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RESEARCH ARTICLES

The role of diet in the metabolism of daidzein by human faecal microbiota sampled from Italian volunteers

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Abstract

The intestinal microbial transformation of daidzein into equol is subject to a wide inter-individual variability. The aim of this study was to investigate *in vitro* this transformation and to evaluate possible correlations between individual diet and equol production. The transformation of daidzein was investigated in anaerobic batch cultures inoculated with mixed fecal bacteria from 90 volunteers. The daidzein metabolism was monitored by liquid chromatography-mass spectrometry, and a chiral column was used to distinguish equol and dihydrodaidzein enantiomers. The obtained results show that daidzein was unchanged (≈27%) or degraded to equol (≈28%), *O*-desmethylangolensin (≈12%) or dihydrodaidzein (≈31%). Furthermore, some subjects (≈2%) are able to produce both equol and *O*-desmethylangolensin. Bacteria represent sub-dominant populations (10^5–10^9 cell/g wet faeces) in "slow" equol producers, while higher counts of equol-producing microorganisms (10^{10}–10^{11} cell/g wet faeces) were found in "quick" equol producers. The *in vitro* test to evaluate equol-producing status is quick and not invasive, and the obtained results are comparable with those reported *in vivo*. Indeed, the only enantiomer present in the batch cultures containing equol was the S-form. No significant correlations between equol production, BMI, age and sex were found. It seems that the equol-producer group consumed less fibre, vegetables and cereals, and more lipids from animal sources.

Keywords: Daidzein; Diet; Equol; Human; *In vitro* metabolism; LC-MS

1. Introduction

Phytoestrogens, particularly the isoflavones derived from soy, such as daidzein (DAI) and genistein, exhibit estrogenic properties in some human tissues [1] and exhibit anti-estrogenic properties in other tissues by competitively inhibiting estrogen binding at estrogen receptor sites [2]. Unlike estrogens, isoflavones seem not to be associated with an increased risk of breast and uterine cancers, and may actually inhibit the development of breast, colon and prostate cancers [3]. Recent studies have determined that soy isoflavones play a role in lowering blood concentrations of total cholesterol and LDL-cholesterol in human [4], limiting the development of atherosclerosis [5] and inhibiting the transcriptional nuclear factor NF-*k*B, the COX2 and aromatase [6]. Moreover, these isoflavones have bone-sparing effects [7].

Isoflavones are present mainly in soy but also in other legumes, fruit, whole grains and nuts, and in these foodstuffs typically occur as glycosides like genistin and daidzin (DAI-glucoside). After ingestion, daidzin and genistin are partially hydrolyzed in the small intestine [8], mostly in the jejunum [9] to the aglycones DAI and genistein, respectively. After DAI consumption, the intestinal bacteria produce dihydrodaidzein (DHD) that can be further metabolized to tetrahydrodaidzein (THD), equol (EQ) and/or *O*-desmethylangolensin (*O*-DMA) [10]. EQ is absorbed more efficiently than its precursor DAI, it is hydroxylated by liver microsomes [11] and it has higher estrogenic activity than DAI [12] and higher antioxidant activity than any other isoflavone [13]. Moreover, it has the ability to reduce the activity of the bone-resorbing cells and to increase bone mineral density in postmenopausal women [14]. EQ can also bind specifically circulating 5-α-

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dihydrotestosterone, but not testosterone, dehydro-epiandrosterone or estrogen. Therefore EQ has antiandrogenic properties and inhibitory effects on the proliferation of benign and malignant prostatic epithelial cells [15]. The human form of EQ produced in the intestine is exclusively the S(−) enantiomer and it was observed that approximately 50–70% of the adult population did not excrete EQ in their urine [16]. This phenomenon has led to the classification of subjects into "EQ producers" or "non-EQ producers", and, recently, a standardized approach to define EQ-producing status based on the $\log_{10}$ transformed urinary S-EQ/DAI ratio has been proposed [17]. Regarding DAI metabolism, it should be pointed out that the conversion of DAI to EQ is a two-step transformation, occurring through the intermediate DHD, carried out by different bacterial strains [18,19]. Moreover, the extent of this metabolism seems to be influenced by some components of the diet [20]. Indeed, it has been reported that EQ production is affected by low-fat and high-carbohydrate diets [21], high-fibre diets [22], long-term ingestion of soy diets [23,24], and meat [23] and vegetables intake [17]. In a recent study, a stable mixed of microbial culture isolated from human faeces was able to convert DAI into EQ [25]. The authors also reported that gaseous hydrogen boosts EQ production. Moreover, propionate and butyrate — known colonic fermentation products from poorly digestible carbohydrate — were also able to promote EQ production, suggesting that a diet rich in carbohydrate can stimulate EQ production. This hypothesis is supported by previous research [26] suggesting that short chain fructo-oligosaccharides (FOS) increase the isoflavones' bioavailability [26,27]. On the other hand, other studies found no effects on EQ production of short-term ingestion of soymilk [28], fermented soymilk [29] or wheat bran [22]. Moreover, the addition of FOS to a medium containing EQ-producing mixed culture inhibits EQ production [25]. Thus, knowledge of the effects of dietary habits on EQ-producing status is scarce and available data often do not overlap. The aim of this work was to evaluate *in vitro* DAI metabolism and the EQ-producing status in Italian subjects, and to evaluate possible correlations between dietary habits and EQ production. Moreover, since the physiological effects of a diet containing DAI could be due not only to the EQ-producing status but also to the efficiency in EQ production, we attempted to evaluate in EQ-producing subjects the number of faecal bacteria able to transform DAI into EQ.

2 Materials and methods

2.1 Chemicals

Analytical standards for DAI, DHD, THD, (R,S)-EQ, S-EQ, dehydroequol (DHE) and *O*-DMA were purchased from Plantech (Reading, UK). Sakuranetin (SAK) was obtained from Extrasynthese (Genay, France). Methanol, acetonitrile and ammonium acetate were from Merck (Darmstadt, Germany). Water was obtained from a MilliQ apparatus (Millipore, Milford, MA, USA).

2.2 Human subjects and dietary intake evaluation

A total of 90 healthy subjects participated in this study. There were 74 females (age 49.6±9.6 years, BMI 24.0±4.3 kg/m^2) and 16 males (age 40.2±14.5 years, BMI 24.2±4.0 kg/m^2).

None of the subjects had any history of gastrointestinal disease or took antibiotics or laxatives during the 3 months before the study. Exclusion criteria for participants also included pregnancy, having food allergy or having a chronic disease. A validated self-administered semi-quantitative food-frequency questionnaire [30] was used to estimate their dietary habits. The study was carried out over a period of 12 months and throughout this time EQ-producing subjects provided two faecal samples. The first was to evaluate the metabolism of DAI and the second, after about 6 months, to determine the number of EQ-producing microorganisms. This study was approved by the local ethics committee, and volunteers were informed of the requirements and of the experimental protocols and gave written informed consent.

2.3 Study design

In the morning, stool specimens were collected in anaerobic bags (Generbag Anaer, bio-Merieux), cooled at +4°C, delivered to the laboratory within 2 h after collection and immediately introduced into an anaerobic cabinet (Forma Scientific, Marietta, OH, USA) under a $N_2/H_2/CO_2$ atmosphere (85:10:5, v/v/v).

2.3.1. Daidzein metabolism

A faecal sub-sample of 1.5 g was homogenized, suspended in 30 ml of reduced brain heart infusion modified medium (BHI) containing DAI (20 μg/ml) and incubated in anaerobic cabinet at 37°C for 72 h. At time 0 and after 3, 6, 24, 48 and 72 h of incubation, 0.5 ml of the faecal suspension was collected in duplicate and diluted to 1 ml with methanol containing the internal standard (10 μg/ml). The methanolic solutions were then centrifuged at 1000×*g* for 1 min and the supernatants diluted twofold in methanol. The resulting solutions were stored at −20°C before LC-MS analysis. The control suspensions (medium, medium plus microflora, medium plus DAI) were also incubated under the same operative conditions and treated as previously described.

2.3.2. Metabolism of DHD enantiomers

A faecal sample from an EQ-producing subject (1.5 g) was suspended in BHI containing S- or R-DHD (20 μg/ml) and incubated in the anaerobic cabinet. At Time 0, every 2 h for 8 h and after 24, 48 and 72 h, aliquots (5 ml) of the mixed fecal cultures were extracted with ethyl acetate (10 ml) and the organic phases dried under N_2. The residues were suspended in 5 ml of methanol and analyzed using a chiral column.

2.3.3. Quantitative evaluation of the EQ-production ability of microorganisms

The number of faecal microorganisms able to convert DAI to EQ was evaluated by the most probable number method. Briefly, the faecal suspensions from the EQ-producing subjects were serially diluted 10-fold in BHI containing DAI (20 μg/ml) and incubated at 37°C. Each dilution was performed in triplicate, and after 7 days in the anaerobic cabinet each dilution was analysed by LC-MS to evaluate the presence of EQ.

2.4 Method validation

The flavanone SAK was used as internal standard to correct the loss of analytes during sample preparation. Calibration curves were constructed for each standard at five concentration levels, and two independent determinations were performed at each concentration. Regression analysis was employed to determine the linearity of the calibration graphs. The LC-MS method was validated for linearity, lower limit of quantization (LLOQ, signal-to-noise ([S/N] ratio of 3) and detection (LLOD, S/N ratio of 6), accuracy, precision and repeatability. Recovery was evaluated by spiking three sterilized faecal material (1.5 g) in BHI with four amounts (2, 5, 10 and 20 μg/ml) of the standard solution mixture containing THD, DAI, DHD, DHE, EQ and *O*-DMA. The spiked faecal cultures were incubated in anaerobic conditions at 37°C for 72 h and then treated as described above. Precision (intra- and inter-day) of the assay was verified by analyzing spiked samples three times on five consecutive days. Repeatability was confirmed by evaluating standard deviations of the retention times and peak areas. The stability was evaluated by incubating in anaerobic conditions at 37°C DAI and its metabolites for 72 h in the medium and the medium containing sterilized faecal suspension. Moreover, EQ was incubated in medium containing faeces from EQ-producing subjects.

2.5 Analytical method

The chromatographic system consisted of an Alliance mod. 2695 (Waters, Milford, MA, USA) equipped with diode array detector mod. 2996 (Waters) and a triple quadrupole mass spectrometer mod. Quattromicro (Micromass, Beverly, MA, USA).

2.5.1. LC-ESI-MS Analysis of DAI and its metabolites

A 3.5-μm C_{18} X-Bridge column (150×2.1 mm, Waters) was used for the separation at a flow rate of 250 μl/min. The eluents were 2 mM CH_3COONH_4 (pH 5.2) (A) and ACN (B), and the linear gradient was as follows: 25% B for 8 min and then from 25% to 50% B in 10 min. The column was maintained at 30°C and 5 μl injected in the LC-MS system. The capillary voltage was set to 3.0 kV, the cone voltage was 15 V, the source temperature was 130 °C and the desolvating temperature was 350°C. The mass spectrometer operated in ESI-negative mode monitoring the ions with $(m/z)^-$ 253 Da (DAI), $(m/z)^-$ 255 Da (DHD), $(m/z)^-$ 257 Da (THD, *O*-DMA), $(m/z)^-$ 241 Da (EQ), $(m/z)^-$ 239 Da (DHE) and $(m/z)^-$ 285 Da (SAK). All data were acquired by Masslink 4.0 software (Micromass). The DAI and its metabolite concentrations were carried out in duplicate. Calibration curves were obtained from DAI, DHD, THD, DHE, EQ, *O*-DMA and SAK stock solutions prepared by dissolving 2 mg of standard powder in 10 ml of methanol. They were measured in the range of 1–10 μg/ml.

2.5.2. Purification of the DHD enantiomers

Two hundred microliters of the racemic DHD mother solution (0.2 mg/ml) was injected into a semi-preparative 250×8 mm Sumichiral OA-7000 column (Sumika Chemical Ltd., Osaka, Japan), and the separation was performed in isocratic mode with water and ACN (60:40 v/v) as eluent at a flow rate of 2 ml/min. The peak solutions of R- and S-DHD were collected individually by a WFC II fraction collector (Waters), evaporated to dryness under N_2 and the residues suspended in the medium to obtain a solution of about 20 μg/ml.

2.5.3. LC-Chiral phase–DAD-MS identification of EQ and DHD enantiomers

A 5-μm Sumichiral OA-7000 250×4.6-mm column was used in isocratic mode with water and ACN (50:50 v/v) as eluent. The flow rate was 2 ml/min and it was split 10:1 before the ESI source. The column was maintained at 35°C and 100 μl was injected in the LC-DAD-MS system. Chromatographic data were acquired in the 200- to 450-nm range and were integrated at 282 nm (EQ) and 275 nm (DHD). The mass spectrometer operated in ESI-negative mode monitoring the ions with $(m/z)^-$ 255 Da (DHD) and $(m/z)^-$ 241 Da (EQ).

2.6. Statistical analyses

Statistical analyses were performed with Excel 2003 for Windows XP, and data were calculated as percentage metabolite produced relative to the amount of incubated precursor.

The possible relationship between food habits and metabolism of DAI was performed using Statistica software (Statsoft, Tulsa, OK, USA). The subjects were divided in five groups depending on their ability to metabolize DAI. Group 1: unable to metabolize DAI; Group 2: main metabolite was DHD; Group 3: main metabolite was EQ; Group 4: main metabolite was *O*-DMA; and Group 5: EQ and *O*-DMA were the main products. The analysis of variance (ANOVA) with food groups or nutrients intake as dependent factors was used. Differences were considered significant for $P<.05$. Nutrient intake values for energy, proteins, lipids, carbohydrates, fibre and food groups were evaluated using WinFood software (Medimatica, Colonnella, TE, I) with the food composition database for epidemiological studies in Italy of the European Institute of Oncology.

3. Results

3.1. Method validation

The accuracy of the extraction for DAI, THD, DHD, DHE, EQ and *O*-DMA from spiked sterilized faecal samples was 96–103% and 97–105% for intra-day and inter-day assay, respectively. The LLOQ was 1 μg/ml for THD, DHD, DHE and EQ, and 0.4 μg/ml for DAI, *O*-DMA and SAK. The LLOD was 0.45 μg/ml for THD, DAI, DHE and EQ, and 0.25 μg/ml for *O*-DMA and DHD. Regarding repeatability, a maximum relative standard deviation of 6% (for DHD and THD) was observed for triplicate injections. The data obtained from DAI stability studies show that no metabolites or changes in the amount of DAI take place. Moreover, the controls did not produce compounds overlapping with DAI, THD, DHD, DHE, EQ or *O*-DMA. On the other hand, in 40 control samples containing the medium plus faecal suspension, a compound (RT 4.6 min) was found with a molecular weight corresponding to THD (RT 3.6 min) or *O*-DMA (RT 15.8 min). Regarding EQ, no changes in its amount were detected after 72 h of incubation with bacteria from faeces of EQ-producing volunteers.

3.2. In vitro DAI metabolism

Fig. 1 shows a typical LC-MS chromatogram obtained by incubating DAI with the faecal culture from an EQ-producing volunteer for 12 h. The identity of DAI, DHD, EQ and *O*-DMA was established by co-chromatography and through their molecular weight. The intermediate compound THD (RT 3.8) and the final product DHE (RT 11) were not found in any faecal culture incubated with DAI. Taking into account these results, Fig. 2 reports the pathway of DAI metabolites produced by human faecal microflora. As shown in Table 1, about 28% of the total faecal batch cultures produced EQ, 27% did not metabolize DAI and 31% caused only the first reductive step of DAI to DHD. Only 2% transformed DAI to both EQ and *O*-DMA. Regarding *O*-DMA and EQ producers, DHD was present as middle passage in more than 55% or 73% of the faecal cultures, respectively. Considering gender differences, the S-EQ producers were 37% and 25% in men and women, respectively. Moreover, about 15% of the tested women were able to produce *O*-DMA as the main metabolite. In contrast, *O*-DMA was never observed as the main metabolite in faecal cultures from males. Fig. 3 shows the time course of *in vitro* metabolism of DAI to EQ by faecal bacteria from EQ-producing volunteers. Inter-individual variability was greater during the first 24 h of incubation and was lowered until the end of the incubation period. The comparison of the different time-course experiments allowed the identification of a sub-group (n=9) of EQ producers that rapidly converted about 50% DAI to EQ within 6 h of fermentation. Little or no increase in EQ concentration occurred up to 48 h of incubation. The other EQ producers (n=16) metabolized DAI more slowly and 50% of the substrate was converted to EQ within 30 h of fermentation.

3.3. Identification of EQ and DHD enantiomers

The results obtained using a chiral column show that S-EQ is the only enantiomer present in the faecal cultures from EQ-producing subjects. Its identity was established by co-chromatography, UV spectra and molecular weight comparison. Moreover, in all the faecal suspensions containing DHD, S-EQ was present in racemic form. The incubation of

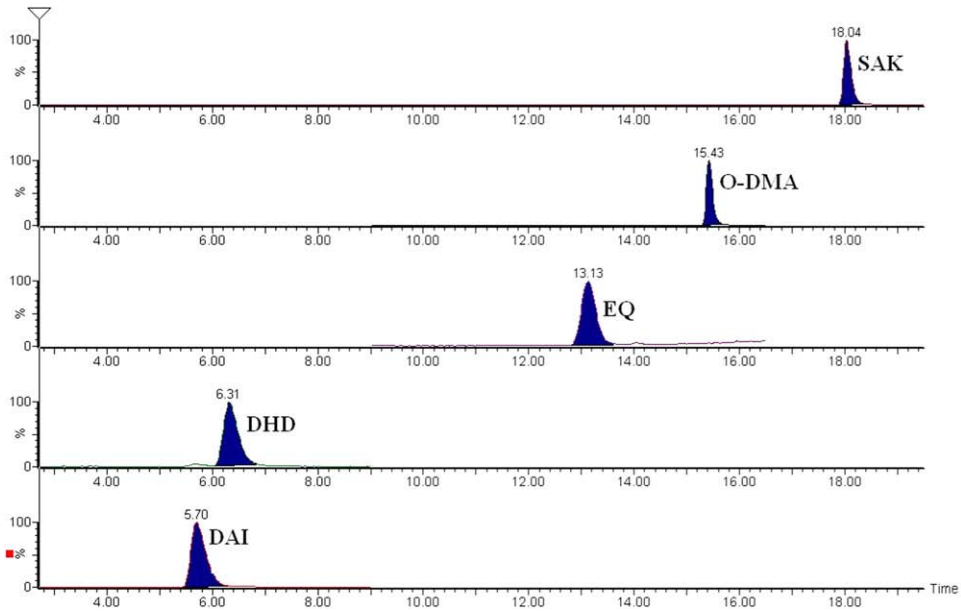

Fig. 1. Typical LC-MS chromatogram (SIR mode) of a faecal suspension from an EQ-producing volunteer incubated for 12 h with DAI. Ions with $(m/z)^-$ 253 Da (DAI), $(m/z)^-$ 255 Da (DHD), $(m/z)^-$ 241 Da (EQ), 257 $(m/z)^-$ Da (*O*-DMA) and 285 $(m/z)^-$ Da (SAK).

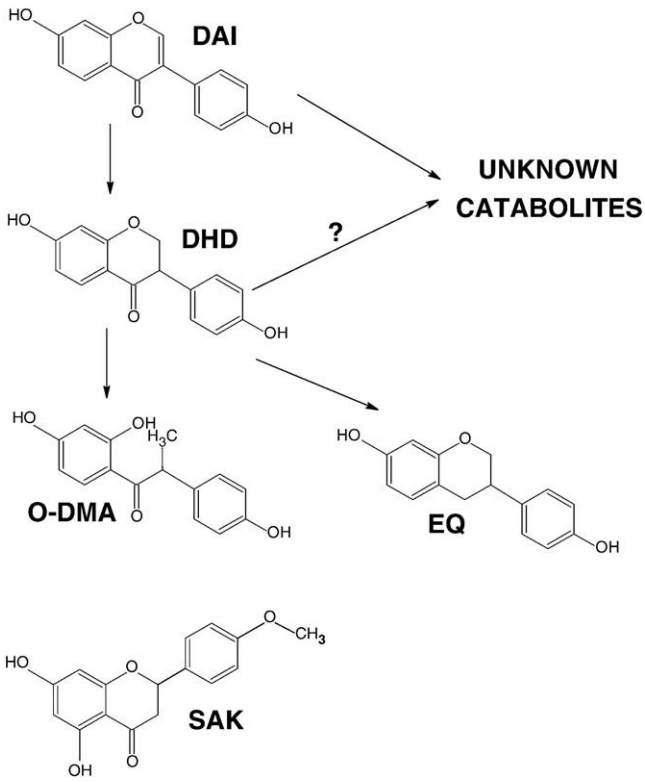

Fig. 2. Proposed pathway of daidzein metabolites produced by human fecal microflora. SAK, the flavanone used as internal standard.

R- or S-DHD with faecal bacteria from an EQ producer produced S-EQ. DHD enantiomers were stable in the medium but the addition of faecal bacteria from an EQ producer produced (R,S)-DHD. Hence it was not possible to establish which DHD enantiomer produced S-EQ.

Table 1
Percentages of DAI and its metabolites in anaerobic batch cultures inoculated with mixed fecal bacteria from 90 volunteers (women=74, men=16)

Group	*n*	%	% DAI[a]	% DHD[a]	% EQ[a]	% *O*-DMA[a]	% Analytes[b]
Women							
1	21	28	81±13	9±10	0±0	0±0	90±8
2	21	28	6±7	73±11	0±0	0±0	79±11
3	19	25	0±0	0±0	48±12	0±0	48±12
4	11	15	0±0	10±13	0±0	60±21	70±17
5	2	3	0±0	0±0	54±31	30±25	84±6
Men							
1	3	19	48±7	2±4	0±0	8±7	58±4
2	7	44	2±6	60±24	0±0	1±2	61±23
3	6	37	0±0	0±0	39±7	3±4	42±8
4	0	0	0±0	0±0	0±0	0±0	0±0
5	0	0	0±0	0±0	0±0	0±0	0±0

Group 1: unable to metabolize DAI; Group 2: main metabolite was DHD; Group 3: EQ producers; Group 4: *O*-DMA producers; and Group 5: EQ and *O*-DMA were the main products.

[a] Percentage after 72 h of incubation calculated as: (analyte/initial DAI)×100.

[b] Σ %DAI+%DHD+%EQ+%ODMA.

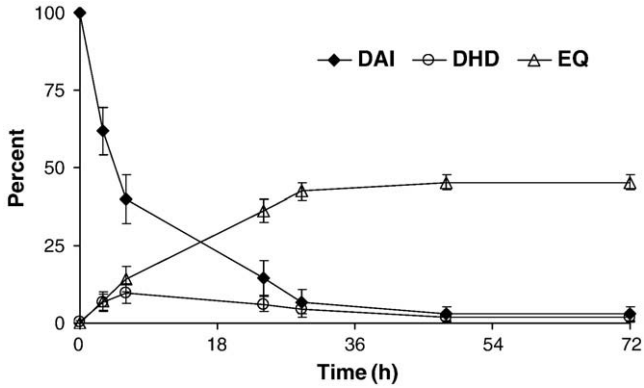

Fig. 3. Kinetics of DAI, EQ and DHD during fermentation experiments using faecal samples from EQ-producing volunteers. Data are presented as means±S.D.

3.4. Dietary habits and DAI metabolism

Table 2 shows the intake of energy, macronutrients (from animal and vegetable sources) and fibre corresponding to the five groups of DAI metabolites. In relation to the EQ-producing group, there were no correlations between sex, age and BMI, but it seems there was a positive association between the consumption of lipids from animal sources and EQ production. Moreover, the fibre intake of the EQ-producing group was lower in the group that did not metabolize DAI. In Table 3, the food consumption pattern corresponding to the five different groups is reported. Regarding EQ producers, it seems that the intake of cereals and pizza was lower in the group that did not metabolize DAI. Moreover, there was a negative correlation between sweets intake and EQ production.

3.5. Number of EQ-producing microorganisms

In 16 EQ-producing volunteers, the number of micro-organisms able to convert DAI in EQ was in the range 10^5–10^9 cells/g wet faeces and for other EQ producers (n=9) these populations were in the range 10^{10}–10^{11} cells/g wet faeces. These volunteers belonged to the sub-group with a high rate of conversion, whereas the other 16 subjects have demonstrated slower EQ production in time-course experiments.

4. Discussion

The present study is the first to investigate the influence of dietary habits on *in vitro* metabolism of DAI by intestinal microbiota of Italian adult men and women.

4.1. DAI Conversion to EQ

About 28% of the tested subjects are “EQ producers”, and the only enantiomer present in the batch cultures containing EQ was the S-form. Thus our *in vitro* results are in agreement with other studies on Western populations which report that only 20–35% of the adult population is

Table 2
Dietary factors in relation to each daidzein-metabolizing phenotype in 90 Italian subjects

Variable	Group 1 (n=24)	Group 2 (n=28)	Group 3 (n=25)	Group 4 (n=11)	Group 5 (n=2)
Age (years)	46.1±1.9	48.5±2.2	53.1±1.7	49.6±3.9	48.5±8.4
BMI (kg/m^2)	23.4±0.8	24.7±1.0	24.3±1.1	22.1±0.9	25.8±5.4
Energy (kcal/day)	1703.3±62.5	1784.6±95.4	1956.7±86.5^a	1743.6±114.2	1386.2±160.9^a
Total proteins (g/day)	70.7±2.2	71.2±3.0^a	79.1±2.2^b	68.2±4.8	46.4±6.6^{ab}
From animals	47.5±2.5	44.0±2.0	50.9±4.7^a	44.0±3.3	28.7±5.4^a
From vegetables	23.1±1.5	27.5±2.1	28.1±1.8	24.2±1.7	17.7±1.3
Total lipids (g/day)	68.4±3.4	63.1±2.8^a	74.9±4.4^{abc}	62.7±4.0^c	47.7±9.5^b
From animals	36.5±2.6	30.5±2.0^a	39.3±4.4^a	31.4±3.4	23.0±3.0
From vegetables	31.8±4.1	32.5±2.0	35.5±3.2	31.2±2.1	24.8±6.4
SFA	24.5±1.3	21.4±1.2^a	26.3±2.1^a	21.4±2.2	16.2±2.1
MUFA	35.5±1.8	32.6±1.3	32.2±1.8	33.9±3.0	43.3±20.4
PUFA	7.1±0.3	7.8±0.9	6.9±0.5	8.0±1.0	9.3±3.0
Cholesterols (mg/day)	193.8±9.8	243.8±19.6	215.1±25.2	219.1±33.9	139.6±10.1
Carbohydrates (g/day)	259.7±16.1	240.4±16.2	209.9±16.0	230.8±12.3	190.7±4.7
From starch	153.1±14.3	138.6±13.3	132.5±12.1	130.6±10.4	108.2±20.0
From sugars	106.4±7.3^a	101.5±8.4^b	76.7±5.2^{ab}	99.8±12.4	82.5±15.3
Fibre (g/day)	27.2±2.7^a	21.0±1.8	19.1±1.6^a	23.8±3.7	17.4±6.1

Values are expressed as mean±S.E.
Values with the same letters (superscript) within the same line are significantly different (P<.05).
SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids.

able to produce EQ from DAI [14,21,22]. Daidzein was rapidly metabolised within the first 6 h of fermentation and its concentration decreased more slowly during the subsequent incubation period, which disappeared completely after 48 h of incubation. Correspondingly, EQ increased rapidly within the first 30 h of incubation, whereas only diminished EQ production was observed during the subsequent hours of fermentation. At the end of the incubation period, about 45% of the initial DAI was converted to EQ, but a percentage of DAI (35–75%) disappeared without being accompanied by an increase in either EQ, DHD or *O*-DMA. This suggests the occurrence of DAI degradation pathways not yet identified. Once formed in the faecal cultures, EQ was stable over 72 h. Some EQ producers converted DAI to EQ within 6 h of fermentation, while most EQ producers reached the same levels of EQ accumulation over 24 h. These results could explain the *in vitro* observation of Possemiers et al. [31] and the conflicting data of clinical studies. The effectiveness of dietary DAI could be, in fact, related not only to EQ-producing status but also to the rate of EQ production from DAI. In this regard, we showed that the different rate of EQ production could be related to inter-individual differences in

Table 3
Food consumption patterns

Food groups	Recommendations[1]	Group 1 (n=24)	Group 2 (n=28)	Group 3 (n=25)	Group 4 (n=11)	Group 5 (n=2)
	g/day					
Milk and yoghurt	375	205.2±26.6	236.0±20.0	181.8±16.4	156.6±8.3	159.6±8.3
Cereal products		43.2±5.4^a	36.1±3.0	19.4±2.7^a	25.7±2.5	3.5±0.6
Pasta	80	50.0±3.7	61.7±5.4	49.9±2.8	64.6±3.3	43.5±3.5
Pizza		39.2±3.9^{ab}	24.4±1.9^a	20.0±2.1^b	23.9±2.1	23.5±2.0
Bread		92.3±8.4^a	58.1±3.2^{ab}	92.0±6.4^b	78.2±4.8	76.5±1.1
Meat	37	51.2±5.0	55.8±3.8	60.0±3.7	67.6±5.9	60.0±4.8
Preserved meat		21.3±2.4^a	36.8±3.5^a	24.9±2.0	29.0±1.9	39.0±2.6
Fish	43	30.9±2.6	24.2±2.4	31.7±3.5	28.4±1.9	20.5±0.6
Eggs	17	5.8±3.9	11.7±1.5	5.8±0.5	9.7±1.9	6.5±0.6
Cheese	32	51.1±5.7	70.0±7.4	50.5±4.0	65.7±5.4	47.0±4.8
Vegetables	500	420.9±35.8	354.7±23.1	303.7±18.5	386.2±26.1	349.0±32.5
Potatoes	57	36.8±3.9	31.9±2.2	34.6±2.7	33.6±2.9	29.0±0.5
Legumes	21	26.8±2.8	23.1±3.0	26.5±3.3	26.2±2.8	4.5±0.8
Fruits	450	261.9±19.6	248.5±15.4	204.7±18.6	344.4±37.6	230.0±28.9
Sweets		40.3±3.8	55.9±4.2^a	35.1±2.9[1]	50.2±3.6	44.5±0.1
Oils and fats		30.5±1.2	24.0±0.8^b	26.5±1.2^a	28.2±1.3	44.0±4.9^{ab}
Alcoholic drink		65.1±10.7	63.1±8.7	70.4±10.7	123.3±19.7	15.5±2.6

Data are expressed as mean±S.E.
[1] Nutritional recommendations (g/day) obtained by multiplying the amount of standard portion by the number of servings [35] recommended by the Italian Nutritional Guidelines [36].
Values with the same letters (superscript) within the same line are significantly different (P<.05).

the number of EQ-producing microorganisms. The status of EQ producer was maintained during this time in all the subjects. Moreover, two volunteers consuming antibiotics (amoxicillin) for 15 days after the first experimental step maintained the ability to produce EQ *in vitro* even when tested 3 months after the antibiotic therapy. Finally, we observed a higher percentage of EQ-producing males compared to females (37% vs. 25%). This result, within the limitations of the low number of males in respect to females, agrees with data reported by Setchell and Cole [17] showing that, among vegetarians, males appeared to be three times more likely to be an EQ producer than females.

4.2. Conversion of DHD enantiomers to EQ

The enantioselectivity of faecal bacteria from EQ producers towards R- or S-DHD could not be evaluated because DHD enantiomers gave racemic DHD in the presence of faecal bacteria. This evidence and the data reported by Wang et al. [32] lead to speculation that S-EQ production is probably due to the enantioselectivity of the EQ-producing bacteria. Briefly, R- or S-DHD is reduced to S-EQ and the untransformed enantiomer, perhaps by way of a spontaneous tautomerization, yields racemic DHD.

4.3. O-DMA Production

O-DMA was the main metabolite in about 15% of faecal samples from women, whereas in none of the faecal cultures from males was it found as the main metabolite. These results confirm an *in vitro* study showing the percentage of *O*-DMA producers ranges between 17% and 25% [25], but they are not in agreement with other studies indicating a higher percentage of *O*-DMA producers [20,31,33]. This discrepancy could be ascribed to different factors such as the potential limitations of *in vitro* models that could induce an imbalance of microbial populations involved in *O*-DMA production, the absence of control faecal suspension and the use of unspecific analytical methods (e.g., LC-UV). *O*-DMA was negatively correlated with the EQ presence in the batch cultures suggesting that probably these metabolites involve the same precursor and different bacterial strains are involved in their production. On the other hand, EQ and *O*-DMA were present in the culture batch of two subjects. In one subject, EQ (85%) was much higher than *O*-DMA (5%), while in the other *O*-DMA (55%) was higher than EQ (23%).

4.4. Diet

Evaluation of the dietary habits by a validated questionnaire showed that food intake was not in agreement with the Mediterranean diet. In fact, protein and fat consumptions were higher than the recommended daily intake and the fibre intake was generally low and higher values were found in the group that did not affect DAI. The energy intake was under 2000 kcal/day and not significantly different between the different groups. Rowland et al. [21] found that the good EQ producers consumed less fat compared with poor EQ producers and more carbohydrates. Similarly, other researchers [22] showed that women who were EQ producers consumed a significantly higher percentage of energy as carbohydrate compared with non-EQ producers, and they also consumed greater amounts of plant protein and dietary fibre. Therefore, it was suggested that, among women, a diet rich in fibre and carbohydrates could promote EQ production. Our results do not support these published results. In fact, our EQ-producing subjects have a diet poor in vegetable products and cereals, and, consequently, lower intake of fibre. Moreover, the intake of lipids from animal sources was higher in the EQ producers while the consumption of mono- and disaccharides was lower. In a recent paper, Setchell and Cole [17] reported that the frequency of EQ producers among vegetarians was 59%, similar to that of the largely soy-consuming Japanese population, and much higher than for nonvegetarian adults (25%), indicating that differences in the composition of the diet could play a key role in intestinal bacterial metabolism of DAI to S-EQ. In our study, between the 90 tested volunteers, five women were vegetarians for 20 years, but anyone was able to produce EQ from DAI. In particular, two were DHD producers and the others did not affect DAI. These data, with the limitations of the low number of subjects, do not confirm the higher frequency of EQ production in vegetarians. Recently, Atkinson et al. [34] have reported a relationship between DAI metabolism and different factors including dietary intake. They found that EQ-producing subjects consumed more vegetables and eggs, but this finding was not statistically significant after correction for the false discovery rate. Our results seem to support these data. Finally, we point out that the relationship between dietary habits and EQ production remains open and that probably future evaluations should be performed by analyzing a longer term dietary pattern.

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