Stinky tofu as a rich source of bioavailable S-equol in Asian diets

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ABSTRACT

The compound S-equol (4',7-dihydroxy-isoflavandiol), a gut bacterial metabolite of isoflavone daidzein, benefits health, but only 20–60% of humans can produce equol after ingesting isoflavones, and it exists only in foods of animal origin in trace amounts. A recent study found a source of stinky tofu contained S-equol. As stinky tofu is a popular traditional fermented soy food in Taiwan, we analyzed S-equol contents of commercial samples, surveyed the intake frequency, and investigated the bioavailability of S-equol by monitoring urinary kinetics following ingestion. Our results showed 91% of the 138 stinky tofu dishes contained S-equol. The mean content per serving (average 198 g) was 2.3 ± 2.5 mg, the highest being 16.3 mg. Stinky tofu eaters on average ingested this food 3.3 times per month. S-equol from ingested stinky tofu appeared in urine within 1 h, reaching maximum excretion at 3.4 h, and 67% of the ingested S-equol was recovered in urine, indicating a rapid and high absorption. Our studies suggest stinky tofu can be a promising dietary source of S-equol for its high content and bioavailability. Further study on the S-equol producing bacteria in stinky tofu is merited for the development of other S-equol rich soy products.

1. Introduction

After ingestion of soy isoflavones, part of daidzein is hydroxylated to dihydrodaidzein, and finally reduced to S-equol (4',7-dihydroxy-isoflavandiol) or ring-cleaved to O-desmethylandogolensin (ODMA) by different gut bacteria (reviewed by Setchell & Clerici, 2010). Only 20–30% of adults living in Western countries, and 50–60% of adults living in Asian countries possess the gut bacteria cable of producing S-equol (so-called equol producer) (reviewed by Setchell & Clerici, 2010). Equol produced by intestinal microflora is exclusively in S-form. S-equol selectively activates estrogen receptor β (ERβ), and has much higher binding affinity to ERβ than does R-form, or its parent daidzein (Setchell et al., 2005). S-equol is also a potent antagonist of dihydrotestosterone (Lund et al., 2004). Epidemiological studies suggested that equol producing ability was associated with a lower incidence of prostate cancer (Akaza et al., 2004; Kurahashi, Iwasaki, Inoue, Sasazuki, & Tsugane, 2008) and a lower risk for breast cancer in premenopausal (Duncan, Merz-Demlow, Xu, Phipps, & Kurzer, 2000) and postmenopausal women (Fuhrman et al., 2008). Our previous study found isoflavone supplementation improved menopausal symptoms only in women with equol producing ability (Jou et al., 2008). Other clinical study also found less postmenopausal bone loss in equol producers than in equol non-producers after 1 year isoflavone supplementation (Wu...
et al., 2007). Moreover, recent studies using S-equol supplementation confirmed its effect on reduction of bone loss (Touisen et al., 2011) and menopausal symptoms (Aso et al., 2012) in postmenopausal women. These findings highlighted the importance of equol producing ability and the variation in this ability might partly explain the inconsistent results in studies of soy benefits.

Long-term habitual diets influence colon bacterial flora which determine the ability to produce equol and remain stable over time (Rowland, Wiseman, Sanders, Adlercreutz, & Bowey, 2000). To improve equol status in humans, especially for those unable to produce equol, direct ingestion of S-equol from diet may be an option. However, S-equol only exists in a few animal foods in trace amounts from the conversion of daidzein in feeds by gut bacteria (Kuhnle et al., 2008). Recently, after analyzing a series of fermented soy products including natto, soy sauce, miso, and tofu-yoh from Japan, douchi, and doufuru from China, Tao-chio and Shiyu-khao from Thailand, and stinky tofu from Taiwan, Abiru, Kumemura, Ueno, Uchiyama, and Masaki (2012) recently found only a source of raw stinky tofu contained S-equol. Stinky tofu, smelling like certain pungent cheese, is a special and popular fermented soy food traditionally consumed in China, Taiwan, and South Asian countries, and is one of the most attractive foods for international tourists. It is made by marinating fresh tofu for hours or days in stinky brine produced by natural fermentation of aged raw food ingredients, such as bamboo shoots, vegetables, and eggs (Chao, Tomii, Watanabe, & Tsai, 2008). Stinky tofu is usually sold at night market vendors or roadside stands, and is deep fried, boiled or steamed before serving.

Information about S-equol contents of ready-to-serve stinky tofu was not available. We therefore examined the contents of S-equol in stinky tofu served at night markets in Taipei and Taichung cities, surveyed the intake frequency, and investigated the bioavailability of S-equol from stinky tofu by measuring urinary excretion kinetics of S-equol after a single ingestion. We performed the kinetics study in equol producers and equol non-producers to compare the response of both groups, and to distinguish the source of urinary S-equol originally existing in stinky tofu from that produced by intestinal bacteria from daidzein of stinky tofu. Isoflavones in urine instead of in blood have been measured to describe the bioavailability of isoflavones (Faughnan et al., 2004; Franke et al., 2009), and the term ‘apparent bioavailability’ was suggested by Franke et al. (2009). From the data of urinary S-equol elimination rate, the extent of absorption, and possibly effective duration of S-equol from stinky tofu can be estimated.

2. Materials and methods

2.1. Collection of stinky tofu samples for equol determination

We collected 138 dishes of stinky tofu from 118 vendors in 16 night markets of Taipei, and Taichung cities. Most of them were deep fried. If a vendor cooked stinky tofu in more than one method, we collected all kinds. After purchase, they were blenderized and stored in −20 °C before analysis.

2.2. Stinky tofu intake information in stinky tofu eaters

Stinky tofu intake frequency and usual intake size in the past 6 months were assessed by using an interviewer-administered quantitative frequency questionnaire. A total of 202 stinky tofu eaters, 87 males and 115 females, aged 20–64 years, randomly recruited from stinky tofu vendors in night markets of Taipei city, responded to the questionnaire.

2.3. Human studies for urine kinetics of S-equol

2.3.1. Screening of equol producing ability of participants

Participants were recruited from the student population at National Taiwan Normal University through advertisement. Inclusion criteria were as follows: (1) habitual intake of stinky tofu; (2) no history of gastrointestinal, liver, or kidney diseases; (3) nonsmoking and no alcohol consumption; (4) not taking antibiotics, isoflavones or probiotics within 1 month prior to enrollment. 74 subjects including 23 males (age, 21.5 ± 2.8 years; height, 172.8 ± 5.6 cm; weight 66.9 ± 10.6 kg) and 51 females (age, 22.2 ± 2.4 years; height, 164.1 ± 4.5 cm; weight, 54.5 ± 6.5 kg) joined this study. They were asked to refrain from soy foods especially stinky tofu during a 5-day run-in period, and 3-day soy isoflavone challenge. During isoflavone challenge period, participants took 1 tablet of an isoflavone supplement (Nature Made Soy Extract, Northridge, CA, USA) thrice daily, on three consecutive days. Each tablet contained daidzein 13.8 mg, genistein 14.4 mg, glycitein 3.0 mg. Daily intakes of foods and isoflavone tablets were recorded by the participants to monitor their compliance. After the 3-day challenge, in the morning of day 4, their first void urine was collected. Urinary isoflavones were analyzed to identify their equol producing ability. Participants with urinary equol levels below the detection limit (100 ng or 0.41 nmol/mL) were considered to be equol non-producers.

2.3.2. Subjects, study design and urine collection for S-equol excretion kinetics

Twenty equol producers and 20 equol non-producers were invited to participate in this experiment. During the 5-day run-in period, the subjects were asked to refrain from soy foods and record their food intake daily. Commercial stinky tofu containing the highest content of S-equol per serving among those we analyzed was given to each participant at its usual serving size. Each participant collected a baseline 1 h urine sample before the ingestion of stinky tofu at 9 am. After ingestion, each participant collected his four 1-h (0–1, 1–2, 2–3, 3–4 h), four 2–h (4–6, 6–8, 8–10, 10–12 h), and three 12–h (12–24, 24–36, 36–48 h) urine samples. Meals without soy products were provided to subjects throughout the urine collection periods. No extra food except water was allowed. All participants stayed in school dining room from 9 am to 9 pm the first day. They left and came back to take meals and handed in urine samples at meal time the following day and the morning of the third day. Urine samples were kept with ice pad before handed in. Human Subject Committee of Taiwan Adventist Hospital approved the procedures for the frequency questionnaire survey and two intervention studies, one for screening equol producing ability and the other for urinary equol kinetics. Written informed consent
was obtained from each participant before inclusion in each study.

2.4. Analytical methods

2.4.1. Stinky tofu isoflavone analysis

Stinky tofu was blenderized with 3-fold its weight of distilled water for 1 min to get a homogeneous suspension. Isoflavone aglycons and glycosides in this suspension were analyzed in accordance with the following two methods. Samples were analyzed in triplicate.

Extraction and measurement of isoflavone aglycons: 1 g of extraction was extracted with 5 mL of n-hexane twice, the hexane layer was discarded. The residue was extracted with 5 mL of ethyl acetate twice. The ethyl acetate layers were pooled and evaporated at 40 °C under vacuum for 2 h. The residue was dissolved in 1 mL of 1:1 mixture of solvent A (18% methanol, 1.8% ethyl acetate, 0.04% phosphate, and 8 μg/mL EDTA-Na in water) and solvent B (2% ethylacetate in methanol). Isoflavone aglycons were measured according to the method described by Tousen et al. (2011) using a HPLC system (Shimadzu, Kyoto, Japan) equipped with Capcell Pak C18 UG120 column (4.6 × 250 mm, Shiseido, Tokyo, Japan). The mobile phase consisted of a mixture of solvent A and B, in a gradient of solvent B from 0% to 70%, and the absorption was measured at 280 nm for equol, and 254 nm for other isoflavones. Daidzein (Extrasynthese, Genay, France), dihydrodaidzein (Toronto Research Chemicals, Toronto, Canada), R/S-equol (Extrasyntese, Genay, France), ODMA (APIN chemicals, Oxfordshire, UK), genistein (Extrasyntese, Genay, France), dihydrogenistein (Plantech, Berkshire, UK), and glycitein (Wako, Osaka, Japan) were used as standards. The detection limits were 100, 25, 25, 125, 25, 200, 75 ng/mL for equol, daidzein, dihydrodaidzein, ODMA, genistein, dihydrogenistein, and glycitein, respectively.

Enantiomeric analysis was conducted by HPLC (Hitachi, Tokyo, Japan) on a 4.6 × 250-mm Chiral CD-Phe column (Shiseido, Tokyo, Japan), following the method of Abiru et al. (2012). We used R- and S-equol (Cayman, Ann Arbor, MI, USA) as standards.

Extraction and measurement of isoavonone glycosides: 1 mL aliquots of suspension were mixed with 9 mL of 70% ethanol, and extracted overnight at room temperature. The mixtures were filtered through polyvinylidenedifluoride membrane (Whatman plc, Maidstone, UK). Determination of isoavonone glycosides in the filtrates was carried out by HPLC (Hitachi, Tokyo, Japan) on a YMC-pack ODS-AM-303 column (4.6 × 250 mm, YMC, Kyoto, Japan) according to the method of Kudou et al. (1991). The mobile phase consisted of a linear gradient of acetonitrile from 15% to 35% in 0.1% acetic acid at a flow rate of 1 mL/min and the absorption was measured at 254 nm. Daidzin, glycitin, genistin, 6'-O-malonyldaidzin, 6'-O-malonylglycitin, 6'-O-malonylgenistin, 6'-O-acetyldaidzin, 6'-O-acetylglycitin and 6'-O-acetylgenistin (Fujicco, Kobe, Japan) were used as standards. The detection limit was 250 ng/mL.

2.4.2. Urine isoflavone analysis

Urine samples were incubated with β-glucuronidase and sulfatase (Sigma–Aldrich, St Louis, MO, USA) to hydrolyze isoavonone glycosides at 37 °C for 30 min. After hydrolysis, the mixture was extracted with 5 mL ethyl acetate and vortexed for 10 min. The extraction was repeated three times to maximize recovery. Isoflavone aglycons and enantiomers were analyzed by HPLC as described above for stinky tofu.

2.5. Calculations and statistics

Data are means ± SD. Noncompartmental pharmacokinetic parameters of urinary equol were calculated using WinNonlin (Pharsight, Mountain View, CA). Data that are not normally distributed, as analyzed by Kolmogorov–Smirnov test, were transformed prior to analysis (see figure caption for details). Comparisons between equol producers and equol non-producers were made by a two-tailed t-test or Mann–Whitney test if the data after transformation were still not normally distributed. Differences were considered significant at p-value <0.05.

3. Results and discussion

3.1. S-equol in commercial stinky tofu

Abiru et al. (2012) first found stinky tofu to be an S-equol rich food, but they analyzed samples only from a single source. Here, the distribution of S-equol contents in 138 dishes of ready-to-serve stinky tofu purchased from Taipei and Taichung night markets is shown in Fig. 1. More than 91% commercial stinky tofu samples contained S-equol, and the mean level was 2.3 ± 2.5 mg/serving, ranging from 0 to 16.3 mg (Fig. 1), or 1.2 ± 1.0 mg/100 g of wet weight (Table 1), comparable to the data (1.4 mg/100 g) reported by Abiru et al. All the equol contained in our samples was S-enantiomer as determined by HPLC. The percentage of isoflavones and their metabolites in aglycone form ranged from 20 to 100, and all the S-equol was aglycone (Table 1). Since S-equol is a gut bacterial metabolite, all the other known food sources of S-equol are of animal origin, mainly dairy and egg products which contain very low levels of equol (1–11 μg per 100 g) (Kuhnle et al., 2008). Nevertheless, in the low-soy-consumption populations, urinary equol concentration correlated strongly with daily consumption (Frankenfeld, 2011), indicating other dietary source of equol is scarce. To the best of our knowledge, stinky tofu is the only food containing a substantial amount of S-equol.

The contents of S-equol varied extremely among the 138 stinky tofu samples (Fig. 1). The most popular cooking method was deep frying (n = 68) (49%) and the next was boiling in spicy hot pot (n = 40) (29%). 20 vendors served stinky tofu by both cooking methods, and the contents of S-equol did not differ significantly between them (2.0 ± 1.6 vs. 1.2 ± 1.7 mg/100 g). However, boiling in water might have caused the loss of S-equol because 20% of the 40 boiled stinky tofu samples did not contain detectable S-equol, while only 3% of the 67 deep fried samples did not.

The variation in S-equol contents also reflected the diversity of manufacturer sources. Most of the raw stinky tofu was made in small local factories. The kinds of bacteria
growing, foods added in the fermented brine, fermentation duration and temperature were possible factors influencing equol production. Up to now, the bacteria identified to have the capacity to convert daidzein to equol were mostly isolated from human feces (reviewed by Setchell & Clerici, 2010). Chao et al. (2008) identified 32 different species of lactic acid bacteria (LAB) in fermented brine used for stinky tofu manufacture in Taiwan. Some LAB such as a mixture of Lactobacillus strains (Lactobacillus plantarum, Lactobacillus fermentum, and Lactobacillus rhamnosus) (Di Cagno et al., 2010), Bifidobacterium breve, and Bifidobacterium longum (Elghali, Mustafa, Amid, & Manap, 2012) can convert daidzein to equol, but none of them were found in fermented brine of stinky tofu (Chao et al., 2008). Regardless of equol production, some LAB in the fermented brine have been proved to have probiotic properties, such as Lactobacillus brevis (Ronka et al., 2003), Lactobacillus

Table 1 – Contents of isoflavones and their metabolites in 100 g of commercial stinky tofu and a serving of the stinky tofu ingested by participants in urinary kinetics study.

<table>
<thead>
<tr>
<th>Component</th>
<th>100 g of commercial stinky tofu (a) (\text{mg}^c (\text{umol}))</th>
<th>A serving of the tested stinky tofu (b) (\text{mg}^c (\text{umol}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total isoflavones</td>
<td>25.6 ± 10.1 (97.1 ± 38.2)</td>
<td>51.7 (200.2)</td>
</tr>
<tr>
<td>Glucosides</td>
<td>10.6 ± 7.7 (40.0 ± 29.0)</td>
<td>3.0 (11.0)</td>
</tr>
<tr>
<td>Genistin</td>
<td>3.8 ± 2.9 (13.9 ± 10.4)</td>
<td>1.1 (4.0)</td>
</tr>
<tr>
<td>Malonylgenistin</td>
<td>2.3 ± 2.1 (8.7 ± 7.8)</td>
<td>1.9 (7.0)</td>
</tr>
<tr>
<td>Acetylgensitnin</td>
<td>0.2 ± 0.5 (0.8 ± 1.8)</td>
<td>–</td>
</tr>
<tr>
<td>Daidzin</td>
<td>2.4 ± 2.2 (9.4 ± 8.6)</td>
<td>–</td>
</tr>
<tr>
<td>Malonyldaidzin</td>
<td>1.4 ± 1.5 (5.7 ± 5.9)</td>
<td>–</td>
</tr>
<tr>
<td>Acetyldaidzin</td>
<td>0.05 ± 0.22 (0.21 ± 0.88)</td>
<td>–</td>
</tr>
<tr>
<td>Glycitin</td>
<td>0.2 ± 0.5 (0.8 ± 1.7)</td>
<td>–</td>
</tr>
<tr>
<td>Acetylglycitin</td>
<td>0.2 ± 0.9 (0.5 ± 3.1)</td>
<td>–</td>
</tr>
<tr>
<td>Aglycones</td>
<td>15.0 ± 8.2 (57.1 ± 31.2)</td>
<td>48.7 (189.1)</td>
</tr>
<tr>
<td>Daidzein</td>
<td>5.5 ± 3.2 (21.7 ± 12.6)</td>
<td>10.4 (40.8)</td>
</tr>
<tr>
<td>Dihydropodaidzein</td>
<td>0.4 ± 0.6 (1.4 ± 2.2)</td>
<td>0.8 (3.0)</td>
</tr>
<tr>
<td>S-equol</td>
<td>1.2 ± 1.0 (4.8 ± 4.3)</td>
<td>16.7 (68.9)</td>
</tr>
<tr>
<td>ODMA</td>
<td>0.01 ± 0.04 (0.04 ± 0.15)</td>
<td>0.1 (0.5)</td>
</tr>
<tr>
<td>Genisteen</td>
<td>6.5 ± 4.2 (24.0 ± 15.4)</td>
<td>14.9 (55.2)</td>
</tr>
<tr>
<td>Dihydrogenisteen</td>
<td>0.6 ± 0.8 (2.0 ± 2.8)</td>
<td>2.3 (8.5)</td>
</tr>
<tr>
<td>Glycitein</td>
<td>0.9 ± 0.5 (3.6 ± 1.9)</td>
<td>3.5 (12.2)</td>
</tr>
</tbody>
</table>

\(a\) Mean ± SD, \(n = 138\).

\(b\) 146 g of the stinky tofu containing the highest amount of S-equol.

\(c\) Weight of aglycone equivalent.

\(d\) Below detectable level.
The mean intake frequency of stinky tofu was 3.3 times/week, and vendors provided stinky tofu containing more than 10 mg S-equol in 7 (5%) samples, while most humans who can produce equol, can produce ODMA too, as observed from this study and reported elsewhere (Atkinson, Newton, Bowles, Yong, & Lampe, 2008; Rowland et al., 2000). Therefore, the microflora in fermented brine might differ from those in human gut.

### 3.2. Stinky tofu intake frequency survey and estimation of S-equol intake from stinky tofu

The mean intake frequency of stinky tofu was 3.3 times/month for 202 stinky tofu eaters recruited from Taipie night markets, and 22.3% of them consumed stinky tofu at least once a week (Table 2). The mean portion size of stinky tofu ingested per time was 195 g (Table 2), similar to the mean serving size of 198 g. The mean content of S-equol in one serving of stinky tofu was 2.3 mg (Fig. 1), therefore stinky tofu eaters on average ingested 253 µg of S-equol each day, much higher than the daily intake amount of 4.0–5.3 µg from dairy and egg products observed in Europe (Ward et al., 2010). Three of the vendors provided stinky tofu containing more than 10 mg S-equol per serving (Fig. 1), a dose reported to be effective for alleviating menopausal symptoms, especially hot flashes and shoulder stiffness (Aso et al., 2012). Therefore, the amount of S-equol ingested from certain stinky tofu can be clinically significant.

### 3.3. Urine pharmacokinetics of S-equol after a single ingestion of stinky tofu

#### 3.3.1. Characteristics of the participants

Among the 74 college students who received soy isoflavone challenge, 30% were identified as equol non-producers, and 70% as equol producers for whom urinary equol concentrations ranged from 191.7 to 3.1 nmol/mL and log_{10} transformed urinary S-equol: daidzein ratio ranged from 0.6 to −1.25, greater than the cut-off value of −1.75 set by Setchell and Cole (2006) to define equol producer status. The proportion of equol producers is higher than the reported ranges in Asians (50–60%) (reviewed by Setchell & Clerici, 2010), and higher than that we previously reported in postmenopausal women (53%) (Jou et al., 2008). The proportion of equol producers was up to 80% in Korean males in their forties (Fujimoto et al., 2008). The variation in methods of defining equol-producer status can influence the rate, but whether the hobby to ingest stinky tofu contributed to the higher rate of equol producers needs further investigation.

Twenty equol producers with higher log_{10} urinary equol: daidzein ratio (≥−0.05) were invited to participate in the study of urine kinetics. But data from two of them were excluded in statistical analysis because equol was detected at their baseline 1 h urine samples. Twenty equol non-producers were invited too, but one declined before start of the study, and another did not complete the urine collections. Subjects whose data were included in kinetic analysis included 18 equol producers (5 males and 13 females; age, 21.9 ± 2.3 years; height, 163.1 ± 6.6 cm; weight, 59.3 ± 13.0 kg; usual intake of stinky tofu, 1.2 ± 1.1 times/month), and 18 equol non-producers (5 males and 13 females; age, 22.4 ± 3.2 years; height, 163.8 ± 6.5 cm; weight, 56.8 ± 9.4 kg; usual intake of stinky tofu, 1.1 ± 1.3 times/month).

#### 3.3.2. Stinky tofu used for urine kinetic study

The stinky tofu containing the highest amount of S-equol was selected for the study of urine equol kinetics. All the participants complete ingestion of a serving (146 g) of stinky tofu,

Table 2 – Characteristics and stinky tofu intake frequency of stinky tofu eaters.

<table>
<thead>
<tr>
<th></th>
<th>All</th>
<th>Male</th>
<th>Female</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>202</td>
<td>87</td>
<td>115</td>
</tr>
<tr>
<td>Age (y)</td>
<td>29.8 ± 9.9</td>
<td>29.4 ± 9.5</td>
<td>30.2 ± 10.3</td>
</tr>
<tr>
<td>Height (cm)</td>
<td>165.8 ± 9.0</td>
<td>173.1 ± 6.5</td>
<td>159.7 ± 5.7</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>60.4 ± 12.9</td>
<td>69.9 ± 11.6</td>
<td>52.1 ± 7.0</td>
</tr>
<tr>
<td>Usual intake of stinky tofu</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Amount (g/time)</td>
<td>195.0 ± 79.6</td>
<td>207.1 ± 71.4</td>
<td>186.8 ± 83.8</td>
</tr>
<tr>
<td>Frequency (times/mo)</td>
<td>3.3 ± 6.4</td>
<td>3.3 ± 4.5</td>
<td>3.2 ± 7.5</td>
</tr>
<tr>
<td>Frequency distribution, n (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&gt;5/wk</td>
<td>5 (2.5)</td>
<td>1 (1.1)</td>
<td>4 (3.5)</td>
</tr>
<tr>
<td>3–5/wk</td>
<td>6 (3.0)</td>
<td>3 (3.4)</td>
<td>3 (2.6)</td>
</tr>
<tr>
<td>1–2/wk</td>
<td>34 (16.8)</td>
<td>22 (25.3)</td>
<td>12 (10.4)</td>
</tr>
<tr>
<td>2–3/month</td>
<td>61 (30.2)</td>
<td>26 (29.9)</td>
<td>35 (30.4)</td>
</tr>
<tr>
<td>1–3/3 months</td>
<td>79 (39.1)</td>
<td>28 (32.2)</td>
<td>51 (44.3)</td>
</tr>
<tr>
<td>1–2/6 months</td>
<td>6 (3.0)</td>
<td>2 (2.3)</td>
<td>4 (3.5)</td>
</tr>
<tr>
<td>&lt;1/6 months</td>
<td>11 (5.4)</td>
<td>5 (5.7)</td>
<td>6 (5.2)</td>
</tr>
</tbody>
</table>

*a Data are means ± SD.*
containing energy 1628 kJ, protein 24.8 g, fat 30.6 g, carbohydrate 3.6 g, and dietary fiber 1.5 g. The amounts of S-equol, daidzein, dihydrodaidzein, and ODMA ingested were 16.7, 10.4, 0.8, and 0.1 mg, respectively; they were all aglycones (Table 1). Total amount of isoflavones ingested was 51.7 mg, and 94.2% of them were aglycones (Table 1).

3.3.3. Urinary kinetics of S-equol over 48 h

3.3.3.1. Excretion of S-equol originally existing in stinky tofu. Urinary S-equol excretion rate and cumulative amounts during each collection period are shown in Figs. 2 and 3A, respectively. Urinary S-equol was derived from two sources after ingesting stinky tofu, one from the consumed stinky tofu itself (68.9 µmol), the other from intestinal bacterial conversion of daidzein (40.8 µmol) and dihydrodaidzein (3.0 µmol) in stinky tofu to S-equol. S-equol from the former source started to appear in urine within the first hour after ingesting stinky tofu (Figs. 2 and 3A), reaching maximum rate at 3.4th h in both groups (Fig. 2), followed by a steep decline (Fig. 2). The rapid appearance of food S-equol in urine reflected rapid absorption of aglycon S-equol in the upper part of small intestine, similar to the absorption and excretion of isoflavones from other aglycone-rich fermented soy food (Okabe, Shimazu, & Tanimoto, 2011). The patterns of urinary excretion of S-equol (Fig. 3A) and sum of equol precursors, daidzein plus dihydrodaidzein (Fig 3B), were similar in equol producers and non-producers in the initial 10 h.

3.3.3.2. Excretion of S-equol produced by gut bacteria. After a sharp decline, a small second rise appeared around 9th h in equol producers, while in equol non-producers, a short-term flat curve occurred (Fig. 2). The second rise in equol producers was mainly the excretion of a second source of S-equol, produced by colon bacteria. The flat curve in equol non-producers represented enterohepatic circulation of the absorbed S-equol, as the behavior of genistein and daidzein (Watanabe et al., 1998; Zubik & Meydani, 2003). The second rise of S-equol excretion rate was much smaller than the first peak partly because the conversion of daidzein and dihydrodaidzein to S-equol was not efficient, but still contributed to a significant difference in excretion rates between both groups after 10 h (Fig. 2). Equol producers excreted significantly higher amounts of S-equol (Fig. 3A) and lower amounts of S-equol precursors (daidzein and dihydrodaidzein) (Fig. 3B) than equol non-producers during 10–12, 12–24, 24–36, and 36–48 h. The excretion pattern of S-equol (Fig. 3A) after 10 h was complementary to the sum of equol precursors (Fig. 3B) within each group.

3.3.3.3. Excretion of summed daidzein and its metabolites. Urinary excretion of summed daidzein and its metabolites (dihydrodaidzein, S-equol, and ODMA) did not significantly differ between both groups throughout each urine collection period and the entire 48 h (Fig. 3C), and the mean overall cumulative recovery in urine was 76.3 ± 8.9% and 75.5 ± 16.4% of those ingested (113.2 µmol calculated from the data in Table 1) for equol producers and non-producers, respectively. These observations provide evidence supporting that the significant difference in S-equol excretion after 10 h between two groups was due to the conversion of daidzein to S-equol in equol producers.

![Fig. 2](image-url) - Urinary S-equol excretion rate over 48 h after a single ingestion of stinky tofu in equol producers and non-producers. Each collection interval spanned several hours, and the point is plotted at the mid-time of each collection interval. Values are means ± SD, n = 18 for each group. Comparisons between two groups at the same time point were made by two-tailed t-test unless otherwise indicated. Data of 7th and 18th h were transformed for square root before statistical analysis. Data of 11th and 42nd h were analyzed by nonparametric Mann–Whitney test. Asterisks indicate different between equol producers and non-producers, *P < 0.01, **P < 0.001.
Fig. 3 – Urinary excretion of S-equol (A), daidzein and dihydrodaidzein (B) and total daidzein (including daidzein, dihydrodaidzein, S-equol, and ODMA) (C) before and at intervals up to 48 h after a single ingestion of stinky tofu in equol producers and non-producers. Values are means ± SD, n = 18 for each group. Comparison between equol producers and non-producers at the same interval were made by two-tailed t-test unless otherwise indicated. Data transformed for square root before statistical analysis: A, 6–8 and 12–24 h; B, 6–8, 12–24 and 24–36 h; C, 2–3, 6–8, 10–12, and 36–48 h. Data analyzed by nonparametric Mann–Whitney test: A, 10–12, 36–48 and 0–48 h; B, 0–1, 4–6, and 36–48 h; C, 0–48 h. Asterisks indicate different between equol producers and non-producers, *P < 0.01, **P < 0.001.
Table 3 – Pharmacokinetic parameters of urine S-equol after intake of stinky tofu.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Equol producer</th>
<th>Equol non-producer</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>18</td>
<td>18</td>
</tr>
<tr>
<td>Stinky tofu intake (g)</td>
<td>148.1 ± 8.6</td>
<td>143.8 ± 8.1</td>
</tr>
<tr>
<td>S-equol intake (μmol)</td>
<td>69.3 ± 4.2</td>
<td>67.8 ± 4.0</td>
</tr>
<tr>
<td>ER_{max} (μmol/h)</td>
<td>6.3 ± 1.2</td>
<td>6.5 ± 1.9</td>
</tr>
<tr>
<td>T_{max} (h)</td>
<td>3.4 ± 0.3</td>
<td>3.3 ± 0.4</td>
</tr>
<tr>
<td>Total excretion (μmol/48 h)</td>
<td>61.5 ± 9.5</td>
<td>45.7 ± 10.4</td>
</tr>
<tr>
<td>Recovery (%)</td>
<td>^b 67.4 ± 14.8</td>
<td></td>
</tr>
</tbody>
</table>

^a Data are means ± SD, ^P < 0.001 analyzed by two-tailed t-test.

^b Data are not shown because not all urinary equol came from ingested equol in equol producers.

3.3.3.4. Urine pharmacokinetics parameters of S-equol. Time to reach maximum excretion rate (ER_{max}) of S-equol was 3.4 h (Table 3), reasonably longer than the reported time to reach maximum plasma S-equol concentration, 2.3 h (Setchell et al., 2005), and 3.17 h (Setchell, Zhao, Jha, Heubi, & Brown, 2009a) after ingesting pure S-equol. The cumulative recovery of S-equol ingested from stinky tofu in 48 h urine was 67.4% calculated from the data of equol non-producers (Table 3), representing that at least 67.4% of stinky tofu S-equol was absorbed into systemic circulation. The value is similar to the reported data of 61% for pure S-equol (Setchell et al., 2009a), but lower than the data of 82% for a patented product manufactured by the fermentation of soy germ with Lactococcus garvieae (Setchell, Zhao, Shoaf, & Rangel, 2009b).

So, the apparent bioavailability of S-equol in stinky tofu was similar to that of pure S-equol, and there may be no matrix effects of stinky tofu. The absence of difference in ER_{max} and T_{max} between groups (Table 3) indicated the absorption, distribution, and excretion of the ingested S-equol were not influenced by gut equol-producing microflora. Since the majority of ingested S-equol excreted during the first 12 h (Fig. 3A), ingestion twice a day is required to maintain a sustained concentration of S-equol. Equol producers can maintain a higher concentration of S-equol by a delayed production of colonic equol from daidzein after 9 h of ingestion (Fig. 2).

4. Conclusions

Our results showed the commercial stinky tofus contained high levels of bioavailable S-equol. We also established that a substantial amount of S-equol had been ingested and absorbed by stinky tofu consumers in Taiwan. As a high-soy-consumption and high-percent-of-equol production population, the influence of long-term S-equol intake on these consumers’ health is unclear, but no apparent adverse effects of stinky tofu intake have been reported. Our findings may extend to other Asian countries. We conclude that stinky tofu can be a promising dietary source of S-equol, especially for those unable to produce S-equol. Further investigation on the S-equol producing bacteria in stinky tofu is merited for the development of other S-equol-rich soy products or supplements.

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after intake of liquid and solid soya foods. British Journal of Nutrition, 102(8), 1203–1210.


