



Microbial and Dietary Factors Are Associated with the Equol Producer Phenotype in Healthy Postmenopausal Women^{1–3}

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Abstract

Equol, a microbial metabolite of daidzein, has been hypothesized as a clue to the effectiveness of soy and its isoflavones but is excreted by only 33% of Caucasians. Microbial and dietary factors associated with the ability to harbor equol-producing bacteria were studied in a randomized dietary intervention trial with 100 healthy postmenopausal women. After a 4-d baseline period, subjects delivered first-void urine, fecal, and breath samples. During the 5-d treatment period, 3 portions of either soymilk or soy germ containing 28.51 and 37.99 mg isoflavone aglycone equivalents/portion, respectively, were administered daily, and on the last day, 24-h urine samples were collected. The urinary recoveries of genistein and daidzein from soymilk were significantly higher than those from soy germ tablets. Because the proportion of equol:(daidzein + metabolites) in the urine did not differ between the treatment groups, subjects were pooled and classified into poor, moderate, and strong equol producers based on this criterion. The strong equol producer phenotype correlated negatively [in vivo, $r = -0.478$ (-0.256 to -0.893), $P = 0.021$; in vitro, $r = -0.576$ (-0.350 to -0.949), $P = 0.030$] with *Clostridium coccoides-Eubacterium rectale* counts and positively [in vivo, $r = 1.158$ (0.971–1.380), $P = 0.048$; in vitro, $r = 1.156$ (1.007–1.327), $P = 0.039$] with the abundance of sulfate-reducing bacteria. Furthermore, persons with a higher PUFA [in vivo, $r = 2.150$ (1.058–4.371), $P = 0.034$; in vitro, $r = 2.131$ (1.144–3.967), $P = 0.017$] and alcohol [in vivo, $r = 1.166$ (0.721–1.887), $P = 0.050$; in vitro, $r = 1.850$ (1.215–2.817), $P = 0.004$] intake were more likely to be strong equol producers. Finally, we validated the daidzein metabolism by fecal cultures as screening assay to identify equol producers without dietary intervention. J. Nutr. 137: 2242–2246, 2007.

Introduction

Considering the results of the Women's Health Initiative (1) and the Million Women Study (2), the North American Menopause Society recommends lifestyle-related strategies and nonprescription remedies such as dietary isoflavones for the relief of mild

menopause-associated vasomotor symptoms prior to prescribing systemic estrogen-containing products (3). Isoflavones are weak agonists of the estrogen receptors; in vitro they have a higher affinity to estrogen receptor- β than $-\alpha$ (4) and are, therefore, often regarded as natural selective estrogen receptor modulators. High levels of isoflavones are found in traditional soy-based foods, soy protein isolate, soy concentrate, and soy flour as well as in dietary supplements that are now widely available.

Although isoflavone ingestion results in increased levels of the parent isoflavones in plasma and urine, these compounds can also be metabolized. The importance of the gut microbiota in the metabolism of isoflavones has been convincingly demonstrated in in vitro and in vivo studies (5). Firstly, because isoflavones derived from our food occur mainly as glycosides, their intestinal absorption requires deglycosylation (6). β -Glucosidases of both the intestinal mucosa and the gut microbiota are involved in this hydrolysis (7,8). Secondly, these bioactive aglycones are extensively

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³ Supplemental Tables 1–3 are available with the online posting of this paper at jn.nutrition.org.

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conjugated during and after absorption through the gut barrier (6). Bacterial enzymes can deconjugate the glucuronides and sulfate esters excreted in the bile and, consequently, enhance the reuptake of isoflavones (5). Finally, specific microbial transformation reactions occur in the colon. Genistein can be converted to dihydrogenistein and 6'-hydroxy-O-desmethyldaidzein or 2,4,6-trihydroxybenzoic acid and *p*-ethyl phenol, whereas the major microbial metabolites of daidzein are dihydrodaidzein (DHD),¹¹ equol, and O-desmethyldaidzein (O-DMA). The extent of this microbial metabolism varies considerably among individuals (9,10).

Equol has gained much attention since the postulation of the equol hypothesis, contending a greater efficacy of soy food diets in so-called equol producers (11). Equol is exclusively formed by intestinal microbiota and only 33% of Caucasians are host to a population of intestinal microorganisms capable of converting daidzein into equol (12). Yet, factors influencing the bioactivation of daidzein *in vivo* remain unclear. Besides the composition and activity of the intestinal microbiota, the diet has been suggested to contribute to the ability to harbor equol-producing bacteria. More in particular, differences in total fat and/or dietary fiber consumption have been highlighted in previous studies (9,10,13,14), but the results are contradictory.

The purpose of this work was to: 1) compare the urinary excretion of soy-derived isoflavones and metabolites after consumption of soymilk and soy germ tablets; 2) evaluate the performance of *in vitro* incubations as an equol-producer phenotyping assay; and 3) identify microbial and dietary factors associated with equol production. Therefore, a randomized dietary intervention trial with 100 healthy postmenopausal Caucasian women was conducted.

Study Design and Methods

Chemicals. Analytical standards for genistein and daidzein, for equol, and for DHD and O-DMA were purchased from Sigma-Aldrich, Extrasynthèse, and Plantech UK, respectively. SoyLife EXTRA, a soy germ powder containing 32.4 mg daidzein/g, was kindly provided by Frutarom Netherlands BV. Type H-1 *Helix pomatia* extract (min. 300 U β -glucuronidase/mg and 15.3 U sulfatase/mg), *p*-nitrophenol, *p*-nitrophenyl- β -D-glucopyranoside, and *p*-nitrophenyl- β -D-glucuronide were obtained from Sigma-Aldrich.

Isoflavone preparations. The isoflavone preparations used in this study were commercially available soymilk derived from whole soybeans (Alpro Soya Drink Nature) in 250-mL cartons and a soy germ powder (SoyLife EXTRA) formulated as tablets. Both preparations were kindly provided by their manufacturers.

One portion of soymilk (250 mL) contained 7.03 ± 0.23 mg glycitein, 16.16 ± 0.19 mg genistein, and 5.32 ± 0.08 mg daidzein aglycone equivalents, of which 1.38, 4.43, and 4.47% were present as aglycones, respectively. Each soy germ tablet contained 12.33 ± 0.70 mg glycitein, 5.18 ± 0.16 mg genistein, and 20.48 ± 1.04 mg daidzein aglycone equivalents, of which 2.53, 2.07, and 2.46%, respectively, were not glycosylated.

Subjects. A total of 100 healthy postmenopausal (≥ 1 y since last menses) Caucasian women aged 44–76 y (57 ± 6 y) participated in this study. Their mean BMI based on self-reported weight and height measurements was 23.9 ± 3.7 kg/m². The exclusion criteria were the use of any exogenous hormone medications, use of antibiotics within the previous month, soy allergy, and a history of gastrointestinal diseases or cancer. Ten participants had taken antibiotics 1–3 mo prior to the study, 7 and 12 subjects finished their last treatment 3–6 mo and 6–12 mo

before recruitment, and 71 women did not use antibiotics during the previous year.

Study design. This study was a randomized dietary intervention trial with a baseline period of at least 4 d and a treatment with soymilk or soy germ tablets during 5 d. Participants consumed their habitual western-type diets but were asked to abstain from soy-based products during the trial. A detailed list of isoflavone-containing foods and dietary supplements was distributed to guide the volunteers in this respect. After the baseline period, subjects delivered first-void urine, fecal, and breath samples. During the treatment period, 3 portions of either soymilk or soy germ were taken daily. On the last day, a 24-h urine sample was collected.

A validated self-administered semiquantitative FFQ was used to estimate the usual fat [total fat, SFA, monounsaturated fatty acid (MUFA) and PUFA], fiber, alcohol, caffeine, and theobromine intake (15). In addition, a general questionnaire including questions about medication use; intake of pre-, pro-, and synbiotics; reproductive factors; sociodemographics; and anthropometric measures was administered.

Sample collection and processing. End-expiratory breath samples were collected using the QuinTron GaSampler system (Ecce Medical) and analyzed immediately by GC-flame ionisation detector (15). The background room air was found to contain < 2 ppm methane. Participants were considered to be methane producers (61%) when their breath methane concentration exceeded 3 ppm (16).

Volumes of the 24-h urine samples were measured and aliquots were stored at -20°C . For the hydrolysis of conjugated isoflavones, a 33-g/L solution of Type H-1 *Helix pomatia* extract in sodium acetate buffer (0.1 mol/L, pH 5) was prepared. One milliliter urine was added to 1 mL sodium acetate buffer and 20 μL β -glucuronidase/arylsulfatase solution and incubated for 1 h at 37°C . The hydrolyzed samples were spiked with 90 μL internal standard (0.4 mol/L 4-hydroxybenzophenone in ethyl acetate) and extracted twice with 5 mL diethyl ether. Finally, the solvent was evaporated at room temperature under a gentle stream of N_2 and the residue was dissolved in 200 μL methanol, transferred into vials, and stored at -20°C prior to HPLC analysis.

The participants were supplied with plastic containers and asked to bring their samples as soon as possible after defecation. Within 6 h of collection, fecal suspensions were prepared by homogenizing 20 g feces with 100 mL phosphate buffer (0.5 mol/L, pH 7) supplemented with 1 g/L sodium thioglycolate. Particulate material was removed by centrifugation at $400 \times g$; 2 min. To assess the daidzein metabolism, fecal cultures were incubated anaerobically for 3 d at 37°C with 4 g/L SoyLife EXTRA in Brain Heart Infusion broth (37 g/L, Oxoid) supplemented with 0.5 g/L L-cysteine HCl and 2 mg/L resazurin, as described Possemiers et al. (17).

Chromatographic methods. Methane concentrations in the alveolar air samples were measured with a Chrompack CP 9000 GC equipped with flame ionization detector (Chrompack) and a NaI-inactivated alumina column (100–120 mesh, 3 mm \times 1.8 m; Alltech Ass.) (15).

Quantitative analyses of the isoflavones genistein and daidzein and the metabolites DHD, O-DMA, and equol were performed by HPLC-UV using a Waters 2695 Alliance separations module and 996 photodiode array detector (Waters) as reported by Maubach et al. (18). The reversed-phase column was an XTerra MS C₁₈ (5 μm , 4.6 mm \times 250 mm; Waters). UV detection was conducted at 260 nm for genistein, 248 nm for daidzein, 277 nm for O-DMA and DHD, and 230 nm for equol. The internal standard was detected simultaneously with and at the same wavelength as described above for each compound.

Microbiological analyses. For the assessment of β -glucosidase and β -glucuronidase activities, fecal suspensions were centrifuged ($5000 \times g$; 5 min), diluted 5-fold in phosphate buffer (0.5 mol/L, pH 7), and incubated aerobically for 30 min at 37°C with *p*-nitrophenyl- β -D-glucopyranoside (2.5 mmol/L) or *p*-nitrophenyl- β -D-glucuronide (2.5 mmol/L), respectively (15). The release of *p*-nitrophenol was recorded with a Tecan Sunrise absorbance reader (Tecan Benelux) at 405 nm before and after incubation.

¹¹ Abbreviations used: DHD, dihydrodaidzein; MUFA, monounsaturated fatty acid; O-DMA, O-desmethyldaidzein.

Total DNA from the fecal suspensions was extracted with QIAamp DNA Stool Mini kits (Qiagen GmbH). The 16S ribosomal RNA genes of bacteria, bifidobacteria, members of the *Clostridium coccoides-Eubacterium rectale* group, *dsrB* genes of sulfate-reducing bacteria, and *mcrA* genes of methanogens were amplified by real-time PCR using an ABI Prism 7000 sequence detection system (PE Applied Biosystems), as described by Possemiers et al. (17) and Van de Wiele et al. (19).

Statistical analyses. All extractions and analyses were performed in triplicate. We used SPSS for Windows version 12.0. Unless reported differently, results were considered significant at an α 2-tailed level of 0.05. Tests for normality of the data and equality of the variances were performed using the Kolmogorov-Smirnov and Levene's test, respectively. Comparison of normally distributed data was performed with Student's *t* test. The nonparametric Mann-Whitney U and Kruskal-Wallis test were used to compare means of nonnormally distributed data. Categorical variables were analyzed using cross-tabulations with χ^2 or Fisher's exact tests. Associations were described using nonparametric Spearman correlations.

Based on the ratios equol:(daidzein+metabolites) excreted in the 24-h urine samples and produced by the fecal cultures, subjects were separated into significantly different groups of in vivo and in vitro producers, respectively, using the TwoStep cluster analysis protocol. Cross-classification analyses and weighted κ statistics, calculated with a linear set of weights (20), were used to measure the level of agreement between the in vivo and in vitro phenotyping method. Associations between the producer phenotypes and subject characteristics, microbial, and dietary variables were evaluated using nominal logistic regression with poor equol producers as reference category.

Ethics approval. Ethical approval was granted from the Ethics Committee of the Ghent University Hospital (EC UZG 2004/044). The volunteers were fully informed on the aims of the study and gave their written consent.

Results

Soymilk vs. soy germ tablets. The soymilk and soy germ groups did not differ in terms of subject characteristics, dietary habits, breath methane excretion, microbial metabolism of daidzein in vivo and in vitro, composition of the intestinal microbiota, and fecal β -glucosidase and β -glucuronidase activities (Supplemental Table 1).

The sum of unconjugated forms and deconjugated glucuronides and sulfates of genistein, daidzein, DHD, equol, and O-DMA in the 24-h urine samples was quantified as daily excretion and dose-percentages recovered (Table 1) and used as proxy for their bioavailability. The recovery of genistein was significantly higher in the soymilk group than the group treated with soy germ. The mean dose-percentages of daidzein detected in the urine differed significantly between the treatment groups only when the daidzein metabolites DHD, equol, and O-DMA were also taken into consideration. For genistein, a significant positive correlation ($r = 0.209$; $P = 0.038$) was found between the ingested amount and the urinary recovery, whereas a stronger negative relation ($r = -0.329$; $P = 0.001$) was observed for daidzein. Neither the fecal β -glucosidase nor the β -glucuronidase activity correlated significantly with the recovery or excretion of genistein and daidzein (Supplemental Tables 2 and 3).

Daidzein metabolism. Based on the percentage daidzein aglycone equivalents excreted in the 24-h urine as equol, the participants were classified as poor (61%), moderate (21%), and strong (18%) in vivo equol producers, respectively. Because almost identical classifications (weighted κ , 0.96; 95% CI, 0.78; 1.14) were obtained when considering the participants of only 1 treatment group, the distinction between the soymilk and soy

TABLE 1 Concentrations of genistein, daidzein, and its metabolites in 24-h urine samples of postmenopausal women after 5 d of soymilk or soy germ consumption^{1,2}

	Soymilk (n = 50)	Soy germ tablet (n = 50)	P
Excretion, mg/d			
Genistein	10.09 \pm 0.76	2.86 \pm 0.32	<0.001
Daidzein	8.20 \pm 0.54	28.18 \pm 2.28	<0.001
DHD	1.46 \pm 0.18	6.76 \pm 0.98	<0.001
Equol	2.81 \pm 0.47	7.42 \pm 1.77	0.943
O-DMA	1.76 \pm 0.26	3.01 \pm 0.51	0.149
Recovery, ³ %			
Genistein (as genistein)	20.96 \pm 1.57	18.42 \pm 2.03	0.037
Daidzein (as daidzein)	51.39 \pm 3.41	45.87 \pm 3.71	0.229
Daidzein (as daidzein + metabolites)	90.50 \pm 4.31	73.86 \pm 4.46	0.002

¹ Values are means \pm SEM, n = 50.

² Intake of 48.48 mg genistein and 15.96 mg daidzein aglycone equivalents per day in the soymilk group; intake of 15.54 mg genistein and 61.44 mg daidzein aglycone equivalents per day in the soy germ group.

³ Recovery = excretion in 24-h urine/daily intake.

germ groups was removed for the further analyses of the daidzein metabolism. The in vitro microbial metabolism of daidzein was highly variable, ranging from 0 to 100% daidzein transformed into equol, which led to a separation of the subjects in poor (51%), moderate (10%), and strong (39%) in vitro equol producers. The in vivo and in vitro phenotyping methods resulted in the same clustering for 65% of the subjects, whereas 10% were assigned to opposite tertiles. The weighted κ was 0.53 (95% CI, 0.37; 0.69). The ratios equol:(daidzein + metabolites) excreted in the 24-h urine samples and produced by the fecal cultures correlated well ($r = 0.845$; $P < 0.001$). Lower correlations were obtained for the proportions daidzein:(daidzein + metabolites) ($r = 0.411$; $P < 0.001$), DHD:(daidzein + metabolites) ($r = 0.443$; $P < 0.001$), and O-DMA:(daidzein + metabolites) ($r = 0.526$; $P < 0.001$) in the urine and incubation broths.

Factors associated with equol production. Age, BMI, smoking, use of antibiotics, fecal β -glucosidase, and β -glucuronidase activity and the intake of caffeine, theobromine, and pre-, pro-, and synbiotic preparations were not significantly associated with the ability to produce equol. However, differences in specific microbial and dietary factors could be linked to the variation in equol production in vivo (Table 2) and in vitro (Table 3). A negative relation was observed between the *Clostridium coccoides-Eubacterium rectale* counts and the strong in vivo ($P = 0.021$) and in vitro ($P = 0.030$) equol producer phenotype. In contrast, the abundance of sulfate-reducing bacteria correlated positively with the strong in vivo ($P = 0.048$) and in vitro ($P = 0.039$) equol producer phenotypes. There was a positive association ($P < 0.050$) between the estimated usual intake of PUFA and the production of equol. In addition, persons who reported higher alcohol consumption were more likely to be strong in vivo ($P = 0.050$) and in vitro ($P = 0.004$) equol producers.

Discussion

The bioavailability of dietary phytoestrogens such as soy isoflavones is characterized by considerable inter-individual variation, which may influence the bioactivity of these compounds

TABLE 2 Microbial and dietary variables in relation to the in vivo equol producer phenotype¹

Characteristics	In vivo equol producer	
	Moderate (n = 21)	Strong (n = 18)
Microbial variables		
Pulmonary methane excretion, ppm	0.497 (0.218–1.134)	0.645 (0.283–1.047)
Fecal microbiota, log ₁₀ cells/g		
Bacteria	0.615 (0.223–1.694)	1.779 (0.681–4.647)
<i>C. coccoides-E. rectale</i> group	0.959 (0.540–1.704)	0.478 (0.256–0.893)*
Sulfate-reducing bacteria	1.008 (0.858–1.184)	1.158 (0.971–1.380)*
Methanogens	0.833 (0.592–1.171)	1.036 (0.700–1.535)
Bifidobacteria	1.164 (0.270–5.018)	2.272 (0.408–12.663)
Dietary variables		
Fat consumption, g/d		
Total fat	0.863 (0.392–1.900)	0.718 (0.343–1.504)
SFA	1.080 (0.456–2.556)	1.316 (0.597–2.901)
MUFA	1.321 (0.482–3.617)	1.362 (0.515–3.602)
PUFA	1.163 (0.556–2.531)	2.150 (1.058–4.371)*
Fiber consumption, g/d		
Fat:fiber ratio	1.016 (0.929–1.110)	1.014 (0.910–1.129)
Fat:fiber ratio	1.635 (1.038–2.576)	0.582 (0.290–1.167)
Alcohol consumption, g/d	1.583 (0.965–2.598)	1.166 (0.721–1.887)*

¹ Values are OR (95% CI) derived from nominal logistic regression with poor in vivo equol producers, n = 61, as reference category. *P ≤ 0.050.

in humans. In this study, differences in microbial and dietary factors could be linked to the variation in equol production in vivo and in vitro. No isoflavones were detected at baseline, as expected from the results of Faughnan et al. (21). The dose-percentages of genistein and daidzein measured in the 24-h urine samples agreed with previous reports (22,23) and were generally higher following soymilk consumption than soy germ ingestion. To determine whether the formulation [chemical composition, dose, and matrix; (5)] was responsible for the difference in daidzein and genistein recovery between the treatment groups, interventions with fully controlled meals are needed.

TABLE 3 Microbial and dietary variables in relation to the in vitro equol producer phenotype¹

Characteristics	In vitro equol producer	
	Moderate (n = 10)	Strong (n = 39)
Microbial variables		
Pulmonary methane excretion, ppm	0.459 (0.147–1.435)	0.874 (0.460–1.659)
Fecal microbiota, log ₁₀ cells/g		
Bacteria	1.227 (0.181–8.315)	1.466 (0.675–3.182)
<i>C. coccoides-E. rectale</i> group	1.018 (0.443–2.337)	0.576 (0.350–0.949)*
Sulfate-reducing bacteria	0.876 (0.670–1.144)	1.156 (1.007–1.327)*
Methanogens	1.528 (0.577–4.043)	1.279 (0.911–1.796)
Bifidobacteria	5.602 (0.378–83.029)	2.051 (0.513–8.202)
Dietary variables		
Fat consumption, g/d		
Total fat	0.343 (0.113–1.044)	0.572 (0.305–1.070)
SFA	3.295 (0.976–11.126)	1.856 (0.938–3.670)
MUFA	3.595 (0.864–14.957)	1.790 (0.800–4.006)
PUFA	3.003 (1.097–8.223)*	2.131 (1.144–3.967)*
Fiber consumption, g/d		
Fat:fiber ratio	0.914 (0.796–1.050)	0.936 (0.857–1.021)
Fat:fiber ratio	1.153 (0.629–2.113)	0.797 (0.517–1.231)
Alcohol consumption, g/d	1.341 (0.717–2.506)	1.850 (1.215–2.817)*

¹ Values are OR (95% CI) derived from nominal logistic regression with poor in vitro equol producers, n = 51, as reference category. *P < 0.050.

In response to the need for a universal and consistent way to phenotype individuals with low soy consumption patterns, Setchell and Cole (24) introduced a new data processing method to define the equol producer phenotype. Although the log₁₀-transformed ratio equol:daidzein excreted in the urine is independent of the precursor intake, this method requires a challenge with soy isoflavones. Furthermore, other daidzein metabolites are not considered, which makes discrimination between the profiles of person A excreting 50 units as daidzein and 50 as equol, and person B excreting 10 units as daidzein, 80 as O-DMA, and 10 as equol, impossible. Here, we propose a different approach to identify equol producers based on the daidzein metabolism by fecal cultures without a dietary intervention.

In this study, both the equol production and the clustering of the subjects into poor, moderate and strong equol producers based on the in vitro experiments correlated well with those estimated from the urinary excretion profiles. The proportion equol:(daidzein + metabolites) in the incubation broths was generally higher than in the urine, accounting for 83% of the cases classified in adjacent tertiles and 90% of the misclassification. Switching to a binary system with low (i.e. poor in vivo and poor and moderate in vitro producers) and high (i.e. moderate and strong in vivo and strong in vitro producers) equol producers resulted in an even better agreement (κ , 0.62; 95% CI, 0.417; 0.82), with 39% high equol producers, as expected from the literature (12). In contrast, using the formula of Setchell and Cole (24), 61% of the subjects would be defined as high equol producers. This discrepancy was mainly due to the contribution of DHD and O-DMA. Although the in vivo bioavailability is the final result of a cascade of complex processes, these results show that 1 fecal sample is sufficient to phenotype an individual as low or high equol producer. Moreover, the same sample could be used to get additional information on the bioactivation of other dietary phytoestrogens such as hop-derived prenylflavonoids (15).

As reviewed by Atkinson et al. (12), the factors triggering the ability to harbor equol-producing bacteria are still unknown. Inter-individual differences in the composition of the gut microbiota as well as the background diet have been suggested to contribute. In this cohort, the strong equol-producer phenotype correlated negatively with *Clostridium coccoides-Eubacterium rectale* counts and positively with the abundance of sulfate-reducing bacteria. Hydrogen gas is formed in the colon by a variety of hydrolytic and saccharolytic bacteria to dispose reducing equivalents during fermentation and is consumed by methanogenic, homoacetogenic, and sulfate-reducing microbiota. Decroos et al. (25) highlighted the importance of H₂ in the production of equol, but further research is needed to explain this interaction.

Several studies reported differences in total fat and/or dietary fiber consumption between low and high equol producers (9,10,13,14). This study reveals that persons with a higher PUFA and alcohol intake were more likely to be strong equol producers. PUFA are concentrated in foods such as fish and seafood, which are not generally consumed on a daily basis in western countries (26). In Japan and Korea, however, the diet is low in red meat and often rich in fish; thus, PUFA accounts for a larger proportion of the total fat intake, which may explain why the prevalence of strong equol producers is higher in these countries (27). To our knowledge, the effect of alcohol on the gut microbiota and, hence, their phytoestrogen metabolism, have not been investigated directly. As it is rapidly and almost completely absorbed in the stomach and the small intestine, alcohol is not expected to importantly affect the intestinal

bacteria, although alcohol consumption has been negatively associated with the microbial *O*-demethylation of isoxanthohumol, a weak phytoestrogen present in hop-derived food supplements and beers (15). No differences were found in the intake of dietary fiber or the use of pre-, pro-, or synbiotic preparations. It was previously shown in several dietary intervention studies that the equol production could not be stimulated with fibers, pre-, or probiotics (28–30).

In summary, we found that the bioavailability of genistein and daidzein, estimated as the dose-percentages recovered in 24-h urine samples, was higher following soymilk consumption than soy germ ingestion. The proportion equol:(daidzein + metabolites) excreted did not differ between the treatment groups. However, differences in specific microbial and dietary factors could be linked to the variation in equol production in vivo and in vitro. Finally, both the equol production and the phenotyping of the subjects based on the daidzein metabolism by fecal cultures correlated well with those estimated from the urinary excretion profiles, showing the potential of fecal incubations to identify equol producers without dietary intervention.

Literature Cited

- Writing group for the Women's Health Initiative investigators. Risks and benefits of estrogen plus progestin in healthy postmenopausal women: principal results of the Women's Health Initiative randomized controlled trial. *JAMA*. 2002;288:321–33.
- Million Women Study Collaborators. Breast cancer and hormone-replacement therapy in the Million Women Study. *Lancet*. 2003;362:419–27.
- The North American Menopause Society. Treatment of menopause-associated vasomotor symptoms: position statement of The North American Menopause Society. *Menopause*. 2004;11:11–33.
- Kuiper GG, Lemmen JG, Carlsson B, Corton JC, Safe SH, van der Saag PT, van der Burg B, Gustafsson JA. Interaction of estrogenic chemicals and phytoestrogens with estrogen receptor beta. *Endocrinology*. 1998;139:4252–63.
- Rowland I, Faughnan M, Hoey L, Wähälä K, Williamson G, Cassidy A. Bioavailability of phyto-estrogens. *Br J Nutr*. 2003;89:545–58.
- Scalbert A, Morand C, Manach C, Rémésy C. Absorption and metabolism of polyphenols in the gut and impact on health. *Biomed Pharmacother*. 2002;56:276–82.
- Day AJ, DuPont MS, Ridley S, Rhodes M, Rhodes MJ, Morgan MR, Williamson G. Deglycosylation of flavonoid and isoflavonoid glycosides by human small intestine and liver beta-glucosidase activity. *FEBS Lett*. 1998;436:71–5.
- Xu X, Harris KS, Wang HJ, Murphy PA, Hendrich S. Bioavailability of soybean isoflavones depends upon gut microflora in women. *J Nutr*. 1995;125:2307–15.
- Lampe JW, Karr SC, Hutchins AM, Slavin JL. Urinary equol excretion with a soy challenge: influence of habitual diet. *Proc Soc Exp Biol Med*. 1998;217:335–9.
- Rowland IR, Wiseman H, Sanders TAB, Adlercreutz H, Bowey EA. Interindividual variation in metabolism of soy isoflavones and lignans: influence of habitual diet on equol production by the gut microflora. *Nutr Cancer*. 2000;36:27–32.
- Setchell KDR, Brown NM, Lydeking-Olsen E. The clinical importance of the metabolite equol: a clue to the effectiveness of soy and its isoflavones. *J Nutr*. 2002;132:3577–84.
- Atkinson C, Frankenfeld CL, Lampe JW. Gut bacterial metabolism of the soy isoflavone daidzein: exploring the relevance to human health. *Exp Biol Med*. 2005;230:155–70.
- Adlercreutz H, Fotsis T, Bannwart C, Wähälä K, Brunow G, Hase T. Isotope dilution gas chromatographic-mass spectrometric method for the determination of lignans and isoflavonoids in human urine, including identification of genistein. *Clin Chim Acta*. 1991;119:263–78.
- Frankenfeld CL, Atkinson C, Thomas WK, Goode EL, Gonzalez A, Jokela T, Wähälä K, Schwartz SM, Li SS, et al. Familial correlations, segregation analysis, and nongenetic correlates of soy isoflavone-metabolizing phenotypes. *Exp Biol Med*. 2004;229:902–13.
- Bolca S, Possemiers S, Maervoet V, Huybrechts I, Heyerick A, Vervarcke S, Depypere HT, De Keukeleire D, Bracke ME, et al. Microbial and dietary factors associated with the 8-prenylnaringenin producer phenotype: a dietary intervention trial with fifty healthy postmenopausal Caucasian women. *Br J Nutr*. In press 2007 [doi: 10.1017/S0007114507749243].
- Soares AC, Lederman HM, Fagundes-Neto U, de Moraes MB. Breath methane associated with slow colonic transit time in children with chronic constipation. *J Clin Gastroenterol*. 2005;39:512–5.
- Possemiers S, Bolca S, Eeckhaut E, Depypere H, Verstraete W. Metabolism of isoflavones, lignans and prenylflavonoids by intestinal bacteria: producer phenotyping and relation with intestinal community. *FEMS Microbiol Ecol*. 2007;61:372–83.
- Maubach J, Bracke ME, Heyerick A, Depypere HT, Serreyn RF, Mareel MM, De Keukeleire D. Quantitation of soy-derived phytoestrogens in human breast tissue and biological fluids by high-performance liquid chromatography. *J Chromatogr B Analyt Technol Biomed Life Sci*. 2003;784:137–44.
- Van de Wiele T, Boon N, Possemiers S, Jacobs H, Verstraete W. Prebiotic effects of chicory inulin in the simulator of the human intestinal microbial ecosystem. *FEMS Microbiol Ecol*. 2004;51:143–53.
- Altman DG. *Practical statistics for medical research*. 1st ed. London: Chapman & Hall; 1991.
- Faughnan MS, Hawdon A, Ah-Singh E, Brown J, Millward J, Cassidy A. Urinary isoflavone kinetics: the effect of age, gender, food matrix and chemical composition. *Br J Nutr*. 2004;91:567–74.
- Richelle M, Pridmore-Merten S, Bodenstab S, Enslin M, Offord EA. Hydrolysis of isoflavone glycosides to aglycones by β -glucosidase does not alter plasma and urine isoflavone pharmacokinetics in postmenopausal women. *J Nutr*. 2002;132:2587–92.
- Tsangalis D, Wilcox G, Shah NP, Stojanovska L. Bioavailability of isoflavone phytoestrogens in postmenopausal women consuming soya milk fermented with probiotic bifidobacteria. *Br J Nutr*. 2005;93:867–77.
- Setchell KDR, Cole SJ. Method of defining equol-producer status and its frequency among vegetarians. *J Nutr*. 2006;136:2188–93.
- Decroos K, Vanhemmens S, Cattoir S, Boon N, Verstraete W. Isolation and characterization of an equol producing mixed microbial culture from a human faecal sample and its activity under gastrointestinal conditions. *Arch Microbiol*. 2005;183:45–55.
- Sioen IA, Pynaert I, Matthys C, De Backer G, Van Camp J, De Henauw S. Dietary intakes and food sources of fatty acids for Belgian women, focused on n-6 and n-3 polyunsaturated fatty acids. *Lipids*. 2006;41:415–22.
- Akaza H, Miyayama N, Takashima N, Naito S, Hirao Y, Tsukamoto T, Fujioka T, Mori M, Kim WJ, et al. Comparisons of percent equol producers between prostate cancer patients and controls: case-controlled studies of isoflavones in Japanese, Korean and American residents. *Jpn J Clin Oncol*. 2004;34:86–9.
- Bonorden MJL, Greany KA, Wangen KE, Phipps WR, Feirtag J, Adlercreutz H, Kurzer MS. Consumption of *Lactobacillus acidophilus* and *Bifidobacterium longum* do not alter urinary equol excretion and plasma reproductive hormones in premenopausal women. *Eur J Clin Nutr*. 2004;58:1635–42.
- Lampe JW, Skor HE, Wähälä K, Howald WN, Chen C. Wheat bran and soy protein feeding do not alter urinary excretion of the isoflavane equol in premenopausal women. *J Nutr*. 2001;131:740–4.
- Nettleton JA, Greany KA, Thomas WK, Wangen KE, Adlercreutz H, Kurzer MS. Plasma phytoestrogens are not altered by probiotic consumption in postmenopausal women with and without a history of breast cancer. *J Nutr*. 2004;134:1998–2003.