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R(-)-O-desmethylangolensin is the main enantiomeric form of daidzein metabolite produced by human *in vitro* and *in vivo*

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Abbreviations: CD, circular dichroism**; DAI**, daidzein; **DHD**, dihydrodaidzein; **e.e.**, enantiomer excess; **IS**, Internal standard; **MRM**, multiple reactions monitoring; **O-DMA**, O-desmethylangolensin; **UPLC**, ultra-performance liquid chromatography; **TDDFT**, time-dependent density-functional theory

Abstract

After ingestion, human intestinal bacteria transform daidzein into dihydrodaidzein, which can be further metabolised to O-desmethylangolensin. This metabolite, unlike daidzein, has a chiral centre and can therefore occur as two distinct enantiomers; however, it is unclear which enantiomer is present in humans. The aim of this study was to define *in vitro* and *in vivo* the structure of O-desmethylangolensin and then to evaluate its pharmacokinetic parameters. Daidzein metabolism was preliminarily investigated in anaerobic batch cultures inoculated with mixed faecal bacteria from O-desmethylangolensin producer volunteers. The transformation was monitored by liquid chromatography-mass spectrometry and a chiral column was used to distinguish dihydrodaidzein and O-desmethylangolensin enantiomers. These were purified, analysed by circular dichroism and the results established R(-)-O-desmethylangolensin as the main product (enantiomer excess 91%). However, both dihydrodaidzein enantiomers were detected. Similar results were obtained by *in vivo* trials. The *in vitro* formation of O-desmethylangolensin seems to be directly correlated with the number of transforming microorganisms. This correlation was found *in vivo* for t_{max} but not for other pharmacokinetic indexes. The pharmacokinetics of daidzein, dihydrodaidzein and O-desmethylangolensin were then evaluated in 11 healthy adult O-desmethylangolensin producers after the single administration of soy milk containing 100 mg daidzein. The conjugated forms of daidzein, dihydrodaidzein and O-desmethylangolensin represent more than 90 and 95% of the plasmatic and urinary forms, respectively. The C_{max}, t_{max} and half-life of O-desmethylangolensin in plasma was 62±53 nM, 28±11 h and 15±6 h, respectively. Relevant inter-individual variations were observed as indicated by the high standard deviations.

Key words: daidzein metabolism; human; microflora; ODMA enantiomers; pharmacokinetics.

1. Introduction

Several studies seem to demonstrate a potentially prophylactic role for isoflavones in prostate [1] and breast cancer [2], cardiovascular disease (CVD) [3], osteoporosis [4], menopausal symptoms [5] and bone-sparing [6]. On the other hand, recent studies do not support the hypothesis that phytoestrogens prevent prostate or colorectal cancer [7] and since 2006 the American Heart Association has not recommended the use of isoflavone supplements to prevent CVD [8]. Thus, the protective effect of isoflavones against some types of cancer and CVD should be verified. Soy isoflavones can reduce the development of atherosclerosis [9] and it seems that they prevent obesity via the inhibition of adipose tissue enlargement [10]. Isoflavones are present mainly in soy as glycosides and after ingestion they are hydrolysed in the small intestine [11], particularly in the jejunum [12], to their respective aglycones, mainly daidzein (DAI) and genistein. Daidzein is converted by anaerobic bacteria in the large intestine to dihydrodaidzein (DHD), O-desmethylangolensin (ODMA), and equol [11]; each of these metabolites has a chiral centre at C-3 due to the reduction of the Δ^{2-3} double bond. Equol is found as its S(-)-equol enantiomer, but the chirality of the other daidzein metabolite, ODMA, is not known. In recent years, some bacterial strains able to produce S-equol have been isolated [13-15]. Regarding ODMA production, fewer single bacterial strains able to cleave the C-ring of daidzein were isolated, for example *Clostridium* HGH 136 [16], *Eubacterium ramulus* [17], *Eubacterium ramulus* Julong 601 [18] and a strictly anaerobic bacteria belonging to the genus *Clostridium* [19]. Recently, a new bacteria belonging to *Lachnospiraceae* was isolated, which appears to metabolise ODMA to resorcinol [20]. Once formed, ODMA is absorbed and conjugated to ODMA-glucuronides or sulphate, which can then be excreted predominantly in the urine [21]. Recently, we have shown in an Italian

population that ODMA was the main daidzein metabolite in about 15% of women, while all males were able to produce ODMA [22]. These results confirm previous studies showing the percentage of ODMA-producers to range between 17 and 25% [23], but they are not in agreement with other studies indicating a higher percentage of ODMA-producers [24]. To equalise the results of *in vitro* and *in vivo* studies, a standardised method should be introduced to define ODMA-producers, similar to that proposed to define equol-producer status [25]. *In vitro,* ODMA possesses several biological activities including anti-carcinogenic and antioxidant activities [26]; it also binds the human oestrogen receptors α and β with a greater affinity than its precursor daidzein, but lower than that of equol [27]. However, ODMA was found to be a more potent inducer of micronucleus formation than equol in mouse lymphoma cells [28]. A comparative study reported the effects of ODMA and equol on bone and lipid metabolism *in vitro* and mice, and the results seemed to suggest that the effects of ODMA were weaker than those of equol [29]. Thus, limited evidence suggests that differences in biological activity may exist between equol and ODMA. In a previous work [22], we evaluated *in vitro* DAI metabolism and the equol-producing status in a group of Italian volunteers and from these selected ODMA-producing subjects to answer topical key questions related to ODMA in humans. The questions were: 1) are intestinal bacteria stereoselective in the synthesis of ODMA; and 2) are the single enantiomers or racemic form of ODMA present in the urine and blood of ODMA-producing subjects after they have ingested a single dose of soy milk containing daidzein? Thus, as a first step we evaluated *in vitro* daidzein conversion to ODMA by UPLC-MS/MS and determined which enantiomers were present in the faecal cultures by LC-chiral phase-MS/MS. After this, their absolute conformation was established by circular dichroism (CD). Finally, we evaluated in the urine and blood of ODMA-producing subjects which enantiomers were present after the ingestion of daidzein and the pharmacokinetics of this process.

2. Materials and methods

2.1 Chemicals

Daidzein, DHD and ODMA were provided by Plantech (Reading, UK). Galangin (IS) was obtained from Extrasynthese (Genay, France) and R-angolensin was from GAIA chemicals (New Milford, CT, US). Brain heart infusion (BHI) was obtained from Difco, while soy milk was purchased from a local market. β-glucuronidase/sulphatase from *Helix pomatia* was obtained from Sigma Aldrich (St. Louis, MO, USA). Methanol, acetonitrile and acetic acid were from Merck (Darmstadt, Germany). Water was obtained from a MilliQ apparatus (Millipore, Milford, MA).

2.3 *In vitro* ODMA production

The volunteers were not taking any supplements, drugs, medication or laxatives. In the morning, stool specimens were collected from 11 ODMA-producing subjects (aged=46.2±8.3 years, BMI=23.1±3.1 Kg/m^2) and treated as previously described [22]. Briefly, a faecal sample of 1.5 g was homogenised, suspended in 30 ml of reduced BHI containing DAI (20 µg/mL) and incubated in an anaerobic cabinet at 37°C for 96 h. At different times of incubation, 0.5 mL of the faecal culture was collected twice and diluted to 1 mL with a methanol-containing internal standard (Galangin, 10 µg/mL). The methanolic mixtures were then centrifuged at 1000 x g for 1 min and the resulting solutions were analysed by UPLC-MS/MS and LC-Chiral phase. The control suspensions were medium, medium plus microflora and medium plus DAI. Furthermore, the quantitative evaluation of ODMA-producing microorganisms was performed according to the most probable number (MPN) method [22].

2.4 Subjects and *in vivo* study design

The healthy ODMA-producing subjects (n=11) employed for the *in vivo* study were recruited to evaluate the presence of ODMA in plasma and urine and to determine which enantiomers were present. The study was approved by the local ethical committee and informal, written consent was obtained from each participant. Volunteers refrained from consuming food and beverages containing isoflavones for 7 days prior to the study and 4 weeks after DAI intake. On the day of the experiment, the fasted subjects were confined to the laboratory and between 6:00 and 6:30 am they received 100 mL of soy milk containing 100 mg of DAI and 4 mg of daidzin. After dinner, the volunteers were allowed to leave the laboratory, returning the next four mornings for blood and urine sampling. Blood samples were collected in tubes containing lithium-heparin at 3, 5, 7, 9, 11, 27, 39, 51, 63, 75, 87 and 99h after the DAI intake. Time zero samples were obtained about 30 min before the milk ingestion. Urine was collected into plastic bottles in five fractions (0–24, 24–48, 48–72 and 72–96 h) and stored at 4°C. The urine volume of each fraction was measured and 10 ml was filtered through a 0.2 μm filter, portioned in glass vials (1 mL) and frozen at -70°C.

2.4.1 Plasma and urine preparation

Heparinised plasma (800 μL) or urine (400 μL) was incubated with 100 μL glucuronidase/sulphatase (1 U/μL) and 100 μL I.S. (50 ng/mL) in 0.1 mol/L CH_3COONa buffer (pH 5.2) at 37°C for 18 h. The reaction mixture was extracted with ethylacetate (2x0.8 mL), vortexed and centrifuged at 1000 x g for 1 min. The supernatants were mixed, dried under N_2 and the residue was dissolved in 100 μl methanol. To evaluate non-conjugated analyte, a plasma sample or urine was incubated with the I.S. and 100 μL of 0.1 mol/L CH_3COONa buffer (pH 5.2) at 37°C for 18 h. The reaction mixture was then treated as described above. All samples were stored at -70°C before analysis.

2.5 Analytical methods

2.5.1 Purification of the ODMA enantiomers

Two hundred microlitres of the racemic ODMA mother solution (0.2 mg/mL) was injected into a semi-preparative 5μm Sumichiral OA-7000 column (250x8 mm, Sumika Chemical Ltd., Osaka, Japan) and the separation was performed in isocratic mode with water and acetonitrile (60:40 v/v) as eluent at a flow-rate of 4 mL/min. The peak solutions of R- and S-ODMA were collected individually by a WFC II fraction collector (Waters, Milford, MA), evaporated to dryness under N_2 and the residues were suspended in methanol. The ODMA enantiomers contained in faecal suspensions (200 mL) were extracted with ethylacetate (2x500mL), the organic phase dried under N_2 and the residue dissolved in 2 ml methanol. The extract was then centrifuged at 1000 x g for 2 min and the enantiomers purified as described above.

2.5.2 Determination of DAI and its metabolites *in vitro* and in plasma and urine

The chromatographic system consisted of an UPLC mod. Acquity (Waters) equipped with triple quadrupole mass spectrometer mod. Quattromicro (Waters). A 1.7 μm BEH C_{18} column (150x2.1 mm, Waters) was used for separation at a flow-rate of 0.5 mL/min and the temperature was maintained at 50°C. The eluents were 0.05% CH_3COOH (A) and CH_3CN (B) and the gradient was as follows: 23% B for 8 min, 23-50% B in 10 min and then 50-80% B in 3 min. The capillary voltage was set to 3.0 kV, the cone voltage was specific for each analyte, the source temperature was 130°C, the desolvating temperature was 350°C and argon was used at $2.5x10^{-3}$ mbar to improve fragmentation in the collision cell. Daidzein and its metabolites were determined in multiple reactions monitoring (MRM) mode and data were acquired by Masslinx 4.0. Capillary, extractor and RF lens voltages were optimised by direct infusion experiments, while precursor and product ions, cone voltage and collision energy

were evaluated by Quan-optimise option. The mass spectrometer was operated in the ESI negative mode and the fragmentation transitions were $(m/z)^-$ 253 → 91 for DAI, 255 → 149 for DHD, 257 → 108, 136 for ODMA and 269 → 77 for IS, with a dwell time of 0.15 sec. To keep the cone clear, the first 2.30 min analysis was discharged by the divert valve.

Calibration curves were obtained from DAI, DHD, ODMA and IS stock solutions prepared by dissolving 2 mg of standard powder in 10 mL methanol. Their quantitative determination in blood and urine was performed in the range 2-400 ng/mL (10 μl injected), while for the *in vitro* test the range was 0.5-10 μg/mL (1 μl injected).

2.5.3 Chirality studies of biosynthesised ODMA by circular dichroism (CD)

The absolute configuration was investigated using the Time-Dependent Density-Functional Theory (TDDFT) calculation [30-31]. CD spectra of R(-)-angolensin, ODMA and DHD enantiomers were obtained in methanol on a Jasco J-810 Spectropolarimeter (Tokyo, Japan) with a scan-speed and band-width of 20 nm/min and 1 nm, respectively. A 1 cm path length cuvette was used for spectra recorded from 200 to 400 nm. The relatively stable conformers (all minima < 2 kcal/mol) were selected after comparisons of the optimised structure energy. To achieve more exact energy values of the stable conformers, the structures were further refined by means of B3LYP/6-311 g++(d,p) density functional theory calculation by the Gaussian 03 program [32]. The specific optical rotatory power $[\alpha]_{589}^{20}$ was determined by a Jasco P-1010 polarimeter equipped with a 1 ml cell maintained at 20°C.

2.5.4 Determination of ODMA and DHD enantiomers in biological samples

The chromatographic system consisted of an UPLC mod. Acquity (Waters) coupled to a triple quadrupole MS mod. Quattromicro (Waters). A 3 μm LUX-cellulose 3 column 150x2.0 mm (Phenomenex, Torrence, CA) was used and the separation was performed in gradient

mode (eluent A, 0.05% CH_3COOH; eluent B, acetonitrile) at a flow-rate of 0.4 mL/min. The gradient was as follows: 32% B for 5 min, 70% B in 10 sec and then 70% B for 3 min. The column and sample were maintained at 30 and 20°C, respectively. The injection volume was 20 µl. The mass spectrometer was operated in MRM mode using the parameters reported above.

2.6 Method validation for the assessment in plasma and urine

External standards were used to quantify DAI, DHD and ODMA in biological samples and galangin was used as an internal standard to correct for the loss of analytes during sample preparation. Calibration curves were constructed for each standard at six concentration levels; three independent determinations were performed at each concentration and regression analysis was employed to determine the linearity of the calibration graphs. LLOQ was defined by the lowest injected inter-day concentration whose RSD% resulted to be <20% [33]. LOD was defined by the lowest concentration that the assay can differentiate from background levels (S/N ratio > 5). The evaluation of the accuracy, precision, recovery, and absence or presence of matrix effect was performed according to Matuszewski et al. [34].

Intra- and inter-day precision of the assay were verified by analysing spiked samples 3 times for 5 consecutive days. Peak purity and identity were confirmed by MS/MS. Precision was confirmed by evaluating standard deviations of the amounts and of retention time.

2.7 Statistical analysis

Statistical analyses were performed with the STATISTICA software (Statsoft Inc., Tulsa, OK, USA). A one-way repeated measure analysis of variance (ANOVA) with the time as the dependent factor was used. The peak plasma concentration (C_{max}) and the time taken to reach peak concentrations (t_{max}) are expressed as mean ± SD. The elimination rate constant (K), the

terminal half-life ($t_{1/2}$), and the area under the curve (AUC) were calculated as previously reported [35].

3. Results

3.1 ODMA production *in vitro*

The accuracy of the extraction for DAI, DHD, ODMA and IS from spiked sterilised faecal samples was 95-102% and 94-103% for intra-day and inter-day assays, respectively. The controls did not produce compounds overlapping with DAI, DHD, ODMA or IS. Regarding ODMA, no changes were detected in its amount after 96 h incubation with bacteria from faeces of ODMA-producing volunteers. The comparison of the different time-course experiments allowed to identify a sub-group (n=4) of ODMA-producers able to reaching the highest ODMA amount in about 12 h and 50 % of DAI was converted to ODMA in about 5 h (Fig. 1A). Poor or no increase in ODMA concentration occurred until 96 h of incubation. In these volunteers, the number of microorganisms able to convert DAI in ODMA was in the range 10^8-10^{10} cells/g wet faeces. The other ODMA-producers (n=7) reached the highest concentration of ODMA between 12 and 22 h (Fig. 1B) and 50% of DAI was converted to ODMA after about 8-9 h of incubation. These volunteers, belonging to the sub-group with a slower rate of conversion, had a number of microorganisms in the range 10^5-10^7 cells/g wet faeces.

3.2 Absolute configuration of ODMA enantiomers

Through the TDDFT calculations, combined with CD spectroscopy of pure ODMA enantiomers and R(-)-angolensin, the absolute configuration of the main ODMA enantiomer obtained from DAI metabolism was determined to be R(-). The CD spectra of the enantiomers of ODMA in methanol exhibited positive and negative cotton effects in the 250-

370 nm regions. In particular, the *R*-form displayed negative cotton at 325 nm and a positive cotton effect at 275 nm. In Fig. 2, the experimental CD spectra of ODMA enantiomers and R(-)-angolensin are shown together with the molecular structure of the R-ODMA and R-angolensin. The $[\alpha]_{589}^{20}$ value of R(-)-ODMA was -135.8°.

3.3 Determination of DAI, DHD and ODMA in plasma and urine

The recovery of the extraction for DAI, DHD, ODMA and I.S. from spiked plasma samples was 94±3, 87±4, 88±3 and 91±4%, respectively. The precision of the method was tested by both intra-day (n=6) and inter-day (5 days, n=6) reproducibility, and the coefficient of variation was below 9%. The DAI, DHD and ODMA concentrations were calculated by external standardisation from calibration curves and their levels in plasma and urine were evaluated before and after the hydrolysis of samples by β-glucuronidase/sulphatase. The conjugated forms of these analytes represented more than 90% and 95% of the plasmatic and urinary forms, respectively. None of the subjects had detectable amounts of DAI or its metabolites at baseline. Mean plasma and urine analyte levels following the intake of 100 mL of milk containing 100 mg of DAI are reported in tables 1 and 2, respectively. The compounds were absorbed by all subjects but there were great inter-individual differences in the $AUC_{(0–75h)}$ and highest plasma level (C_{max}) values. The C_{max} of ODMA was reached in about 28 h and it was not found in plasma 75 h after the DAI ingestion. Table 3 reports the mean values of the kinetics indexes calculated from the DAI, DHD and ODMA plasma levels and are expressed as apparent bioavailability measured as the area under the curve (AUC). The half-life ($t_{1/2}$) for DAI, DHD and ODMA was about 21, 16 and 14 h, respectively.

3.4 Evaluation of ODMA and DHD enantiomers in plasma and urine

Figure 3 shows an example of DAI, DHD, ODMA and IS peaks detected by LC-chiral phase-MS/MS in an enzymatically hydrolysed plasma sample obtained 11 h after soy milk intake. For ODMA and DHD enantiomers, the separation factor (α) was 1.3 and 1.2, respectively.

4. Discussion

To the best of our knowledge, this is the first paper reporting the *in vitro* and *in vivo* determination of the absolute configuration of ODMA produced by humans via daidzein metabolism. Moreover, the direct correlation between production rate of ODMA and the number of bacteria able to metabolise daidzein was established both *in vitro* and *in vivo*.

4.1 ODMA production *in vitro*

All of the tested subjects were "ODMA-producers" and the main enantiomer present (e.e. 91%) in the batch cultures containing ODMA was the R(-)-form. Daidzein was rapidly metabolised within the first six to nine hours of fermentation and its concentration decreased more slowly during the subsequent incubation period, and disappeared completely after 12 h of incubation. Correspondingly, ODMA increased rapidly after about three hours of incubation and the maximum concentration was reached in the range 12-22 hours. At the end of the incubation period (96 h), but already after 24 h, about 70% of the initial DAI was converted to ODMA and once formed it was stable over 96 h. On the other hand, a percentage of DAI (25-35%) disappeared without the production of known metabolites. This phenomenon was previously observed monitoring the equol production from daidzein *in vitro* [22]. Thus, stoichiometric assessments suggest that the DAI degradation pathway responsible for forming ODMA is not yet fully identified. Among the 11 ODMA-producing volunteers, 4 were able to convert 50% of DAI to ODMA within about 5 hours of fermentation, while the other 7 ODMA-producers reached the same level within about 8 hours. The different rates of

formation seem to depend on the number of bacterial able to metabolise DAI to ODMA. Indeed, in the faster ODMA-producers, the number of microorganisms able to convert DAI in ODMA was 10^8 (n=3) and 10^{10} cells/g wet faeces (n=1). These volunteers belonged to the sub-group with the higher rate of conversion. For other ODMA-producers, the bacterial populations were 10^6 (n=5), 10^5 (n=1) and 10^7 cells/g wet faeces (n=1). These volunteers, who demonstrated slower ODMA production in time-course experiments, belonged to the sub-group with the lower rate of conversion. In this regard, we showed that the different rate of ODMA production could be related to inter-individual differences in the number of ODMA-producing microorganisms.

4.2 Evaluation of ODMA and DHD enantiomers

Regarding ODMA, the R(-)-enantiomer was the main end-product of the daidzein degradation found in the faecal suspensions, urine and plasma of ODMA-producers. On the contrary, in the samples containing DHD, it was present in racemic form with a slight predominance of the S(+) enantiomer (e.e. 54%). The presence of both enantiomers could be explained with a tautomerisation reaction favoured to the presence of a carbonyl α-hydrogen or, less likely, to the presence of a racemase. ODMA and DHD enantiomers were separated using a new chiral stationary phase based on 4-methylbenzoate linked to cellulose. The separation of these analytes was preliminarily performed with different chiral columns but performance in terms of the peak shape, speed of analysis and separation factor (α) was obtained with the said column. Furthermore, the use of a 3 μm column instead of the traditional 5 μm column avoided the split of the flow before the ESI source.

4.3 Pharmacokinetics of DAI, DHD and ODMA in plasma and urine

The pharmacokinetic data show that all healthy ODMA-producers absorb DAI rapidly and the time taken to attain peak plasma concentrations after ingestion is about 8 h, while its

metabolites, DHD and ODMA, reach maximum plasma concentrations at about 20 and 28 h, respectively. At these times, the mean maximum plasma concentration (Cmax) of DAI, DHD and ODMA was 787 ng/mL (3.1 μM), 26 ng/mL (0.1 μM) and 16 ng/mL (0.06 μM), respectively. Thus, this underlines that DAI C_{max} was about 30- and 50-times higher than that of DHD and ODMA, respectively.

Pharmacokinetic analysis of the plasma curves showed that DAI, DHD and ODMA have relatively long half-lives of elimination, which were 21, 16 and 14 h, respectively.

ODMA, an important intestinal bacterial metabolite of daidzein, was detected in the plasma and urine of all the subjects. The plasma profiles showed that there is a time lag in its appearance and that, after a single DAI ingestion, it takes at least 7–11 h before ODMA appears in plasma. This observation is consistent with a colonic origin for its formation. In particular, our data suggest a direct correlation between the conversion rate of the DAI to ODMA and the number of bacteria that are able to perform the transformation. In fact, the t_{max} was 27 and 11 h for the subjects with lower (subjects 1-6, Table 3) and higher (subjects 8-11, table 3) conversion rates, respectively. One volunteer (subject 7) belonging to the subgroup with the lower rate conversion had t_{max} of 51 h, which was significantly different to that of other subjects of the same sub-group. It should be emphasised, however, that the latter had lower number of microorganisms able to convert DAI to ODMA, C_{max} and AUC than all of the other subjects. It is therefore possible that this subject was not an ODMA-producer. As a result, it would be desirable to introduce a standardised method, like that used to define equol-producers status [25], to evaluate whether a subject is an ODMA-producer or not. Regarding C_{max}, AUC and $t_{1/2}$, it seems that there is no correlation between these parameters and the number of bacteria that are able to produce ODMA.

Most DAI is excreted into the urine within the first 24 h, with a high individual variability, ranging from 15 to 42% of the ingested amount. Thus, the recovery of DAI in urine was low,

also considering that the excretion of DHD and ODMA produced by catabolism of DAI did not account for more than 10% of the dose ingested.

5. Conclusion

After incubation with intestinal microflora, daidzein was metabolised to the optically active compounds DHD and ODMA. Circular dichroism analysis established that R(-)-O-desmethylangolensin was the main product, while both DHD enantiomers were detected. The *in vitro* formation of ODMA seems to be directly correlated with the number of ODMA-producing bacteria. This correlation was found *in vivo* for t_{max} but not for the other pharmacokinetic indexes evaluated. ODMA was detected in the plasma and urine of all subjects, with high inter-individual variations. After ingestion, daidzein was excreted in the urine but its recovery was no higher than 55-60%, even when also including its metabolites. This suggests that the daidzein degradation pathway resulting in the formation of ODMA is not yet fully identified.

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Legends to the figures

Figure 1. Kinetics of DAI, ODMA and DHD during fermentation experiments using faecal samples from ODMA-producing volunteers. Data are presented as means.

A: sub-group at higher rate conversion (n=4) - 50% DAI conversion in about 5 h.

B: sub-group at lower rate conversion (n=7) - 50% DAI conversion in about 8 h.

Figure 2. CD spectra of ODMA enantiomers (A) and R(-)-Angolensin (B).

Figure 3. Typical LC-MS/MS chromatograms of a plasma sample collected 27 h after the intake of 100 mL soy milk containing 100 mg Daidzein.

DAI, Daidzein: RT 3.2 min, $(m/z)^-$ 253 $\rightarrow$ 91

DHD 1, S(+)-Dihydrodaidzein: RT 3.1 min, $(m/z)^-$ 255 $\rightarrow$ 149

DHD 2, R(-)-Dihydrodaidzein: RT 4.0 min, $(m/z)^-$ 255 $\rightarrow$ 149

ODMA 1, R(-)-O-Desmethylangolensin: RT 4.5 min, $(m/z)^-$ 257 $\rightarrow$ 136

ODMA 2, S(+)-O-Desmethylangolensin: RT 5.4 min, $(m/z)^-$ 257 $\rightarrow$ 136

IS, Galangin: RT 6.4 min, $(m/z)^-$ 269 $\rightarrow$ 77

Table 1. Daidzein and related metabolites concentrations (mean ± s.d.) in plasma sample before (t=0) and after drinking milk containing 100 mg daidzein.

t (h)	DAI (μM)	DHD (nM)	ODMA (nM)
0	0.0±0.0	0.0±0.0	0.0±0.0
3	1.7±0.7	4.9±14.8	0.0±0.0
5	2.2±1.0	23.1±56.5	0.2±0.6[a]
7	2.7±1.3	38.0±79.8	1.0±3.0
9	2.4±0.7	42.6±74.2	1.8±5.0
11	2.0±0.5	46.6±66.0	9.7±20.9
27	0.8±0.4	54.4±49.4	52.5±66.3
39	0.0±0.0	31.9±32.5	33.4±40.2
51	0.0±0.0	5.0±15.1	14.2±18.5
63	0.0±0.0	0.0±0.0	4.1±3.3
75	0.0±0.0	0.0±0.0	0.0±0.0

[a] Detected only in two subjects.

Table 2. Amount of DAI, DHD and ODMA (mean ± s.d.) excreted in the 24 h urine before (t=0) and after drinking milk containing 100 mg DAI.

t (h)	DAI	DHD	ODMA
0	0.0±0.0	0.0±0.0	0.0±0.0
24	21.0±12.5	2.1±3.0	1.1±1.1
48	4.6±3.0	1.1±0.5	1.4±0.9
72	2.3±5.3	0.0±0.1	0.2±0.3
96	0.0±0.0	0.0±0.0	0.0±0.0
Total mg	27.9±9.4	3.3±3.0	2.7±2.2

Table 3. Plasma kinetic indexes in 11 healthy volunteers for DAI, DHD and ODMA after ingestion of soy milk containing 100 mg DAI.

Subject	DAI				DHD				ODMA			
	C_{max}	t_{max}	AUC	$t_{1/2}$	C_{max}	t_{max}	AUC	$t_{1/2}$	C_{max}	t_{max}	AUC	$t_{1/2}$
1	4.7	7	62.2	17	91.6	27	2.4	17.8	47.6	27	0.9	9.1
2	3.6	7	61.9	24.3	147.6	27	4.4	18.1	88.3	27	2.5	20.2
3	2.5	11	45.3	25.3	61.9	11	1.4	16.8	112.1	27	2.8	18.8
4	3.6	7	33.2	16.3	170.6	9	4.3	19.8	32.5	27	0.6	10.3
5	2.2	7	36.3	18.0	21.6	27	0.6	15.5	192.3	27	5.0	15.2
6	2.6	8	38.5	21.0	88.2	27	0.7	16.2	57	27	1.0	19.4
7	1.5	11	36.7	26.4	5.1	27	0.1	19.2	9.2	51	0.2	4.8
8	3.0	9	60.1	29.7	73.1	27	2.1	18.5	26.2	11	0.4	8.1
9	2.8	8	60.8	20.1	86.2	27	2.4	17.2	27.4	11	0.5	21.3
10	3.8	7	55.6	19.9	130.3	9	2.8	19.3	29.1	11	0.7	18.4
11	3.3	5	48.6	14.1	238.7	7	3.2	6.0	60.1	11	1.3	13.7
mean±sd	3.1±0.9	7.9±1.8	49.0±11.5	21.1±4.8	101.4±67.2	20.5±9.1	2.2±1.4	16.7±3.8	62.0±52.6	28±11	1.4±1.5	14.5±5.7

C_{max}, peak plasma concentration (nM for DHD and ODMA, μM for DAI); t_{max}, time taken to reach peak concentration (h); AUC, area under the curve [0-75h (μM x h)/ml]; $t_{1/2}$, terminal half-life (h).
Subjects 1-7: sub-group at ODMA lower rate conversion. Subjects 8-11: sub-group at ODMA higher rate conversion.

*Highlights (for review)

Research Highlights

Daidzein metabolism in ODMA-producers subjects

Daidzein and its metabolites by UPLC-MS/MS

Enantiomers separation by a new chiral column

R(-)-O-desmethylangolensin the main enantiomer *in vitro* and *in vivo*

ODMA-producing bacteria number affects the t_{max}

Figure
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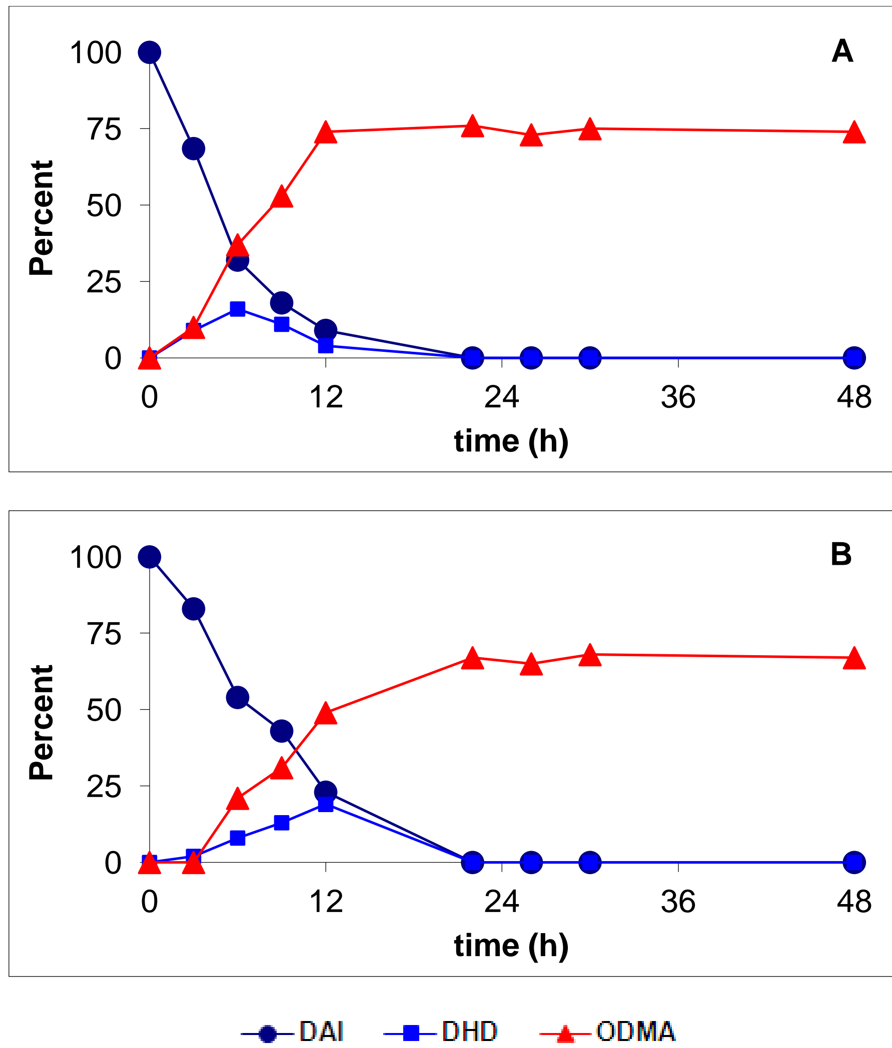
Figure 1



Figure
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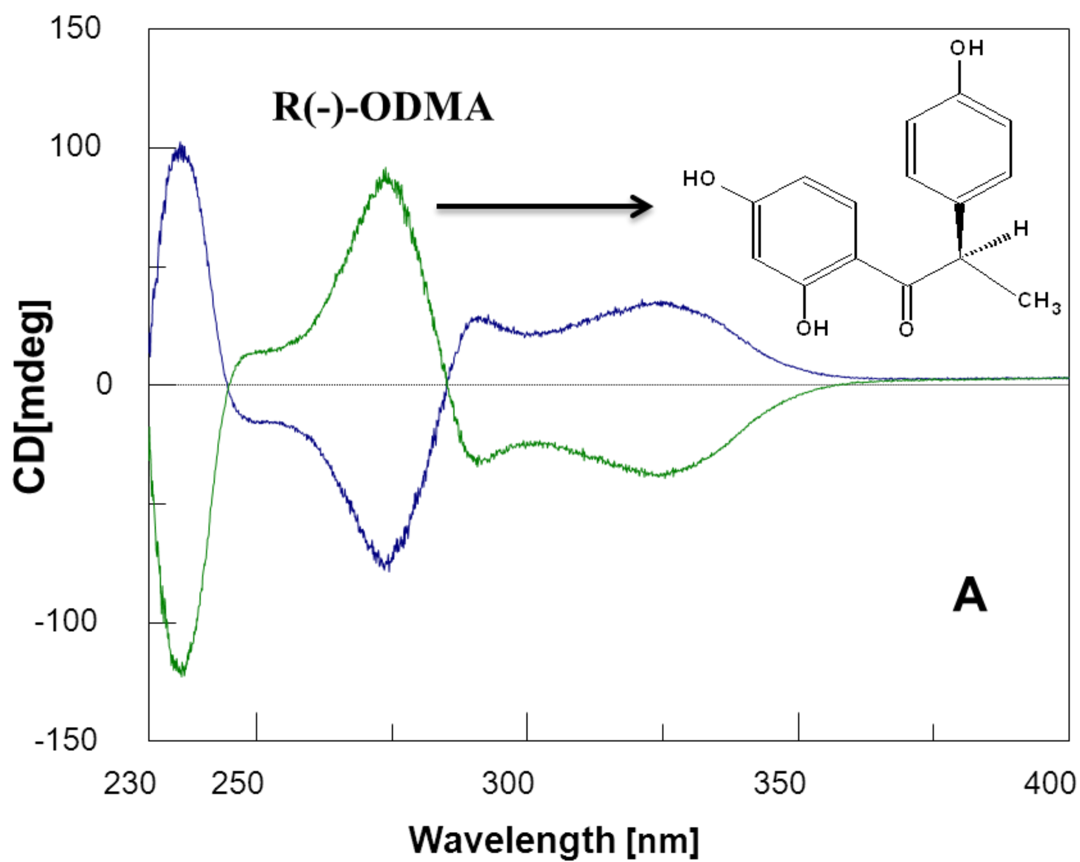
Figure 2



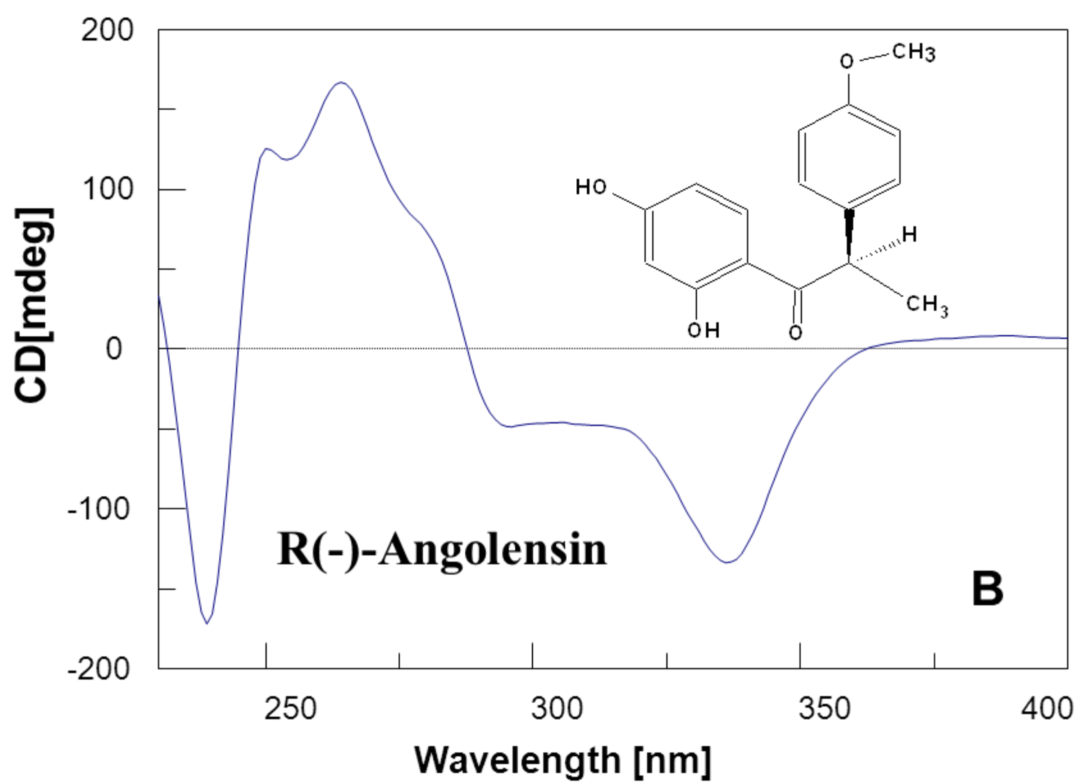


Figure 3