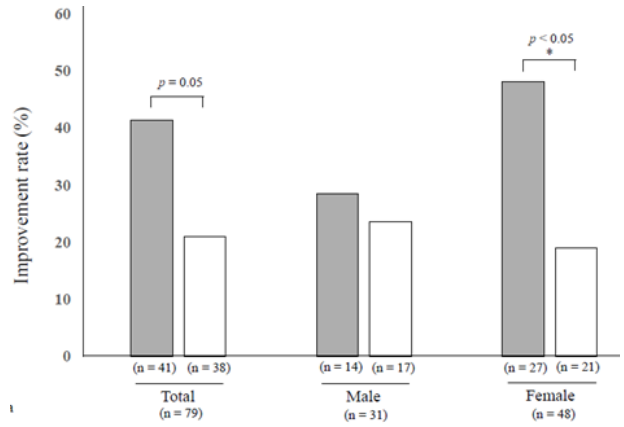


**Figure 1:** Alleviation of constipation after consuming tofu or p-tofu. Overall constipation improvement rate in the Tofu (n=41) and p-Tofu (n=38) groups. The overall constipation improvement rate was 41% and 21% in the Tofu group and p-Tofu group, respectively (p=0.05). The improvement rate in females was significantly higher in the Tofu group (48%) than the p-Tofu group (19%; p<0.05).

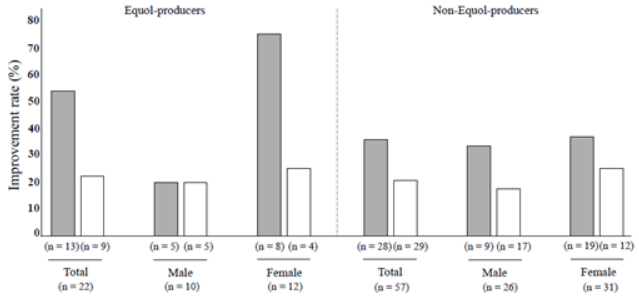


**Figure 2:** Alleviation of constipation after consuming tofu or p-tofu. Differences in constipation improvement rate between equol-producers (n=22) and non-equol-producers (n=57) in the Tofu and p-Tofu groups. The overall constipation improvement rate was higher in Tofu group than the p-Tofu group in both equol producers and non-equol producers. However, the largest improvement rate was found in female equol-producers in the Tofu group, although there were no significant differences because of the small sample number.

Note: Shaded bar, Tofu group; open bar, p-Tofu group; * p<0.05 according to the chi-square test.

Equol, the isoflavone-derived aglycone with the highest physiological activity, generated from daidzein through intestinal microbiota, was detected in 13 and 9 participants in the Tofu and p-Tofu groups, respectively. The proportion of equol-producers in this study was 21/79 (26.5%), which is low compared with the average of around 30-50% in Asian countries [14]. In equol-producers, pre- and post-intervention equol values were similar in the Tofu group; by contrast, in the p-Tofu group, equol values after the intervention decreased to approximately one-third of pre-intervention values, although this was not significant (p<0.1). Similarly, in the p-Tofu group, equol conversion efficiency from daidzein post-intervention was 50% of pre-intervention levels, while post-intervention daidzein levels were around 70% of pre-intervention levels, although these changes were not statistically significant (p<0.1). Therefore, the decrease in equol levels in equol-producers from the p-Tofu group appears to have resulted from both lower daidzein levels and lower conversion efficiency.

Next, the changes in urinary isoflavone levels in participants were analyzed with a focus on resolving constipation (Table 3). Only participants in the Tofu group with improved constipation had a considerable increase in daidzein and genistein postintervention compared with pre-intervention values; in particular, daidzein was significantly higher after the intervention (p<0.05). By contrast, equol-producers in the p-Tofu group whose constipation did not improve tended to have lower equol levels and a significantly lower conversion efficiency after the intervention than at baseline (p<0.05).

## Discussion

Among many reports concerned with the health effect of soybean isoflavones [1,2], few studies report the beneficial effects on the intestinal microbiota such as inhibition of obesity-related bacteria growth [3]. To our knowledge, whether isoflavone intake affects abdominal conditions (i.e., constipation) has not been clinically studied. Here, we found that particularly in females, constipation was alleviated after consumption of tofu, but not p-tofu. This indicates that isoflavones influence the intestinal environment. In males, the tendency for constipation improvement in the Tofu group was not statistically significant. Although non-significant, the difference in constipation improvement rates was larger in equol-producers than in non-equol-producers; however, when analyzed by sex, it was clear that this difference was observed only in female equol-producers. These sex differences suggests that females may be more sensitive to isoflavones including equol. In fact, isoflavones are estrogen-like diphenolic molecules, and

| | Tofu | | | | Placebo-Tofu | | | |
|---|---|---|---|---|---|---|---|---|
| | Pre-Intervention | | Post-intervention | | Pre-Intervention | | Post-Intervention | |
| **All Subjects** | (n=41) | | | | (n=38) | | | |
| Daidzein (mg/g-Cre) | 1.00 | (0.32-2.37) | 1.79 | (0.66-3.84) | 1.49 | (0.54-2.35) | 1.04 | (0.30-1.59) |
| Genistein (mg/g-Cre) | 0.97 | (0.46-2.43) | 1.69 | (0.46-4.47) | 1.12 | (0.39-2.93) | 0.88 | (0.25-2.19) |
| **Male** | (n=14) | | | | (n=22) | | | |
| Daidzein (mg/g-Cre) | 0.59 | (0.30-2.17) | 1.65 | (0.73-2.07) | 1.17 | (0.35-2.20) | 1.31 | (0.44-2.26) |
| Genistein (mg/g-Cre) | 1.24 | (0.68-2.38) | 2.15 | (0.53-4.03) | 0.89 | (0.39-2.84) | 1.63 | (0.30-2.99) |
| **Female** | (n=27) | | | | (n=16) | | | |
| Daidzein (mg/g-Cre) | 1.03 | (0.34-2.24) | 2.25 | (0.61-4.64) | 1.66 | (0.68-2.87) | 0.77 | (0.29-1.13) |
| Genistein (mg/g-Cre) | 0.84 | (0.36-2.48) | 1.63 | (0.63-4.52) | 2.09 | (0.66-2.84) | 0.68 | (0.23-1.07) |
| **All Equol-Prodecers** | (n=13) | | | | (n=9) | | | |
| Daidzein (mg/g-Cre) | 1.73 | (0.48-2.46) | 1.79 | (0.66-3.84) | 1.83 | (1.46-3.59) | 1.28 | (0.71-1.87) |
| Genistein (mg/g-Cre) | 1.79 | (0.58-2.79) | 2.47 | (0.46-3.92) | 0.93 | (0.36-3.52) | 1.64 | (0.83-2.20) |
| Equol (mg/g-Cre) | 1.03 | (0.57-2.46) | 0.77 | (0.49-1.37) | 1.66 | (0.92-2.19) | 0.56 | (0.28-1.03) |
| Equol (g/g-E+D) | 0.47 | (0.32-0.54) | 0.36 | (0.11-0.76) | 0.32 | (0.24-0.64) | 0.18 | (0.16-0.44) |
| **Male** | (n=5) | | | | (n=5) | | | |
| Daidzein (mg/g-Cre) | 1.18 | (0.39-2.46) | 1.66 | (0.66-1.79) | 1.83 | (1.46-3.77) | 1.43 | (0.71-4.75) |
| Genistein (mg/g-Cre) | 2.43 | (2.23-2.79) | 2.60 | (0.46-2.73) | 1.31 | (0.93-3.62) | 1.64 | (1.59-1.95) |
| Equol (mg/g-Cre) | 0.82 | (0.59-2.90) | 0.57 | (0.37-0.69) | 1.78 | (0.94-2.19) | 0.57 | (0.28-1.27) |
| Equol (g/g-E+D) | 0.54 | (0.33-0.68) | 0.25 | (0.14-0.36) | 0.60 | (0.24-0.68) | 0.18 | (0.17-0.45) |
| **Female** | (n=8) | | | | (n=4) | | | |
| Daidzein (mg/g-Cre) | 1.92 | (0.87-2.45) | 2.81 | (1.16-4.42) | 2.46 | (1.36-3.25) | 1.25 | (0.94-1.43) |
| Genistein (mg/g-Cre) | 0.88 | (0.52-2.65) | 1.74 | (0.82-4.50) | 0.53 | (0.23-1.50) | 1.42 | (0.64-2.99) |
| Equol (mg/g-Cre) | 1.08 | (0.50-1.48) | 1.37 | (0.77-4.68) | 1.03 | (0.31-2.66) | 0.44 | (0.24-0.68) |
| Equol (g/g-E+D) | 0.40 | (0.28-0.52) | 0.58 | (0.11-0.87) | 0.31 | (0.28-0.40) | 0.24 | (0.16-0.35) |

**Table 2:** The values of urinary biomarkers in the two intervention groups.

Note: *, p<0.05; The data was shown as median and IQR in parentheses; non-parametric analysis (Wilcoxon rank sum test).

| | Tofu | | | | Placebo-Tofu | | | |
|---|---|---|---|---|---|---|---|---|
| | Pre-Intervention | | Post-Intervention | | Pre-Intervention | | Post-Intervention | |
| **All Subjects** | (n=41) | | | | (n=38) | | | |
| Daidzein (mg/g-Cre) | 1.00 | (0.32-2.37) | 1.79 | (0.66-3.84) | 1.49 | (0.54-2.35) | 1.04 | (0.30-1.59) |
| Genistein (mg/g-Cre) | 0.97 | (0.46-2.43) | 1.69 | (0.46-4.47) | 1.12 | (0.39-2.93) | 0.88 | (0.25-2.19) |
| **Improvement** | (n=17) | | | | (n=8) | | | |
| Daidzein (mg/g-Cre) | 1.60 | (0.48-2.11) | 2.28 | (1.46-8.12) | 1.98 | (1.28-2.56) | 0.98 | (0.63-1.37) |
| Genistein (mg/g-Cre) | 0.97 | (0.58-2.25) | 2.64 | (0.73-6.90) | 0.82 | (0.67-2.52) | 1.21 | (0.20-1.75) |
| **Non-Improvement** | (n=24) | | | | (n=30) | | | |
| Daidzein (mg/g-Cre) | 0.57 | (0.30-2.82) | 1.24 | (0.38-2.54) | 1.00 | (0.41-2.16) | 1.06 | (0.25-1.74) |
| Genistein (mg/g-Cre) | 1.16 | (0.43-2.51) | 1.58 | (0.44-2.45) | 1.34 | (0.39-3.10) | 0.88 | (0.26-2.58) |
| **All Equol-Prodecers** | (n=13) | | | | (n=9) | | | |
| Daidzein (mg/g-Cre) | 1.73 | (0.48-2.46) | 1.79 | (0.66-3.84) | 1.83 | (1.46-3.59) | 1.28 | (0.71-1.87) |
| Genistein (mg/g-Cre) | 1.79 | (0.58-2.79) | 2.47 | (0.46-3.92) | 0.93 | (0.36-3.52) | 1.64 | (0.83-2.20) |
| Equol (mg/g-Cre) | 1.03 | (0.57-2.46) | 0.77 | (0.49-1.37) | 1.66 | (0.92-2.19) | 0.56 | (0.28-1.03) |
| Equol (g/g-E+D) | 0.47 | (0.32-0.54) | 0.36 | (0.11-0.76) | 0.32 | (0.24-0.64) | 0.18 | (0.16-0.44) |
| **Improvement** | (n=7) | | | | (n=2) | | | |
| Daidzein (mg/g-Cre) | 2.11 | (1.11-2.53) | 3.35 | (1.97-4.99) | 4.35 | (3.74-4.95) | 0.99 | (0.85-1.14) |
| Genistein (mg/g-Cre) | 1.79 | (0.87-4.00) | 2.73 | (1.74-5.08) | 2.84 | (1.84-3.85) | 1.24 | (1.04-1.44) |
| Equol (mg/g-Cre) | 1.13 | (0.80-1.80) | 0.86 | (0.53-5.27) | 3.72 | (2.75-4.69) | 0.80 | (0.68-0.91) |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Equol (g/g-E+D) | 0.33 | (0.23-0.51) | 0.25 | (0.11-0.58) | 0.44 | (0.34-0.54) | 0.45 | (0.44-0.45) |
| **Non-Improvement** | **(n=6)** | | | | **(n=7)** | | | |
| Daidzein (mg/g-Cre) | 1.09 | (0.54-2.14) | 0.46 | (0.25-1.51) | 1.79 | (0.90-2.71) | 1.43 | (0.90-3.31) |
| Genistein (mg/g-Cre) | 1.41 | (0.50-2.83) | 0.36 | (0.22-2.07) | 0.93 | (0.30-2.42) | 1.95 | (0.93-2.85) |
| Equol (mg/g-Cre) | 0.70 | (0.36-2.19) | 0.73 | (0.45-1.21) | 0.94 | (0.66-1.92) | 0.31 | (0.15-0.92) |
| Equol (g/g-E+D) | 0.53 | (0.38-0.64) | 0.56 | (0.19-0.82) | 0.32 | (0.26-0.64) | 0.17 | (0.15-0.25) |

**Table 3:** The values of urinary biomarkers in the two intervention groups that focus on relieving constipation.

Note: *, $p<0.05$; The data was shown as median and IQR in parentheses; non-parametric analysis (Wilcoxon rank sum test); ↑, increase; ↓, decrease.

their beneficial effects are observed in a variety of female diseases including hormonal disorders, cancer, and osteoporosis [10,15-20]. It may be that a larger sample number is required to observe any effect of isoflavones on constipation in males.

Isoflavones are digested by intestinal microbiota and the physiologically functional aglycones produced are then absorbed into the bloodstream. In particularly, equol is produced from daidzein by specific bacteria in the microbiota; therefore, it is important to consider the efficiency of the digestion and absorption of these isoflavones when analyzing their effects on human health. This study found that urinary isoflavones were unchanged in the Tofu group during the intervention. However, even in the short study period, isoflavones were considerably decreased in the p-Tofu group, suggesting that consumption of isoflavone-containing tofu at least maintained functional isoflavones levels in Japanese individuals. The consumption of tofu is a common dietary habit to obtain isoflavones for health in the Japanese population. The European Food Safety Authority concluded that the bioavailability of isoflavones from tofu is low [21], although there was no estimation of the bioavailability given. Isoflavones have a biphasic appearance in the plasma and urine following ingestion [10], and the bioavailability of daidzein and genistein are similar in both the plasma and urine [22]. However, there is little clinical data on urinary isoflavone values.

Equol, the isoflavone metabolite with the highest physiological activity, is generally not reported as a corrected value in urine analysis, although a few studies report absolute values (e.g., ng/mL) [23,24]. However, this value cannot be used for inter individual comparisons because it is affected by the amount of urine collected. Using creatinine corrected values, we found that the tofu intervention resulted in decreased levels of all three isoflavones, especially in females in the p-Tofu group. These results indicate that even during short periods of not consuming isoflavone-containing tofu, Japanese females may be more susceptible to decreases in isoflavone levels than males. Combining our urinary equol data with that of 90 healthy adult equol-producers from previous clinical studies [12,13], gives an average value of 1.98mg/g Cre (male (n=40), 2.24mg/g Cre; female (n=50), 1.77mg/g Cre), indicating that urinary equol is lower in female equol-producers than male equol-producers. Therefore, females with a relatively low equol level may be more sensitive to reductions in isoflavone intake, even over a short period. However, values were not analyzed by age or menopausal status, which may have affected the results in females. Thus, the equol reference value is necessary for understand the relationship between changed values and beneficial effects. The establishment of an equol reference range for plasma and urine would be helpful to investigate the effects of equol on human health both scientifically and clinically. Our study shows that approximately 70% of participants were non-equol producers (defined as urinary equol <200ng/mL, as measured by HPLC). However, a more sensitive method would lead to higher detection rates and enable the creation of more specific reference values.

According to the Ministry of Health, Labor and Welfare's report (2002), the intake of soy isoflavone aglycones, as estimated from the intake of soy and soy products, is 16 to 22 mg/day in Japan [25]. However, the daily intake of isoflavone aglycones is decreasing because of the Westernization of food in Japan. Recently, the Ministry of Agriculture, Forestry and Fisheries (2020) recommended a daily intake of isoflavone aglycones of 40-50mg, and an upper limit of 70-75mg, although it is unlikely a person would be able to overdose when eating soy products [26]. The amount of isoflavone aglycones absorbed by the body is affected by an individual's intestinal microbiota. This study, where participants consumed tofu containing 26.4 mg of isoflavones, showed that intake of tofu improved constipation and maintained urinary isoflavones compared with intake of p-tofu containing no isoflavones. Unlike miso and soy sauce, tofu is an unfermented soybean product and has almost no isoflavone aglycones [27]. Therefore, any beneficial effects were presumably due to isoflavone aglycones derived from microbial metabolism in the intestine. This indicates that tofu consumption influenced the intestinal environment and contributed to human health. However, more investigation is needed to clarify the relationship between the amount of absorbed isoflavone aglycones and health benefits, including clinical parameters measured in more participants.

There were some limitations to our study. First, equol-producers were defined as participants with ≥200ng/mL urinary equol. In addition, some variables in this study were not able to undergo statistical analysis because of the small number of participants.

This study demonstrated that consumption of prepared tofu, including 26.4mg of isoflavones, alleviated constipation and maintained urinary isoflavone aglycon level, suggesting that it positively affected the intestinal environment. These effects were mainly observed in females.

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## Ethics Approval and Consent to Participate

The study was ethically approved and was conducted in accordance with the ethical standards described in the 1964 Declaration of Helsinki and its later amendments. This study was conducted with written informed consent from all participants.

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