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Microbial and dietary factors associated with the 8-prenylnaringenin producer phenotype: a dietary intervention trial with fifty healthy post-menopausal Caucasian women

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Hop-derived food supplements and beers contain the prenylflavonoids xanthohumol (X), isoxanthohumol (IX) and the very potent phyto-oestrogen (plant-derived oestrogen mimic) 8-prenylnaringenin (8-PN). The weakly oestrogenic IX can be bioactivated via *O*-demethylation to 8-PN. Since IX usually predominates over 8-PN, human subjects may be exposed to increased doses of 8-PN. A dietary intervention trial with fifty healthy post-menopausal Caucasian women was undertaken. After a 4 d washout period, participants delivered faeces, blank urine and breath samples. Next, they started a 5 d treatment with hop-based supplements that were administered three times per d and on the last day, a 24 h urine sample was collected. A semi-quantitative FFQ was used to estimate fat, fibre, alcohol, caffeine and theobromine intakes. The recoveries of IX, 8-PN and X in the urine were low and considerable inter-individual variations were observed. A five-fold increase in the dosage of IX without change in 8-PN concentration resulted in a significant lower IX recovery and a higher 8-PN recovery. Classification of the subjects into poor (60 %), moderate (25 %) and strong (15 %) 8-PN producers based on either urinary excretion or microbial bioactivation capacity gave comparable results. Recent antibiotic therapy seemed to affect the 8-PN production negatively. A positive trend between methane excretion and 8-PN production was observed. Strong 8-PN producers consumed less alcohol and had a higher theobromine intake. From this study we conclude that *in vivo* *O*-demethylation of IX increases the oestrogenic potency of hop-derived products.

Phyto-oestrogens: Hops: 8-Prenylnaringenin: Post-menopausal women

Phyto-oestrogens are polyphenolic, non-steroidal plant-derived compounds, which are structurally related to 17β-oestradiol[1]. Predominant phyto-oestrogens in the Western diet belong to the subclasses of isoflavones, lignans and prenylflavonoids[2]. Depending on the endogenous oestrogen levels and the target tissue, these compounds can mimic or modulate the activity of circulating oestrogens resulting in potential health effects[3]. Binding of phyto-oestrogens to the human oestrogen receptors α and β is not the only common feature of this quite diverse group. Their bioavailability is characterized by considerable inter-individual variation, which is partly due to differences in metabolism by the gut microbiota[4] and the background diet[5,6].

Prenylflavonoids including xanthohumol (X), isoxanthohumol (IX) and 8-prenylnaringenin (8-PN), are found in hops (*Humulus lupulus* L. Cannabaceae) and hop-derived products such as beers and herbal preparations claiming sedative effects, relief of menopausal complaints or breast enhancement[7,8]. Whereas X and IX have no or weak oestrogenic activity, 8-PN is substantially more oestrogenic than other dietary phyto-oestrogens[9]. 8-PN is also unique in respect of its oestrogen receptor specificity, as it binds preferably to oestrogen receptor-α[10].

The weak phyto-oestrogen IX gained much attention recently, since it can be *O*-demethylated into 8-PN *in vitro*

Abbreviations: 8-PN, 8-prenylnaringenin; IX, isoxanthohumol; X, xanthohumol.

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by the human faecal microbiota[11] and by mammalian cytochrome P450 enzymes[12,13]. As its concentration in hop-derived products exceeds that of 8-PN by over ten-fold[14], bioactivation of IX might increase the exposure of man to 8-PN. However, analysis of fifty-one faecal samples revealed large inter-individual variation in the IX conversion capacity of the human intestinal bacteria, which led to a separation of individuals in poor, moderate and high 8-PN producers[15]. Until now, this bioactivation has not been studied extensively *in vivo*. Schaefer *et al.*[16] detected 8-PN in the urine of two men after a single dose of 10 mg IX mixed into a high-alcoholic beverage. In a small *in vivo* trial involving three women, Possemiers *et al.*[15] found a clear relationship between the *in vitro* transformation potential of faecal microbial cultures and the urinary 8-PN excretion after IX consumption.

Understanding the factors influencing the bioactivation of IX *in vivo* is important to estimate the 8-PN exposure after oral administration of hop-based products. In addition to differences in the composition and activity of the intestinal microbiota, dietary factors may also affect the bioavailability of phyto-oestrogens[4]. Macronutrient intake can selectively modify the growth of gut bacteria involved in the bioactivation of phyto-oestrogens[17] by changing the intestinal pH, redox potential or transit time or by influencing the availability of substrates[18]. In particular, differences in total fat and dietary fibre consumption have been highlighted in previous studies[5,6,17,19]. The purpose of the present work was: (1) to examine the extent of inter-individual variation in urinary excretion of hop-derived prenylflavonoids; (2) to assess the prevalence of the different 8-PN producer phenotypes; (3) to evaluate the importance of microbial activation of IX towards 8-PN exposure; (4) to identify microbial and dietary factors that are associated with *in vivo* 8-PN production. Therefore, a dietary intervention trial with fifty healthy postmenopausal Caucasian women was conducted.

Materials and methods

Chemicals and reagents

The isolation of X from spent hops (i.e. the vegetative residue left after liquid or supercritical CO_2 extraction of hop cones; NATECO2, Wolnzach, Germany), isomerization of X into IX and preparation of 8-PN were performed as described by Possemiers *et al.*[11]. A stock solution of 15 mM-IX in absolute ethanol was prepared. 4-Hydroxybenzophenone (internal standard) was obtained from Fluka Chemie (Buchs, Switzerland). 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 (Bornem, Belgium). All solvents were purchased from Biosolve (Valkenswaard, The Netherlands).

Study design and population

An intervention trial with fifty healthy post-menopausal Caucasian women was undertaken to study the microbial metabolism of phyto-oestrogens from hops *in vivo* and *in vitro*. Women were eligible for participation if they were not using any exogenous hormone medications, not suffering from a gastrointestinal disease and free of cancers. Women who were receiving an antibiotic therapy (*n* 2) were scheduled at least 1 month after antibiotic therapy completion. Subject information such as date of birth, weight, height, use of pre-, pro-, syn- or antibiotics, smoking and time of last vaginal bleeding were collected upon recruitment.

Participants consumed their habitual Western-type diets, but were asked to abstain from products based on hops during the trial. A list of prenylflavonoid-containing foods was distributed in order to help the volunteers. After a 4 d washout period, subjects delivered a faecal sample for incubation purposes and microbiological phenotyping, a blank urine sample and two end-expiratory breath samples. Subsequently, three hop-derived dietary supplements per d were administered during five consecutive days and on the fifth treatment day a 24 h urine sample was collected. A self-administered semi-quantitative FFQ was developed to estimate the usual fat, fibre, alcohol, caffeine and theobromine consumption. This FFQ included questions on the average consumption (frequency, daily portion size) of seventy-six food items during the past year and some additional questions involving more detailed information about some product groups. The inclusion of food items was based on knowledge from previously conducted population dietary surveys in Belgium. The validity and reproducibility of this instrument are reported elsewhere (Bolca *et al.*, unpublished data).

The present study was given ethical approval by the Ethics Committee of the Ghent University Hospital (EC UZG 2005/022). The volunteers were fully informed on the aims of the study and gave their written consent.

Sample collection and processing

End-expiratory breath samples were collected using the Quin-Tron GaSampler system (Ecce Medical, Schoten, Belgium). The alveolar air samples were analysed immediately by GC-Flame ionisation detector (FID). The background room air was found to contain less than 2 ppm methane. Participants were considered to be methane producers when their breath methane concentration exceeded 3 ppm[20].

Volumes of the 24 h urine samples were measured and aliquots were stored at −20°C. For hydrolysis of conjugated prenylflavonoids, a 33 g/l solution of Type H-1 *Helix pomatia* extract in sodium acetate buffer (0·1 M, pH 5) was prepared. Unfrozen urine (15 ml) was added to sodium acetate buffer (15 ml) and β-glucuronidase/arylsulfatase solution (30 μl) and the samples were mixed and incubated for 1 h at 37°C. The hydrolysed samples were spiked with 90 μl internal standard (0·4 M-4-hydroxybenzophenone in ethyl acetate) before extraction. The solid-phase extraction Bond Elut® C18 silica columns (5 ml, 500 mg, Varian, St.-Katelijne-Waver, Belgium) were pre-conditioned with 5 ml methanol, 5 ml water and 5 ml sodium acetate buffer (0·1 M, pH 5), consecutively. After sample application, the cartridges were rinsed with 5 ml water and 5 ml methanol/water (2:3) and the compounds of interest were eluted with 2 ml methanol using a VacMaster 20 sample processing unit (IST, Hengoed, Mid Glamorgan, UK). Finally, the solvent was evaporated at room temperature under a gentle stream of N_2 and the residue was dissolved in 100 μl methanol, transferred into HPLC vials and stored at −20°C prior to analysis.

Faecal suspensions were prepared by homogenizing 20 g faeces with 100 ml phosphate buffer (0·5 M, pH 7) supplemented with 1 g/l sodium thioglycolate. Particulate material was removed by centrifugation at 400 ***g*** for 2 min. To assess the 8-PN production capacity of each faecal culture, *in vitro* experiments were set up following the incubation and extraction protocols developed by Possemiers *et al.*[11]. Aliquots of faecal suspensions were also stored at −80°C for measurement of bacterial β-glucosidase and β-glucuronidase activities.

Hop-derived dietary supplements

Two different batches of hop-based capsules (MenoHop®, Biodynamics bvba, Ostend, Belgium), were used: twelve women (24 %) were given BD01 capsules; thirty-eight women (76 %) ingested BD02 capsules. Composition and manufacturing information have been described by Heyerick *et al.*[8]. The concentrations of prenylflavonoids were measured by HPLC-UV after extracting 200 mg of the capsules with 10 ml methanol and diluting the supernatant five times in methanol. BD01 contained 0·23 ± 0·01 mg IX, 0·10 ± 0·01 mg 8-PN and 1·38 ± 0·03 mg X per capsule; BD02 contained 1·20 ± 0·04 mg IX, 0·10 ± 0·01 mg 8-PN and 2·04 ± 0·06 mg X per capsule.

Quantification of methane by GC-FID

Alveolar air samples were taken using a Pressure-Lok precision analytical syringe (Alltech Ass., Deerfield, IL, USA). The methane concentrations were measured with a Chrompack CP 9000 GC equipped with a flame ionization detector (Chrompack, Middelburg, The Netherlands), based on the protocol of Boeckx *et al.*[21]. The analyses were carried out using the following conditions: injection temperature 65°C; oven temperature 35°C; detector temperature 250°C. A mixture of 50·3 (SD 1·5) ppm CH_4 in Ar was used as standard gas (L'Air Liquide, Liege, Belgium).

Quantification of prenylflavonoids by HPLC-UV

Quantitative analyses of the prenylflavonoids extracted from the urine samples, incubation media or capsules were performed by HPLC-UV using a Waters 2695 Alliance separations module (Waters, Milford, MA, USA) equipped with a Waters 996 photodiode array detector and Waters Millenium software v3.20 as reported by Possemiers *et al.*[11]. Detection was done simultaneously at 295 nm (for IX, 8-PN and 4-hydroxybenzophenone) and at 370 nm (for X). Peaks were identified by comparison of the retention times and UV spectra with those of authentic isolated reference compounds. Concentrations were calculated based on peak area integration of the analytes and the internal standard.

Measurement of faecal enzyme activity

Unfrozen faecal suspensions were centrifuged at 5000 ***g*** for 5 min and diluted five times in phosphate buffer (0·5 M, pH 7)[22]. For the assessment of β-glucosidase and β-glucuronidase activities, the suspensions were incubated aerobically for 30 min at 37°C with *p*-nitrophenyl-β-D-glucopyranoside (2·5 mM) or *p*-nitrophenyl-β-D-glucuronide (2·5 mM), respectively. Release of *p*-nitrophenol was recorded with a Tecan Sunrise™ absorbance reader (Tecan Benelux, Mechelen, Belgium) at 405 nm before and after incubation. The absorbance of a series of different concentrations of *p*-nitrophenol was used to calculate the enzymatic activities, according to the method of Berg *et al.*[23].

Statistical approach

All extractions and analyses were performed in triplicate means and SD were calculated. The Statistical Package for the Social Sciences for Windows version 12.0 (SPSS Inc., Chicago, IL, USA) was used to carry out all statistical analyses. Unless reported differently, a P value of 0·05 was used as threshold for significance. Two-sided significance levels are quoted. 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 or ANOVA. The non-parametric Mann–Whitney U and Kruskal–Wallis test were used to compare means of non-normally distributed data. Partial Pearson correlation coefficients adjusted for the type of hop treatment were computed to measure associations between urinary parameters.

Subjects were separated into statistically different groups using the TwoStep cluster analysis protocol. Associations between the producer phenotype and subject characteristics, urinary, microbial and dietary parameters were evaluated using nominal logistic regression with poor 8-PN producers as reference category. Cross-classification analysis calculated the agreement between the *in vivo* and *in vitro* data.

Results

Subject characteristics and diet

The mean age of the subjects was 57 years with a range from 46 to 74 years. Twenty women (40 %) were classified as overweight (BMI ≥25 kg/m^2); five (25 %) of these were obese (BMI ≥30 kg/m^2). Four women (8 %) were smokers. The majority had not used antibiotics during the past year and consumed less than one pre-, pro- or synbiotic preparation per month (Table 1). Forty-nine good-quality FFQ (98 %) were included in the analysis. The average consumption of total fat, SFA, MUFA, PUFA, fibre and alcohol was compared with the guideline daily amounts for seniors proposed by the Belgian Health Council[24] (Table 2). Only 10 % of the study population reported a daily fibre intake of at least 30 g/d, as recommended. While the Belgian Health Council advises not to drink any alcohol on a regular basis, the Eurodiet

Table 1. Time since last antibiotic therapy and frequency of consumption of pre-, pro- or synbiotic preparations, expressed as a percentage of individuals (n 50)*

Antibiotics (%)		Pre-, pro- or synbiotics (%)	
≤1 month	0	≥once per week	14
1–3 months	4	once per week	2
3–6 months	8	2–3 times per month	2
9–12 months	10	once per month	16
≥1 year	78	never or less than once per month	66

* For details of subjects and procedures, see Materials and methods.

Table 2. Actual and guideline daily intakes and percentage of the FFQ reports*†

(Mean values and standard deviations for forty-nine subjects)

	Consumption (g/d)			
	Actual			
Food item	Mean	SD	Guideline	Subjects (%)
Total fat	54·18	20·83	<67	72
SFA	23·15	9·46	<22	47
MUFA	27·00	10·40	>22	27
PUFA	9·11	3·91	12–22	25
Fibre	19·61	6·77	>30	10
Alcohol	8·46	12·11	0	27
Caffeine	0·15	0·10	US	US
Theobromine	0·03	0·04	US	US

* FFQ reports are in agreement with the recommendations for seniors proposed by the Belgian Health Council[24].
† For details of subjects and procedures, see Materials and methods.
US, unspecified.

guideline allows up to 12 g/d[24]. The estimated alcohol intake of thirty-seven participants (76 %) was less than 12 g/d; thirteen (35 %) of these reported to drink less than one alcoholic beverage per month. Twenty-eight subjects (57 %) consumed at least two cups of regular coffee per d (223·82 (SD 61·13) mg caffeine/d), while twenty-one (43 %) consumed not more than one cup (125 ml) of regular coffee per d or drank coffee with reduced caffeine content, decaffeinated or surrogate coffee (45·52 (SD 40·00) mg caffeine/d).

Pulmonary methane excretion

Thirty-three (66 %) volunteers had methane (4·05–27·95 ppm) in their alveolar air samples and were included in the group of methane producers. The other participants (34 %) did not exceed the selected methane threshold in their breath (1·20–3·00 ppm). Age, BMI, use of pre-, pro-, syn- or antibiotics, smoking and dietary parameters (total fat, SFA, MUFA, PUFA, fibre, alcohol, caffeine and theobromine intake) were comparable for both methane producers and non-producers.

Urinary excretion and recovery of prenylflavonoids

IX, 8-PN and X in the 24 h urine samples were quantified as daily excretion and percentage of the daily doses recovered (Table 3). The inter-individual variations in urinary excretion were eighteen-fold for IX, thirteen-fold for 8-PN and six-fold for X between subjects of the BD01 group; between the subjects of the BD02 group, the inter-individual variations were thirty-fold for IX, forty-fold for 8-PN and twenty-seven-fold for X. A thirteen-fold variation in the renal excretion of total prenylflavonoids was observed in both treatment groups. The recoveries of IX, 8-PN and X were low, especially for X. Less than 1 % of the dosed X was found in the urine. A 1·5-fold increase in X dosage resulted in an equivalent rise in urinary X excretion. In contrast, multiplying the ingested amount of IX with a factor five decreased the recovery of IX significantly ($P=0·005$). Although the capsules contained the same amount of 8-PN, the average 8-PN excretion was higher in the BD02 group, but the difference was statistically not significant. When controlling for the type of hop treatment, the urinary recovery of IX correlated modestly with the recovery of 8-PN (R 0·293; $P=0·041$). There was a stronger correlation between the recoveries of X and IX (R 0·445; $P=0·001$) and of X and 8-PN (R 0·605; $P<0·001$), respectively. Neither the faecal β-glucosidase (24·6 ± 19·9 μmol *p*-nitrophenol/h per g faeces) nor the β-glucuronidase (15·5 (SD 9·9) μmol *p*-nitrophenol/h per g faeces) activity correlated with the excretion of IX, 8-PN or X.

Based on the ratio 8-PN:(8-PN + IX) excreted in the urine and the type of hop treatment, three different groups were formed (Fig. 1(A)). Thirty-one (62 %), twelve (24 %) and seven (14 %) women were classified as poor, moderate and strong *in vivo* 8-PN producers, respectively. The average urinary recovery of 8-PN ($P=0·014$) and IX ($P=0·002$) were significantly different between these groups, while the average recovery of X ($P=0·374$) was similar.

Microbial metabolism of prenylflavonoids

The microbial metabolism of IX was highly variable ranging from 0 up to 100 % IX transformed into 8-PN and led to a separation of the subjects in poor (61 %), moderate (27 %) and strong (12 %) *in vitro* 8-PN producers (Fig. 1(B)). These clusters had significantly different average urinary recoveries of 8-PN ($P=0·027$) and X ($P=0·032$), but not for IX. The percentages IX transformed and 8-PN produced by the faecal cultures correlated inversely (R −0·650, $P<0·001$). The prevalence of poor, moderate and strong *in vitro* 8-PN producers was not significantly influenced by the type of hop treatment.

Table 3. Concentrations of isoxanthohumol (IX), 8-prenylnaringenin (8-PN) and xanthohumol (X) in 24 h urine samples of post-menopausal women who ingested a hop-based food supplement BD01 (*n* 12) or BD02 (*n* 38) three times per d for 5 d*

(Mean values and standard deviations)

	Excretion (μg/d)						Recovery (%)					
	IX[a]		8-PN		X		IX[b]		8-PN		X	
Food supplement	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
BD01	187·3	162·1	35·5	23·6	14·2	9·2	26·9	24·6	11·6	7·8	0·33	0·22
BD02	287·8	200·7	51·8	38·5	19·3	10·9	8·0	5·6	16·3	12·2	0·32	0·18

[a] $P=0·054$, BD01 *v.* BD02.
[b] $P=0·005$, BD01 *v.* BD02.
* For details of subjects and procedures, see Materials and methods.

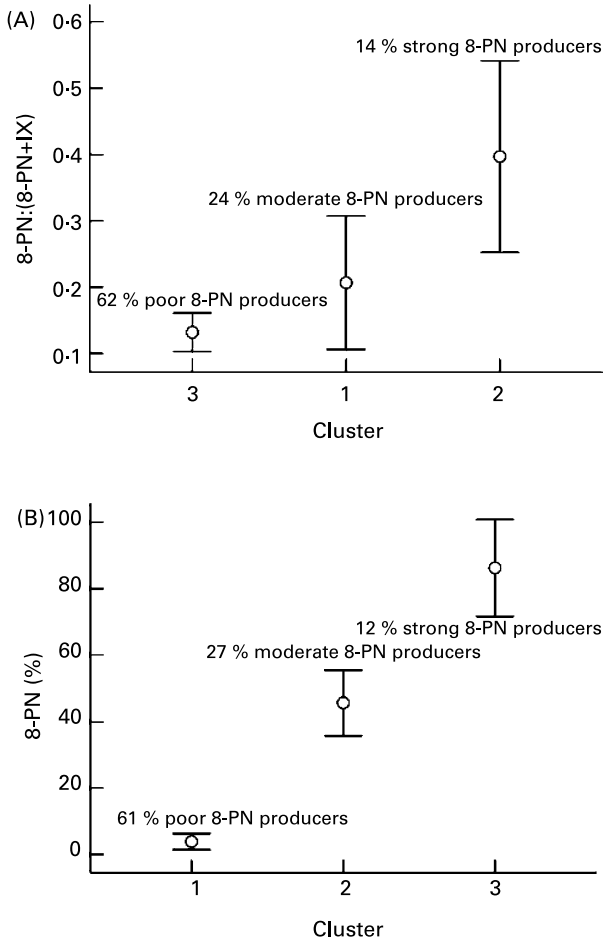

Fig. 1. Clustering of individuals (n 50) in three significantly different groups, namely, poor, moderate and strong 8-prenylnaringenin (8-PN) producers, based on (A) the ratio 8-PN:(8-PN + isoxanthohumol (IX)) excreted in the 24 h urine and the type of hop treatment; (B) the capacity of the faecal microbiota to convert IX in 8-PN. Values are means with their standard deviations represented by vertical bars. For details of subjects and procedures, see Materials and methods.

Factors associated with 8-prenylnaringenin production

Associations between the *in vivo* 8-PN producer phenotype and subject characteristics, microbial and dietary parameters are presented in Table 4. Individuals who had not taken antibiotics at least 6 months before participation were more likely to be strong *in vivo* 8-PN producers ($P=0{\cdot}035$). There was some indication ($0{\cdot}050 < P < 0{\cdot}075$) that pulmonary methane excretion was positively related with *in vivo* 8-PN production. A lower reported alcohol consumption ($P=0{\cdot}039$) and a higher intake of theobromine ($P=0{\cdot}050$) were associated with the strong *in vivo* 8-PN producer phenotype.

Most of the subject characteristics, urinary, microbial and dietary parameters were not strongly associated with the *in vitro* 8-PN producer phenotype (Table 5). However, positive trends ($0{\cdot}050 < P < 0{\cdot}075$) between urinary excretion and recovery of X and *in vitro* 8-PN production were observed. Lower estimated alcohol ($P=0{\cdot}041$) and higher theobromine intake ($P=0{\cdot}047$) correlated with the *in vitro* strong 8-PN producer phenotype.

As alcohol consumption differed between the 8-PN producer phenotypes, this topic was examined more thoroughly. Alcoholic beverages were included in four items of the FFQ: aperitif; wine and champagne; beer; liqueurs and spirits. In addition to the frequency and daily portion size of beer consumption, questions on the type of beer (no or low alcoholic, pilsner type or strong beer) were included to estimate the usual intake of both alcohol and prenylflavonoids. Neither *in vivo* nor *in vitro* 8-PN production correlated significantly with the usual beer consumption. Twenty-six women (53 %) reported to drink less than 200 ml beer per month, whereas the others had an average estimated beer consumption of 56 (SD 70) ml/d. Habitual X, IX, 8-PN and total prenylflavonoid intakes were similar in all 8-PN producer phenotypes and ranged from 0 to 102 μg/d for X, 0 to 630 μg/d for IX, 0 to 21 μg/d for 8-PN and 0 to 753 μg/d for total prenylflavonoids.

Cross-classification analyses for 8-PN producer phenotypes estimated from *in vivo* and *in vitro* data indicated that six women (12·2 %) were misclassified, while twenty-six subjects (53·1 %) were classified correctly and forty-three participants (87·7 %) were classified correctly or in the adjacent category.

Discussion

The prenylflavonoids X, IX and 8-PN are found in hops (*Humulus lupulus* L. Cannabaceae) and hop-derived products, including beers and food supplements. As bioactivation of IX to the potent phyto-oestrogen 8-PN might increase the oestrogenic potency of such products, the metabolism of prenylflavonoids was studied *in vivo* and *in vitro*. In order to work with the target population for phyto-oestrogen therapy and to minimize the variations in circulating oestrogen concentrations and their possible interactions with the phyto-oestrogen metabolism[25] and liver enzymes[26], only healthy hormone-naive women were included. The recoveries of the dosed IX, 8-PN and X were low and considerable inter-individual variations were observed. Classification of the volunteers into poor (60 %), moderate (25 %) and strong (15 %) 8-PN producers based on either urinary excretion or microbial bioactivation capacity of faecal samples gave comparable results. Recent antibiotic therapy seemed to affect the 8-PN production negatively. A positive trend between methane excretion and 8-PN production was observed. A lower alcohol and a higher theobromine intake were associated with the strong 8-PN producer phenotype.

Less than 1 % of the administered X was excreted in the urine and a 1·5-fold increase in X dosage resulted in an equivalent rise in urinary excretion. After oral administration of 20 to 500 mg/kg body weight X as a pure compound or as a hop extract, Avula *et al.*[27] found 0·06–0·49 % of the dosed X in the urine and more than 99·5 % in the faeces of rats. These results suggest that X is poorly absorbed through the intestinal wall, thereby resulting in a low oral bioavailability. This feature may be an important bottleneck in the development of X as a novel broad-spectrum cancer chemopreventive agent[28]. Based on the apparent permeability coefficients assessed in the Caco-2 cell monolayer model, Nikolic *et al.*[29] predicted a rather efficient intestinal absorption via passive diffusion for 8-PN. This rapid enteral absorption was also found after a single oral administration of 8-PN to healthy postmenopausal women[30], as well as in the present study. The recoveries of 8-PN and IX in the 24 h urine samples

Table 4. Subject characteristics, microbial and dietary parameters in relation to the *in vivo* 8-prenylnaringenin (8-PN) producer phenotype, expressed as OR and upper and lower 95 % CI derived from nominal logistic regression with poor *in vivo* 8-PN producers as reference category*

	In vivo 8-PN producer					
	Moderate			Strong		
		95 % CI			95 % CI	
	OR	Upper	Lower	OR	Upper	Lower
Subject characteristics						
Age (years)	0·986	0·870	1·118	0·945	0·792	1·128
BMI (kg/m^2)	1·097	0·919	1·310	0·999	0·780	1·278
Smoking						
Yes	0·000	0·000	0·000	0·000	0·000	0·000
No	1·000	Reference		1·000	Reference	
Use of antibiotics						
1–3 months	2·500	0·142	43·97	0·000	0·000	0·000
3– 6 months	0·833	0·077	8·995	0·000	0·000	0·000
6– 12 months	0·000	0·000	0·000	9·375	1·174	74·84
≥ 1 year	1·000	Reference		1·000	Reference	
Microbial parameters						
Pulmonary methane excretion	1·472	0·554	3·912	1·121	0·326	3·858
Enzyme activities (μmol/h per g)						
β-glucuronidase	1·056	0·970	1·151	0·935	0·825	1·061
β-glucosidase	1·039	0·987	1·094	1·065	1·002	1·132
Faecal metabolism						
Degradation of IX	1·033	0·990	1·079	0·989	0·946	1·034
Production of 8-PN	1·041	0·988	1·096	1·011	0·957	1·067
Dietary parameters						
Fat consumption (g/d)						
Total fat	1·538	0·565	4·189	0·700	0·192	2·558
SFA	0·523	0·169	1·618	1·670	0·383	7·291
Unsaturated fatty acids	0·638	0·246	1·658	1·327	0·386	4·564
MUFA	1·326	0·556	3·165	0·934	0·383	2·277
PUFA	1·042	0·878	1·236	0·926	0·726	1·180
Fat consumption (%)						
SFA	0·822	0·492	1·374	1·115	0·620	2·007
Unsaturated fatty acids	0·939	0·634	1·392	0·992	0·607	1·622
Fibre consumption (g/d)	1·103	0·991	1·227	1·051	0·927	1·191
Fat:fibre ratio	0·617	0·317	1·202	0·665	0·312	1·416
Alcohol consumption (g/d)	0·799	0·350	1·821	0·257	0·071	0·933
Methylxanthine intake (mg/d)						
Caffeine	1·000	0·991	1·009	0·998	0·987	1·010
Theobromine	0·757	0·395	1·451	2·041	0·793	5·255
Pre-, pro-, synbiotics						
≥ once per week	1·500	0·224	10·04	1·313	0·115	15·03
once per week	6·6 E8	6·6 E8	6·6 E8	1·016	1·016	1·016
2–3 times per month	0·000	0·000	0·000	0·000	0·000	0·000
once per month	1·200	0·189	7·628	1·050	0·095	11·56
≤ once per month	1·000	Reference		1·000	Reference	

* For details of subjects and procedures, see Materials and methods.
IX, isoxanthohumol.

were comparable to those reported for the isoflavones genistein (8–16 %) and daidzein (15–50 %)[31–33].

The observed inter-individual variation in urinary excretion of prenylflavonoids was considerable and higher than the 5·5-fold variation in 24 h renal excretion of 8-PN reported by Schaefer *et al.*[16] in a small trial involving six subjects. In comparison, concentrations of the isoflavones genistein and daidzein in 24 h urine samples varied between subjects twelve- to twenty-four-fold and thirteen to eighty-five-fold, respectively[5,32,34]. Lampe *et al.*[35] and Rowland *et al.*[17] reported a wider range of excretion of the microbial daidzein metabolites equol and *O*-desmethylangolensin and the enterolignans enterolacton and enterodiol in human subjects.

The urinary recovery data indicated that the excretion of IX and 8-PN was dependent on the ingested amount of IX. A five-fold increase in IX dosage decreased the recovery of IX significantly. Simultaneously, the average 8-PN excretion increased although the ingested amount of 8-PN was unchanged. This is probably due to the conversion of IX into 8-PN *in vivo*. Urinary excretion of 8-PN after consumption of IX has also been reported by Schaefer *et al.*[16] and Possemiers *et al.*[15], and in both studies inter-individual differences were noticed. In addition, the urinary recovery of 8-PN was significantly different between poor, moderate and strong 8-PN producers. As the prevalence of the different 8-PN producer phenotypes was equivalent in both treatment groups, these results suggest that the extent of the conversion

Table 5. Subject characteristics, urinary, microbial and dietary parameters in relation to the *in vitro* 8-prenylnaringenin (8-PN) producer phenotype, expressed as OR and upper and lower 95 % CI derived from nominal logistic regression with poor *in vitro* 8-PN producers as reference category*

	In vitro 8-PN producer					
	Moderate			Strong		
		95 % CI			95 % CI	
	OR	Upper	Lower	OR	Upper	Lower
Subject characteristics						
Age (years)	1·117	0·986	1·265	1·104	0·938	1·300
BMI (kg/m^2)	1·043	0·880	1·236	0·992	0·789	1·247
Smoking						
Yes	2·265	0·100	51·41	0·000	0·000	0·000
No	1·000	Reference		1·000	Reference	
Use of antibiotics						
1– 3 months	1·171	0·041	33·52	0·000	0·000	0·000
3– 6 months	0·417	0·020	8·920	0·000	0·000	0·000
6– 12 months	2·872	0·311	26·55	2·646	0·177	39·50
≥ 1 year	1·000	Reference		1·000	Reference	
Urinary parameters						
Urinary excretion (μg/d)						
IX	1·000	0·996	1·004	0·993	0·982	1·004
8-PN	0·965	0·926	1·006	0·978	0·930	1·027
IX	0·985	0·874	1·109	1·165	0·980	1·384
Urinary recovery (%)						
IX	0·950	0·465	1·943	0·882	0·334	2·330
8-PN	0·900	0·789	1·027	0·943	0·820	1·086
X	0·272	0·000	199·0	689·0	0·269	1·7 E6
Microbial parameters						
Pulmonary methane excretion	0·771	0·287	2·071	0·599	0·143	2·502
Enzyme activities (μmol/h per g)						
β-glucuronidase	1·015	0·948	1·087	1·023	0·931	1·123
β-glucosidase	0·999	0·965	1·034	0·999	0·943	1·058
Dietary parameters						
Fat consumption (g/d)						
Total fat	1·224	0·473	3·163	0·224	0·036	1·412
SFA	0·820	0·279	2·410	6·392	0·821	49·76
Unsaturated fatty acids	0·604	0·200	1·821	4·485	0·847	23·74
MUFA	1·583	0·600	4·180	0·901	0·341	2·381
PUFA	0·644	0·329	1·264	1·092	0·643	1·853
Fat consumption (%)						
SFA	0·956	0·593	1·539	2·088	0·919	4·746
Unsaturated fatty acids	0·921	0·659	1·350	1·732	0·882	3·402
Fibre consumption (g/d)	1·025	0·927	1·132	1·065	0·940	1·208
Fat:fibre ratio	1·045	0·656	1·665	1·170	0·674	2·033
Alcohol consumption (g/d)	0·893	0·396	2·014	0·152	0·021	1·092
Methylxanthine intake (mg/d)						
Caffeine	1·002	0·995	1·009	0·991	0·980	1·003
Theobromine	0·923	0·479	1·779	1·450	0·552	3·811
Pre-, pro-, synbiotics						
≥ once per week	0·425	0·042	4·352	2·125	0·283	15·97
once per week	0·000	0·000	0·000	0·000	0·000	0·000
2–3 times per month	0·000	0·000	0·000	0·000	0·000	0·000
once per month	0·243	0·026	2·272	0·000	0·000	0·000
≤ once per month	1·000	Reference		1·000	Reference	

* For details of subjects and procedures, see Materials and methods.
IX, isoxanthohumol; X, xanthohumol.

depends on the IX dose administered. Cross-over studies with different IX doses are warranted to investigate this in more detail.

The 8-PN production was estimated from *in vivo* as well as *in vitro* data. The study population was separated into poor (62 %), moderate (24 %) and strong (14 %) *in vivo* 8-PN producers. Based on the microbial bioactivation capacity of the faecal cultures, a similar classification prevailed. This is in quite good agreement with the incubation experiments of Possemiers *et al.*[15], which separated faecal samples into slow (63 %), moderate (21 %) and high (16 %) IX converters. The hypothesis that microbial and dietary factors give rise to these different 8-PN producer phenotypes was tested.

The inverse relationship between use of antibiotics and 8-PN production indicates that intestinal microbiota are involved. Pulmonary methane excretion may serve as an indicator of methane production by intestinal microbiota[36]. All humans harbour methanogens in the colon, but methane

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only appears in the breath if the concentration of *Methanobrevibacter smithii* exceeds 10^8/g dry weight faeces[37]. Age-related increases in transit time and carbohydrate malabsorption promote methanogenesis[38] and may explain the high prevalence of methane producers in the present study. H_2 gas is formed in the colon by a variety of hydrolytic and saccharolytic bacteria to dispose reducing equivalents during fermentation[39] and is consumed by methanogenic, homoacetogenic and sulphate-reducing microbiota. Comparison of the prevalence of the different H_2-consuming microbiota in faecal samples of poor, moderate and strong 8-PN producers will unravel the relationship between pulmonary methane excretion and 8-PN production.

Several observational studies have reported differences in dietary intake, particularly fat and fibre, in relation to equol and enterolignan production capacities[5,6,17,19]. The present study did not reveal significant differences. To our knowledge, the effects of alcohol, caffeine, and theobromine consumption on the gut microbiota and, hence, their phyto-oestrogen metabolism have not been investigated directly. As these compounds are rapidly and almost completely absorbed in the stomach and the small intestine, they are not expected to importantly affect the intestinal bacteria, although caffeine intake has been associated with the microbial metabolism of daidzein[25,40]. There is some suggestive evidence for an inhibitory effect of alcohol on the activity of CYP1A2[41,42], the mammalian enzyme system that has been shown to demethylate IX into 8-PN *in vitro*[12]. Thus, the involvement of hepatic enzymes in the bioactivation of IX as suggested by Schaefer *et al.*[16] may explain why a lower reported alcohol consumption was associated with the likelihood of being a strong *in vivo* 8-PN producer. CYP1A2 activity accounts for almost 95 % of the demethylation of caffeine[43], but contributes only partially to the elimination of theobromine[44]. Although low and high caffeine consumers were equally present in the current study population, intake of this CYP1A2 inducer could not be linked to 8-PN production. The mechanism of the positive correlation between theobromine consumption and the strong 8-PN producer phenotype remains unclear. As a constituent of cacao, theobromine is consumed by a large proportion of the population, but it possesses little pharmacological activity. It is important to note that only 10 % of the study population had a daily fibre intake above 30 g, as recommended for seniors by the Belgian Health Council[24]. As a consequence, the effect of fibre consumption or the fat: fibre ratio on the conversion of IX into 8-PN may have been overlooked in this study. Similarly, the influence of the well-known induction of CYP1A2 activity by tobacco smoking could not be clarified, as only four participants (8 %) were smokers. Even though the present study was not specifically designed to address this question, it is unlikely that prior consumption of hop-derived products influences the ability to convert IX into 8-PN, since no differences in the habitual intake of prenylflavonoids were found between the different 8-PN producer phenotypes. Analogously, soya intervention studies failed to stimulate equol production in low equol producers[45,46].

Cross-classification analyses for 8-PN producer phenotype estimated from *in vivo* and *in vitro* data indicated that 12·2 % of the women were misclassified, while 53·1 % were classified correctly. This shows that the *in vitro* incubation experiments give a good indication of the 8-PN producer phenotype and, additionally, stresses the important contribution of the intestinal microbiota towards the *in vivo* bioactivation of IX. Although the *in vivo* bioavailability is the final result of intestinal absorption, human and microbial metabolism, cellular retention, distribution and excretion, these results show the potential of faecal incubations as an appropriate screening assay.

Although biotransformation is generally regarded as a process of metabolic inactivation prior to excretion, there are many well-known examples of metabolic activation. Demethylation by cytochrome P450 enzymes or by gut microbiota represents a common metabolic pathway for activation of pro-oestrogens, such as the isoflavone formononetin[47,48] and the synthetic steroid mestranol[49]. Similarly, *in vivo* *O*-demethylation of IX into 8-PN increased the oestrogenic potency of the hop-derived food supplements in this study, since inter-individual differences in daily excretion of more than 200 μg 8-PN or 2–20 μg 17β-oestradiol equivalents[50] were observed.

In summary, we showed that individuals can be phenotyped as poor (60 %), moderate (25 %) or strong (15 %) 8-PN producers based on either urinary excretion data or faecal incubation experiments. This inter-individual variation in 8-PN production could be linked to differences in microbial and dietary factors. From this study, we conclude that the oestrogenic potency of hop-derived products depends on the 8-PN producer phenotype and the concentration of IX.

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References

1. Adlercreutz H (2002) Phyto-oestrogens and cancer. *Lancet Oncol* **3**, 364–373.
2. Fletcher RJ (2003) Food sources of phyto-oestrogens and their precursors in Europe. *Br J Nutr* **89**, S39–S43.
3. Ososki AL & Kennely EJ (2003) Phytoestrogens: a review of the present state of research. *Phytother Res* **17**, 845–869.
4. Rowland IR, Faughnan M, Hoey L, Wähälä K, Williamson G & Cassidy A (2003) Bioavailability of phyto-estrogens. *Br J Nutr* **89**, S45–S58.
5. Blakesmith SJ, Lyons-Wall PM, Joannou GE, Petocz P & Samman S (2005) Urinary isoflavonoid excretion is inversely associated with the ratio of protein to dietary fibre intake in young women. *Eur J Nutr* **59**, 284–290.
6. Horner NK, Kristal AR, Prunty J, Skor HE, Potter JD & Lampe JW (2002) Dietary determinants of plasma enterolactone. *Cancer Epidemiol Biomarkers Prev* **11**, 121–126.
7. Coldham NG & Sauer MJ (2001) Identification, quantitation and biological activity of phytoestrogens in a dietary supplement for breast enhancement. *Food Chem Toxicol* **39**, 1211–1224.

8. Heyerick A, Vervarcke S, Depypere HT, Bracke ME & De Keukeleire D (2006) A first prospective, randomized, double-blind, placebo-controlled study on the use of a standardized hop extract to alleviate menopausal discomforts. *Maturitas* **54**, 164–175.
9. Milligan SR, Kalita JC, Heyerick A, Rong H, De Cooman L & De Keukeleire D (1999) Identification of a potent phytoestrogen in hops (*Humulus lupulus* L.) and beer. *J Clin Endocrinol Metab* **83**, 2249–2252.
10. Schaefer O, Hümpel M, Fritzemeier K-H, Bohlmann R & Schleuning W-D (2003) 8-prenylnaringenin is a potent ERα selective phytoestrogen present in hops and beer. *J Steroid Biochem Mol Biol* **84**, 359–360.
11. Possemiers S, Heyerick A, Robbens V, De Keukeleire D & Verstraete W (2005) Activation of pro-estrogens form hops (*Humulus lupulus* L.) by intestinal microbiota; conversion of isoxanthohumol into 8-prenylnaringenin. *J Agric Food Chem* **53**, 6281–6288.
12. Guo J, Nikolic D, Chadwick LR, Pauli GF & van Breemen RB (2006) Identification of human hepatic cytochrome P450 enzymes involved in the metabolism of 8-prenylnaringenin and isoxanthohumol form hops (*Humulus lupulus* L.). *Drug Metab Dispos* **34**, 1152–1159.
13. Nikolic D, Li Y, Chadwick LR, Grubjesic S, Schwab P, Metz P & van Breemen RB (2004) Metabolism of 8-prenylnaringenin, a potent phytoestrogen from hops (*Humulus lupulus*), by human liver microsomes. *Drug Metab Dispos* **32**, 272–279.
14. Stevens JF, Taylor AW & Deinzer ML (1999) Quantitative analysis of xanthohumol and related prenylflavonoids in hops and beer by liquid chromatography-tandem mass spectrometry. *J Chromatogr A* **832**, 97–107.
15. Possemiers S, Bolca S, Grootaert C, Heyerick A, Decroos K, Dhooge W, De Keukeleire D, Rabot S, Verstraete W & Van de Wiele T (2006) Isoxanthohumol from hops (*Humulus lupulus* L.) is activated into the potent phytoestrogen 8-prenylnaringenin *in vitro* and in the human intestine. *J Nutr* **136**, 1862–1867.
16. Schaefer O, Bohlmann R, Schleuning W-D, Schulze-Forster K & Hümpel M (2005) Development of a radioimmunoassay for the quantitative determination of 8-prenylnaringenin in biological matrices. *J Agric Food Chem* **53**, 2881–2889.
17. Rowland IR, Wiseman H, Sanders TAB, Adlercreutz H & Bowey EA (2000) Interindividual variation in metabolism of soy isoflavones and lignans: influence of habitual diet on equol production by the gut microflora. *Nutr Cancer* **36**, 27–32.
18. Cummings JH & Macfarlane GT (1991) The control and consequences of bacterial fermentation in the human colon. *J Appl Bacteriol* **70**, 443–459.
19. Frankenfeld CL, Patterson RE, Horner NK, Neuhouser ML, Skor HE, Kalhorn TF, Howald WN & Lampe JW (2003) Validation of a soy food frequency questionnaire and evaluation of correlates of plasma isoflavone concentrations in postmenopausal women. *Am J Clin Nutr* **77**, 674–680.
20. Soares ACF, Lederman HM, Fagundes-Neto U & de Morais MB (2005) Breath methane associated with slow colonic transit time in children with chronic constipation. *J Clin Gastroenterol* **39**, 512–515.
21. Boeckx P, Van Cleemput O & Villaralvo I (1996) Methane emission from a landfill and the methane oxidising capacity of its covering soil. *Soil Biol Biochem* **28**, 1397–1405.
22. Hoey L, Rowland IR, Lloyd AS, Clarke DB & Wiseman H (2004) Influence of soya-based infant formula consumption on isoflavone and gut microflora metabolite concentrations in urine and on faecal microflora composition and metabolic activity in infants and children. *Br J Nutr* **91**, 607–616.
23. Berg JO, Nord CE & Wadstrom T (1978) Formation of glycosidases in batch and continuous cultures of *Bacteroides fragilis*. *Appl Environ Microbiol* **35**, 269–273.
24. Superior Health Council (2006) *Recommended daily allowances in Belgium: revisions November 2006*, 1st ed. Brussels: Federal Public Service Health, Food Chain Safety Environment.
25. Frankenfeld CL, McTiernan A, Tworoger SS, *et al.* (2004) Serum steroid hormones, sex hormone-binding globulin concentrations and urinary hydroxylated estrogen metabolites in post-menopausal women in relation to daidzein-metabolizing phenotypes. *J Steroid Biochem Mol Biol.* **88**, 399–408.
26. Pollock BG, Wylie M & Stack JA (1999) Inhibition of caffeine metabolism by estrogen replacement therapy in postmenopausal women. *J Clin Pharmacol* **39**, 936–940.
27. Avula B, Ganzera M, Warnick JE, Feltenstein MW, Sufka KJ & Khan IA (2004) High-performance liquid chromatographic determination of xanthohumol in rat plasma, urine and fecal samples. *J Chromatogr Sci* **42**, 378–382.
28. Gerhaüser C, Alt A, Heiss E, *et al.* (2002) Cancer chemopreventive activity of xanthohumol, a natural product derived from hop. *Mol Cancer Ther.* **1**, 959–969.
29. Nikolic D, Li Y, Chadwick LR & van Breemen RB (2006) *In vitro* studies of intestinal permeability and hepatic and intestinal metabolism of 8-prenylnaringenin, a potent phytoestrogen form hops (*Humulus lupulus* L.). *Pharm Res* **23**, 864–872.
30. Rad M, Hümpel M, Schoemaker RC, Schleuning W-D, Cohen AF & Burggraaf J (2006) Pharmacokinetics and systemic endocrine effects of the phyto-oestrogen 8-prenylnaringenin after single oral doses to postmenopausal women. *Br J Clin Pharmacol* **62**, 288–296.
31. Setchell KDR, Brown NM, Desai PB, Zimmer-Nechimias L, Wolfe B, Jakate AS, Creutzinger V & Heubi JE (2003) Bioavailability, disposition, and dose-response effects of soy isoflavones when consumed by healthy women at physiologically typical dietary intakes. *J Nutr* **133**, 1027–1035.
32. Wiseman H, Casey K, Bowey EA, Duffy R, Davies M, Rowland IR, Lloyd AS, Murray A, Thompson A & Clarke DB (2004) Influence of 10 wk of soy consumption on plasma concentrations and excretion of isoflavonoids and on gut microflora metabolism in healthy adults. *Am J Clin Nutr* **80**, 692–699.
33. Xu X, Wang H, Murphy PA, Cook L & Hendrich S (1994) Daidzein is a more bioavailable soymilk isoflavone than is genistein in adult women. *J Nutr* **124**, 825–832.
34. Karr SC, Lampe JW, Huchins AM & Slavin JL (1997) Urinary isoflavonoid excretion in humans is dose dependent at low to moderate levels of soy-protein consumption. *Am J Clin Nutr* **66**, 46–51.
35. Lampe JW, Karr SC, Hutchins AM & Slavin JL (1998) Urinary equol excretion with a soy challenge: influence of habitual diet. *Proc Soc Exp Biol Med* **217**, 335–339.
36. Bond JH, Engel RR & Levitt MD (1971) Factors influencing pulmonary methane excretion in man. *J Exp Med* **133**, 572–588.
37. Miller TL & Wolin MJ (1986) Methanogens in human and animal intestinal tracts. *Syst Appl Microbiol* **7**(2-3), 223–229.
38. Fernandes J, Wolever TMS & Rao AV (2000) Interrelationships between age, total dietary fiber intake and breath methane in humans. *Nutr Res* **20**, 929–940.
39. Calloway DH, Colasito DJ & Mathews RD (1966) Gases produced by human intestinal microflora. *Nature* **212**, 1238–1239.
40. Frankenfeld CL, Atkinson C, Thomas WK, Goode EL, Gonzalez A, Jokela T, Wähälä K, Schwartz SM, Li SS & Lampe JW (2004) Familial correlations, segregation analysis, and nongenetic correlates of soy isoflavone-metabolizing phenotypes. *Exp Biol Med* **229**, 902–913.
41. LeMarchand L, Franke AA, Custer LJ, Wilkens LR & Cooney RV (1997) Lifestyle and nutritional correlates of cytochrome CYP1A2 activity: inverse associations with plasma lutein and alpha-tocopherol. *Pharmacogenetics* **7**, 11–19.

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42. Rizzo N, Hispard E, Dolbeault S, Dally S, Leverge R & Girre C (1997) Impact of long-term ethanol consumption on CYP1A2 activity. *Clin Pharmacol Ther* **62**, 505–509.
43. Higdon JV & Frei B (2006) Coffee and health: a review of recent human research. *Crit Rev Food Sci Nutr* **46**, 101–123.
44. Gates S & Miners O (1999) Cytochrome P450 isoform selectivity in human hepatic theobromine metabolism. *J Clin Pharmacol* **47**, 299–305.
45. Lampe JW, Skor HE, Wähälä K, Howald WN & Chen C (2001) Wheat bran and soy protein feeding do not alter urinary excretion of the isoflavan equol in premenopausal women. *J Nutr* **131**, 740–744.
46. Vedrine N, Mathey J, Morand C, Brandolini M, Davicco MJ, Guy L, Remesy C, Coxam V & Manach C (2006) One-month exposure to soy isoflavones did not induce the ability to produce equol in postmenopausal women. *Eur J Clin Nutr* **60**, 1039–1045.
47. Batterham TJ, Shutt DA, Hart NK, Braden AWH & Tweeddale HJ (1971) Metabolism of intraruminally administered [4-^{14}C]formononetin and [4-^{14}C]biochanin A in sheep. *Aust J Agric Res* **22**, 131–138.
48. Lundh TJO, Pettersen H & Kiessling K (1988) Demethylation and conjugation of formononetin and daidzein in sheep and cow liver microsomes. *J Agric Food Chem* **36**, 22–25.
49. Schmider J, Greenblatt DJ, Von Moltke LL, Karsov D, Vena R, Friedman HL & Shader RI (1997) Biotransformation of mestranol to ethinyl estradiol *in vitro*: the role of cytochrome P-450 2C9 and metabolic inhibitors. *J Clin Pharmacol* **37**, 193–200.
50. Milligan SR, Kalita JC, Pocock V, Heyerick A, De Cooman L, Rong H & De Keukeleire D (2002) Oestrogenic activity of the hop phyto-oestrogen, 8-prenylnaringenin. *Reproduction* **123**, 235–242.

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