

# Nutrient Physiology, Metabolism, and Nutrient-Nutrient Interactions

## The Prenylflavonoid Isoxanthohumol from Hops (*Humulus lupulus* L.) Is Activated into the Potent Phytoestrogen 8-Prenylnaringenin In Vitro and in the Human Intestine<sup>1</sup>

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**ABSTRACT** Hops, an essential beer ingredient, are a source of prenylflavonoids, including 8-prenylnaringenin (8-PN), one of the most potent phytoestrogens. Because 8-PN concentrations in beers are generally low, its health effects after moderate beer consumption were considered negligible. However, human intestinal microbiota may activate up to 4 mg/L isoxanthohumol (IX) in beer into 8-PN. Depending on interindividual differences in the intestinal transformation potential, this conversion could easily increase the 8-PN exposure 10-fold upon beer consumption. Here, we present a further investigation of the process both in vitro and in vivo. In vitro experiments with the dynamic SHIME model showed that hop prenylflavonoids pass unaltered through the stomach and small intestine and that activation of IX into 8-PN (up to 80% conversion) occurs only in the distal colon. In vitro incubations of 51 fecal samples from female volunteers with IX enabled us to separate the fecal microbiota into high (8 of 51), moderate (11 of 51) and slow (32 of 51) 8-PN producers, clearly illustrating an interindividual variability. Three women, selected from the respective groups, received a daily dose of 5.59 mg IX for 4 d. Intestinal IX activation and urinary 8-PN excretion were correlated ( $R^2 = 0.6417$ ,  $P < 0.01$ ). These data show that intestinal conversion of IX upon moderate beer consumption can lead to 8-PN exposure values that might fall within the range of human biological activity. *J. Nutr.* 136: 1862–1867, 2006.

**KEY WORDS:** • phytoestrogens • hops • 8-prenylnaringenin • intestinal bacteria • SHIME

Phytoestrogens are plant constituents that structurally or functionally mimic female estrogens. Some display selective estrogen receptor modulating activity and could therefore play a beneficial role in the prevention of osteoporosis, menopausal complaints, or cancers (1). Multiple studies now indicate negative side effects of hormone replacement therapy (HRT)<sup>3</sup> (2,3), an effective method for treating various menopausal

symptoms (4,5). Because there is a rapidly growing body of literature on the health benefits of phytoestrogens, many women now view them as a more natural or “herbal” alternative to HRT (6,7).

For centuries, hops (*Humulus lupulus* L.) were used primarily as an essential ingredient in the beer brewing process. Yet, hops have been studied since 1953 as a possible source of estrogenically active compounds (8), and 8-prenylnaringenin (8-PN) was identified as a very potent phytoestrogen (9). In vitro and animal data suggest that 8-PN might exhibit several biological activities (10–14), and hop-containing dietary supplements are marketed to reduce menopausal complaints and used for breast enhancement (15).

An important factor influencing the bioavailability and activity of phytoestrogens is their metabolic fate upon ingestion. In general, after reaching the colon, flavonoids are partially degraded, depending on their structure, thereby leading to lower bioavailability (16). However, microbial transformation

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<sup>3</sup> Abbreviations used: BHI, brain heart infusion; HRT, hormone replacement therapy; IX, isoxanthohumol; 8-PN, 8-prenylnaringenin; SCFA, short-chain fatty acids; SHIME, simulator of the human intestinal microbial ecosystem; X, xanthohumol.

in the colon may also increase the biological activity of the ingested compounds, a process that has been described for different phytoestrogens (17). For hop prenylflavonoids, a similar phenomenon was observed recently. Isoxanthohumol (IX) is the prevailing prenylflavonoid in beer and is 10–30 times more abundant as 8-PN (18). Recently, Schaefer et al. (19) noted the activation of IX into the phytoestrogen 8-PN inside the human body. Our previous research indicated that the intestinal microbial community might be responsible for this production of 8-PN after IX consumption (20).

Up to now, hop prenylflavonoids were not considered to be relevant phytoestrogens in the human diet because 8-PN concentrations in beer were considered too low to affect human health. However, microbial *O*-demethylation of IX in the human intestine could readily increase intestinal 8-PN concentrations 10-fold (20), leading to the uptake of active estrogen doses after moderate beer consumption that might fall within the range of biological activities (21). Therefore, the importance of this microbial process was further investigated in vitro in the Simulator of the Human Intestinal Microbial Ecosystem (SHIME), in which the transformation of IX into 8-PN was studied in the different parts of the human gut. Based on 51 fecal samples, interindividual differences were assessed and a small in vivo trial was set up to relate the in vitro activation data with the excretion of 8-PN after IX consumption.

## MATERIALS AND METHODS

### Chemicals

The isolation of xanthohumol (X) from spent hops, isomerization of X into IX, and chemical synthesis of 8-PN were performed as described earlier by Possemiers et al. (20). Stock solutions of the different prenylflavonoids (X, IX, 8-PN) were prepared in dimethyl sulfoxide at a concentration of 5 g/L.

### Simulator of the Human Intestinal Microbial Ecosystem (SHIME)

**Experimental design.** The reactor set-up was adapted from the SHIME, representing the different parts of the adult human gut (22), and consisting of a succession of 5 reactors. The first 2 reactors were based on the fill-and-draw principle to simulate different steps in food uptake and digestion, with peristaltic pumps adding a defined amount of SHIME feed (140 mL, 3 times/d) and pancreatic and bile liquid (60 mL, 3 times/d), respectively, to the stomach (V1) and duodenum (V2) compartment and emptying the respective reactors after specified intervals. The last 3 compartments were continuously stirred reactors with constant volume and pH control, specific for each compartment. Upon inoculation with fecal microbiota, these reactors simulate the ascending (V3), transverse (V4), and descending (V5) colon. Inoculum preparation, retention time, pH, temperature settings, and reactor feed composition were described previously (23). The fecal sample to start this SHIME run was derived from an individual who had an IX-converting intestinal community. After reactor start-up, the system was allowed to stabilize for 3 wk before the start of the experiment (23).

**Stability in the upper intestine.** To test the stability of IX, X, and 8-PN in the upper intestine, the compounds were added to the SHIME feed (in 3 separate experiments) and transferred through the system toward the first colon compartment as described previously (23). Samples were taken before and after each incubation step. The specific cyclization of X to form IX was also monitored in water containing 0.05% HCl (pH 2) and incubation at 37°C for 1 h.

**Batch experiments.** All fermentation experiments were performed in Brain Heart Infusion (BHI) broth (37 g/L, Oxoid) with 0.5 g/L L-cysteine-HCl under anaerobic conditions, according to Possemiers et al. (20). Three weeks after the inoculation of the SHIME system, a 5-mL sample was withdrawn from SHIME reactors 3, 4, and 5 and

transferred to penicillin flasks containing 45 mL BHI. At the start of the incubation, ~70  $\mu\text{mol/L}$  of X, IX, or 8-PN (5  $\mu\text{L}$  stock solution/mL batch culture) was added to each flask. Cultures were incubated at 37°C for 6 d. At the start and after 1, 2, 3, and 6 d, samples were taken using syringes. All experiments were performed in triplicate.

**SHIME run.** The SHIME experiment consisted of 3 phases: a 2-wk control period in which standard feed was dosed to the SHIME, a treatment period (d 15–29) in which 70  $\mu\text{mol/L}$  IX was supplemented to the SHIME feed, and, finally, a 1-wk wash-out period in which the SHIME was fed again with standard SHIME feed. During these phases, samples from the different colon reactors were taken at different time intervals and analyzed for prenylflavonoids, short-chain fatty acids, and microbial community composition.

**HPLC analysis.** The prenylflavonoids were extracted by liquid-liquid extraction using ethyl acetate (20). Quantitative analyses were effected by HPLC using a Waters 2695 Alliance Separations Module (Waters) equipped with a Waters 996 Photodiode Array Detector and Waters Millennium Software v3.20 (20). An extra confirmation of the identity of the compounds in the samples was achieved based on the typical fragmentation patterns in LC-MS-MS analysis using a Waters Quattro Micro instrument with electrospray ionization (24).

**In Vitro estrogenicity testing.** The estrogenic properties of the prenylflavonoids were assessed using a receptor bioassay based on a genetically modified *Saccharomyces cerevisiae*, according to the protocol developed by Routledge and Sumpter (25). Estrogenic activity was quantified through  $\beta$ -galactosidase expression in the yeast and the conversion of chlorophenol red- $\beta$ -D-galactopyranoside into chlorophenol red, measured at 540 nm. Dose-response curves for  $\beta$ -galactosidase activity were calculated using SigmaPlot (v4, SPSS) by a 4-parameter logistic regression using the Marquardt-Levenberg algorithm (26).

**Evaluation of microbial activity and structure.** Liquid samples (10 mL) from each colon reactor were collected and frozen at  $-20^\circ\text{C}$  for subsequent analysis. Microbial activity was monitored by its short-chain fatty acid (SCFA) production. The SCFA were extracted from the samples with diethyl ether and determined with a Di200 GC (Shimadzu) (27).

The microbial structure of the SHIME community was determined by real-time PCR. Total DNA extractions of 1-mL liquid SHIME samples were performed using the method described by Boon et al. (28). Using an ABI Prism SDS 7000 instrument (Applied Biosystems) and the qPCR<sup>T</sup> core kit for Sybr<sup>T</sup> Green I (Eurogentec), 10 times diluted general bacterial DNA as well as specific DNA from *Lactobacillus* spp., *Bifidobacterium* spp., *Atopobium* spp., and the *Clostridium coccoides-Eubacterium rectale* group, were quantified by real-time PCR. The 16S rRNA genes for all members of the bacteria were amplified using primer PRBA338f and P518r (28). The primer sets, annealing temperature, and Mg concentration used to quantify DNA of specific groups were described by Rintilä et al. (29). Their PCR protocol was adapted as follows: 2 min at 50°C, 5 min at 95°C and 40 cycles of 20 s denaturation at 94°C, 30 s primer annealing at the specific temperature, and 1 min primer extension at 60°C. Standard curves were constructed with DNA from representative species of the different groups in a concentration range from  $10^{10}$  to  $10^2$  DNA copies/ $\mu\text{L}$  and DNA from at least 10 nontarget species was used as a negative control to test amplification specificity. All reactions were specific for target species.

### Interindividual differences in the conversion of isoxanthohumol

**Study design.** Healthy women ( $n = 51$ ; 24–55 y old) participated voluntarily in this study. None of the women had a history of gastrointestinal disease and had not taken antibiotics during the 3 mo before the study. All participants delivered a fecal sample for incubation purposes. Samples were homogenized in phosphate buffer (20% wt:v) and incubated in triplicate in BHI broth (10% v:v) with 70  $\mu\text{mol/L}$  IX for 72 h according to Possemiers et al. (20); the conversion of IX into 8-PN was assessed.

Based on the results of this in vitro study, 3 participants, designated A, B, and C, were selected (aged 24, 25, and 26 y with BMI of 21, 18, and 21  $\text{kg/m}^2$ , respectively). Before the study, the participants delivered a fresh fecal sample, which was incubated for 13 d; samples

were taken at the start and after 3, 8, and 13 d. Then, for 4 consecutive days, these participants ingested each morning a capsule containing  $5.59 \pm 0.97$  mg IX and cellulose. Subjects consumed their habitual diets but were asked not to drink any beer at least 5 d before and during the study. The study was given ethical approval by the Ethics Committee of the Ghent University Hospital (EC UZG 2005/022) and written informed consent was obtained from each participant.

**Sample collection and preparation.** Blank urine samples were collected before the study and participants made four 24-h urine collections during the study. The urine volumes were quantified and aliquots were stored at  $-20^{\circ}\text{C}$ . Then, 15 mL sodium acetate buffer (0.1 mol/L, pH 5.0) and  $300 \mu\text{L}$   $\beta$ -glucuronidase/arylsulfatase (Sigma Aldrich) were added to a 15-mL sample. The samples were incubated for 1 h at  $37^{\circ}\text{C}$ , followed by solid-phase extraction with a C18 silica columns (Bond Elut<sup>®</sup>, Varian) and methanol. The solvent was evaporated under nitrogen gas and the residue was redissolved in  $100 \mu\text{L}$  methanol. Recoveries of IX and 8-PN were  $99.7 \pm 7$  and  $99.4 \pm 6\%$ , respectively.

### Statistical analysis

Normality of the data and equality of the variances were assessed using the Kolmogorov-Smirnov test and the Hartley test, respectively. Comparison of means on nonnormally distributed data were evaluated with the nonparametric Kruskal-Wallis test. Comparison of normally distributed data was performed with ANOVA; when ANOVA indicated significant differences, means were compared using the Student-Newman-Keuls multiple comparison test. Comparison of the SHIME IX concentrations with the theoretical mean IX concentration was performed with a 1-sample Student's *t* test. The incubation data from the 51 fecal samples were separated into different groups of 8-PN production levels using the 2-step cluster analysis protocol. Differences were considered significant at  $P < 0.01$ . Calculations were performed using the Statview software (version 5.0, SAS Institute).

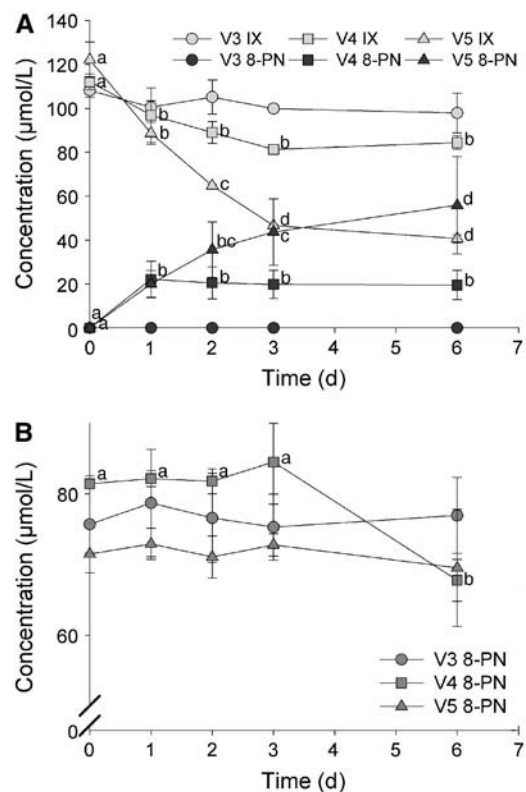
## RESULTS

### Transformation of hop prenylflavonoids in the SHIME

**Stability in the upper intestinal tract.** None of the compounds was affected (ANOVA,  $P > 0.01$ ) during the passage through the upper gut, indicating that, without taking absorption from the small intestine into account, IX, X, and 8-PN reach the large bowel unaltered upon oral ingestion.

**Intestinal transformation of IX and 8-PN.** In the ascending colon compartment (V3), no conversion occurred (Fig. 1A). In the distal colon parts (V4 and V5), however, transformation of IX occurred ( $P < 0.001$ ) with a conversion of  $20 \pm 8$  and  $46 \pm 23\%$ , respectively, of the incubated IX into 8-PN after 6 d. For this specific microbial community, almost no degradation of 8-PN occurred (Fig. 1B). Only in the transverse colon, degradation of  $16 \pm 3\%$  of the originally incubated 8-PN ( $P < 0.01$ ) occurred after long-term incubation.

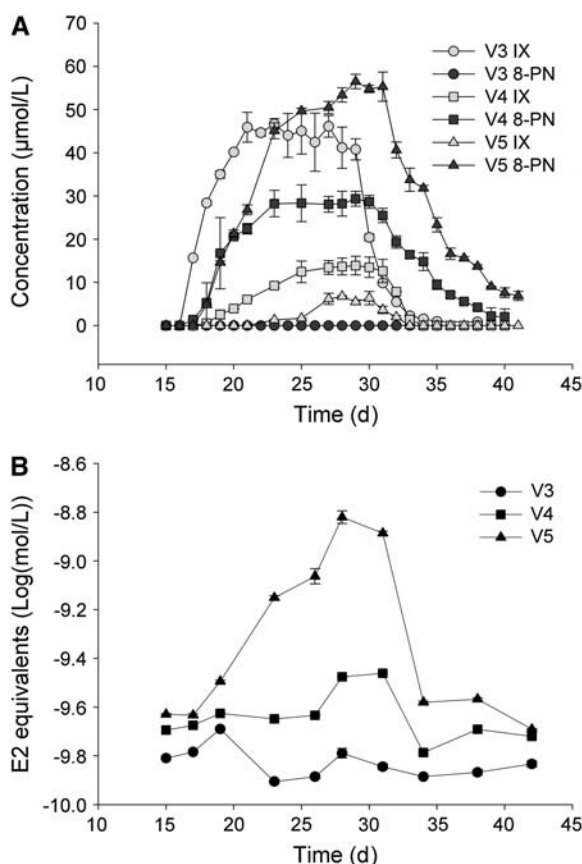
**Activation of IX into 8-PN in the SHIME.** During the treatment period, the IX concentration in the first colon vessel increased, approaching a steady-state concentration of  $\sim 45 \mu\text{mol/L}$  (Fig. 2A) and no conversion of IX into 8-PN occurred in this part. Through the overflow connection between V3 and V4, the IX concentration in the simulated transverse colon also rapidly reached a steady-state concentration of  $\sim 14 \mu\text{mol/L}$ . However, this concentration was much lower than that of V3 because IX was partially converted into 8-PN ( $\pm 29 \mu\text{mol/L}$ ). Finally, in the simulated descending colon, very low IX ( $\pm 6 \mu\text{mol/L}$ ) and high 8-PN concentrations ( $\pm 56 \mu\text{mol/L}$ ) were recovered. When IX supplementation was stopped, the 8-PN concentration decreased at a slower rate than the IX concentration in both V4 and V5.



**FIGURE 1** Conversion of IX into 8-PN (A) and degradation of 8-PN (B) in samples taken from the simulated ascending (V3), transverse (V4), and descending (V5) colon of the SHIME (batch experiment). Values are means  $\pm$  SD,  $n = 3$ . Data were analyzed by ANOVA for repeated measurements, followed by the Student-Newman-Keuls multiple comparison test. For each curve, means without a common letter differ,  $P < 0.01$ .

To evaluate whether the increase in 8-PN conversion toward the distal end of the colon also led to a parallel increase of the estrogenic properties of the SHIME matrix, filter-sterile SHIME supernatant was tested in the yeast estrogen screen (Fig. 2B). A transient increase in the estrogenic properties of V4 and especially V5 was noted, and after the wash-out period, the estrogenic values were similar to the values at the start of the experiment. Although a fraction of the 8-PN was lost due to the filter sterilization, the yeast estrogen screen data were correlated with the HPLC data ( $R^2 = 0.8156$ ,  $P < 0.001$ ), showing that at least a fraction of the 8-PN produced was available for biological activity.

Taking into account the mass balance of incoming and outgoing IX concentrations, the theoretical IX concentrations in the respective colon compartments of the SHIME were calculated over the course of the IX supplementation and wash-out periods. Conversion of IX into 8-PN was not considered and the theoretical steady-state IX concentration in each colon compartment was  $45 \mu\text{mol/L}$ . The sum of the IX and 8-PN concentrations in the ascending and transverse colon compartments corresponded to the theoretical IX concentrations (Student's *t* test,  $P > 0.01$ ), indicating that IX was converted only to 8-PN and that the latter was not further degraded by the colon microbiota. In the descending colon, however, the sum of the IX and 8-PN concentrations was higher than the theoretical IX concentration (Student's *t* test,  $P < 0.01$ ), indicating an accumulation in the latter colon compartment. These data showed that IX was converted microbially to 8-PN for 65% conversion in the transverse and 85% in the descending colon compartment.

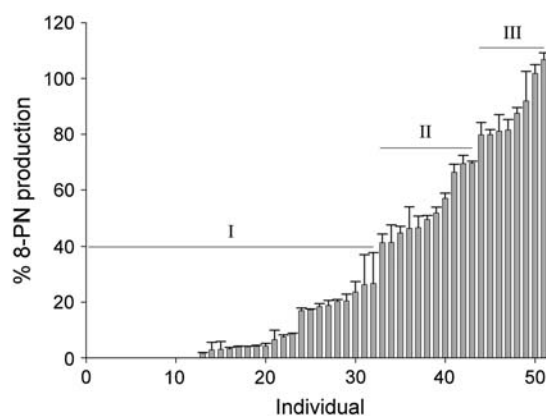


**FIGURE 2** Conversion of IX into 8-PN in the simulated ascending (V3), transverse (V4) and descending (V5) colon of the SHIME (continuous experiment) after IX was fed from d 15 to 29. Concentrations of IX and 8-PN (A) and as equivalent concentrations of  $17\beta$ -estradiol after quantification of the estrogenic properties of the SHIME supernatant (B) are shown. Values are means  $\pm$  SD,  $n = 3$ . The increase in estrogenic properties of the different SHIME compartments was correlated with the increase in 8-PN concentrations in the respective vessels ( $R^2 = 0.8156$ ,  $P < 0.001$ ).

**Effect on microbial community activity and structure.** In V3, only acetic (25 mmol/L), butyric (2 mmol/L), and propionic acid (7 mmol/L) were produced. In V4 and V5, next to acetic (37 and 45 mmol/L, respectively), butyric (6 mmol/L) and propionic acid (14 mmol/L) and small amounts of isobutyric, isovaleric, and valeric acid were recovered in the range of 2 mmol/L. However, all fatty acid concentrations remained constant throughout the experimental period (42 d), indicating no changes in the microbial activity due to the supplementation of IX (ANOVA for repeated measurements,  $P > 0.01$ ). Although the abundance of *Lactobacillus* spp. and *Atopobium* spp. remained constant in the range of 1–5%, an increase ( $P < 0.01$ ) in the abundance of the *C. coccoides*-*E. rectale* group and *Bifidobacterium* spp. from ~5 to 20% was noted in the transverse and descending colon.

#### Interindividual differences in the conversion of IX into 8-PN

**Incubation of fecal bacteria with IX.** The 51 fecal samples were incubated with IX immediately after defecation, and 8-PN production was monitored after 72 h (Fig. 3). Three different ( $P < 0.01$ ) groups were formed, separating individuals in high



**FIGURE 3** Conversion of IX into 8-PN by intestinal bacteria from 51 different human individuals. The individuals were arranged by increasing 8-PN production. Values are means  $\pm$  SD,  $n = 3$ . The 3 groups (designated I, II and III) with significantly different means ( $P < 0.01$ , Kruskal-Wallis) were identified by 2-step cluster analysis.

(8/51), moderate (11/51), and slow (32/51) IX converters, with a mean 8-PN production of 78.8, 48.5, and 6.9%, respectively.

Based on these results, high, moderate, and slow IX-converting individuals ( $n = 1$  each) were selected (designated A, B and C) and new fecal samples were incubated (Table 1). This confirmed that individual A rapidly converted all incubated IX into 8-PN, whereas the microbial community from person B activated IX only partially and IX remained unaltered in incubation C throughout the entire incubation period.

**Recovery of IX and 8-PN in urine.** Although only IX was consumed, high amounts of 8-PN (190  $\mu$ g/d on d 4) were detected in the urine of individual A (Table 1). Based on the quantification of IX in the urine, 8-PN constituted  $13\% \pm 2$  of the total amount of prenylflavonoids excreted in urine on d 1 up to  $41 \pm 1\%$  on d 4. In contrast, much lower 8-PN ratios were recovered in the urine of subjects B (on d 3 and 4) and C (only on d 4). Clearly, in parallel with the *in vitro* incubations, interindividual differences determine the activation of IX, as quantified by the 8-PN excretion. Moreover, because the

**TABLE 1**

*Relation between in vitro IX conversion in fecal samples and in vivo exposure to 8-PN after 4 d of IX consumption (5.59 mg/d) by 3 individuals*

	Time, d	Individual		
		A	B	C
Fecal data <sup>2</sup>	3	100.0 $\pm$ 5.4	7.8 $\pm$ 1.3	ND <sup>1</sup>
	8	100.0 $\pm$ 0.9	30.8 $\pm$ 1.7	ND
	13	100.0 $\pm$ 0.7	39.7 $\pm$ 5.1	ND
Urinary excretion <sup>3,4</sup>	1	13.1 $\pm$ 2.5	ND	ND
	2	23.1 $\pm$ 0.1	ND	ND
	3	19.4 $\pm$ 0.2	8.4 $\pm$ 0.4	ND
	4	40.6 $\pm$ 0.6	13.9 $\pm$ 2.1	4.5 $\pm$ 0.8

<sup>1</sup> ND, 8-PN not detected.

<sup>2</sup> Fecal data are presented as the mean  $\pm$  SD % conversion of IX into 8-PN,  $n = 3$ .

<sup>3</sup> In the 24-h-pooled urine samples, the ratio  $\pm$  SD of 8-PN relative to IX + 8-PN was quantified,  $n = 3$ .

<sup>4</sup> The urinary excretion of 8-PN was correlated with the intestinal microbial activation potential of IX, as expressed by the percentage of 8-PN production in the fecal samples on d 13 ( $R^2 = 0.6417$ ,  $P < 0.01$ ).

highest IX-converting fecal sample was derived from individual A with the highest urinary 8-PN excretion, the in vitro and in vivo data were correlated ( $R^2 = 0.6417$ ,  $P < 0.01$ ), indicating the importance of the microbial community in this process.

## DISCUSSION

Using the SHIME, it was shown that IX and other prenylflavonoids remained unaltered after passage through the stomach and small intestine. The microbial transformations in the colon were site specific because activation of IX into 8-PN occurred only in the distal parts with up to 80% conversion. Analysis of 51 fecal samples separated individuals into high, moderate, and slow IX converters. Because only one-third of the individuals were identified as moderate or high 8-PN producers and because a small in vivo trial indicated a clear relation between urinary 8-PN excretion and the intestinal microbial transformation potential of IX, interindividual differences in microbial IX transformation potential may have a crucial effect on 8-PN exposure after IX consumption.

In general, monomeric flavonoids reach the small intestine unaltered (30). Our results confirm these data because IX, 8-PN, and X were fully recovered after passage through the simulated stomach and small intestine. However, Nikolic et al. (31) reported a cyclization of X into IX in 0.05% HCl following a first-order kinetic, indicating that X may also act as a proestrogen due to transformation into IX in the acid stomach. Because X was neither transformed into IX in our simulated stomach nor in a repetition of the latter researcher's experiment, X was not considered further in this research.

The SHIME is an ideal model with which to study the activation of IX into 8-PN, allowing us to study the microbial community and its activity in great detail in the different parts of the intestine that differ community structure and activity (22,23,32). The conversion of IX was limited to the simulated transverse and descending colon, leading to a strong increase in estrogenic properties. Schaefer et al. (19) noted that urinary 8-PN excretion after beer consumption was slower than expected (up to several days). Our data indicate that this is probably due to delayed conversion of IX into 8-PN because beer is an important source of IX and the ingested IX first has to reach the distal colon, possibly after absorption and enterohepatic recirculation, a transfer that can take up to 48 h (33).

Because the uptake of prenylflavonoids may also affect the intestinal microbiota themselves, shifts in the community structure or activity during the SHIME experiments were investigated. No changes in SCFA profiles were noted, but a relative increase in the *C. coccoides-E. rectale* cluster and *Bifidobacterium* spp. was noted. Although further research is necessary, the mentioned cluster contains a number of homoacetogenic bacteria with enzyme systems for the demethylation of methoxylated phenols (34). Furthermore, 8-PN exerted antibacterial activity in vitro (data not shown), indicating that differences in sensitivity to 8-PN could explain possible shifts in community structure.

Simons et al. (16) studied the degradation of different flavonoids by intestinal bacteria and separated the subjects into high, moderate, and slow degraders. The statistical analysis of the 51 fecal samples indicates that this separation is also important for the demethylation of IX. Because only one-third of the subjects could produce moderate or high amounts of 8-PN, an important part of the population would not benefit from this reaction. In parallel with the noted interindividual differences in the conversion of daidzein into equol (17), these results stress the importance of the gut microbial community toward

the final biological activity of phytoestrogens, a factor that should not be neglected when determining the final active dose inside the body.

Based on the in vitro data concerning interindividual variability, a preliminary in vivo trial was designed as proof of principle of the importance of the gut microbiota toward in vivo exposure to 8-PN. One individual from each of the high, moderate, and low IX-converting groups, was administered IX for 4 d, and a fresh stool sample was obtained to confirm their intestinal transformation potential. The excretion of 8-PN was significantly correlated with the in vitro data from the fecal samples. Nevertheless, the small size of this trial and the careful selection of the individuals based on the in vitro data encourage further research in this domain because the importance of the microbial activation reaction was further confirmed.

Until recently, possible health effects after moderate beer consumption related to the presence of 8-PN were considered negligible because concentrations in beers are generally very low. Furthermore, for hop-containing supplements, only the 8-PN content is taken into account. This study shows that this approach may no longer be valid because IX can act as a proestrogen in beers and supplements. Because woman A excreted up to 40% 8-PN after IX consumption, even a 50% loss in the feces could still lead to an 8-PN exposure in the range of 1–2 mg/d after daily moderate beer consumption; this could fall within the range of biological activity. Further in vivo trials are being conducted to evaluate the possible health effects of moderate beer consumption or supplements.

We conclude that human exposure to the biologically active 8-PN depends not only on the initial 8-PN concentrations in beers but also on the combination of exposure to IX and the microbial bioactivation potential in the gut.

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## LITERATURE CITED

- Ososki AL, Kennelly EJ. Phytoestrogens: a review of the present state of research. *Phytother Res*. 2001;17:845–69.
- Beral V; Million Women Study Collaborators. Breast cancer and hormone-replacement therapy in the Million Women Study. *Lancet*. 2003;362:419–27.
- Rossouw JE, Anderson GL, Prentice RL, LaCroix AZ, Kooperberg C, Stefanick ML, Jackson RD, Beresford SAA, Howard BV, et al. Risks and benefits of estrogen plus progestin in healthy postmenopausal women—principal results from the Women's Health Initiative randomized controlled trial. *JAMA*. 2002;288:321–33.
- Wagner JD, Anthony MS, Cline JM. Soy phytoestrogens: research on benefits and risks. *Clin Obstet Gynecol*. 2001;44:843–52.
- Brzezinski A, Debi A. Phytoestrogens: the "natural" selective estrogen receptor modulators? *Eur J Obstet Gynecol Reprod Biol*. 1999;85:47–51.
- Gainford MC, Simmons C, Nguyen H, Verma S, Clemons M. A practical guide to the management of menopausal symptoms in breast cancer patients. *Support Care Cancer*. 2005;13:573–8.
- MacGregor CA, Canney PA, Patterson G, McDonald R, Paul J. A randomised double-blind controlled trial of oral soy supplements versus placebo for treatment of menopausal symptoms in patients with early breast cancer. *Eur J Cancer*. 2005;41:708–14.
- Koch W, Heim G. Estrogens in hops and beer; preliminary report. *Munch Med Wochenschr*. 1953;95:845.
- Milligan SR, Kalita JC, Heyerick A, Rong H, De Cooman L, De Keukeleire D. Identification of a potent phytoestrogen in hops (*Humulus lupulus* L.) and beer. *J Clin Endocrinol Metab*. 1999;84:2249–52.
- Effenberger KE, Johnsen SA, Monroe DG, Spelsberg TC, Westendorf JJ. Regulation of osteoblastic phenotype and gene expression by hop-derived phytoestrogens. *J Steroid Biochem Mol Biol*. 2005;96:387–99.
- Hümpel M, Isaksson P, Schaefer O, Kaufmann U, Ciana P, Maggic A, Schlemming WD. Tissue specificity of 8-prenylnaringenin: protection from ovariectomy induced bone loss with minimal trophic effects on the uterus. *J Steroid Biochem Mol Biol*. 2005;97:299–305.

12. Diel P, Thomae RB, Caldarelli A, Zierau O, Kolba S, Schmidt S, Schwab P, Metz P, Vollmer G. Regulation of gene expression by 8-prenylnaringenin in uterus and liver of Wistar rats. *Planta Med.* 2004;70:39–44.
13. Pepper MS, Hazel SJ, Humpel M, Schleuning WD. 8-Prenylnaringenin, a novel phytoestrogen, inhibits angiogenesis in vitro and in vivo. *J Cell Physiol.* 2004;199:98–107.
14. Zierau O, Morrissey C, Watson RWG, Schwab P, Kolba S, Metz P, Vollmer G. Antiandrogenic activity of the phytoestrogens naringenin, 6-(1,1-dimethylallyl)naringenin and 8-prenylnaringenin. *Planta Med.* 2003;69:856–8.
15. Coldham NG, Sauer MJ. Identification, quantitation and biological activity of phytoestrogens in a dietary supplement for breast enhancement. *Food Chem Toxicol.* 2001;39:1211–24.
16. Simons AL, Renouf M, Hendrich S, Murphy PA. Human gut microbial degradation of flavonoids: structure-function relationships. *J Agric Food Chem.* 2005;53:4258–63.
17. Rowland I, Wiseman H, Sanders T, Adlercreutz H, Bowey E. Metabolism of oestrogens and phytoestrogens: role of the gut microflora. *Biochem Soc Trans.* 1999;27:304–8.
18. Stevens JF, Taylor AW, Deinzer ML. Quantitative analysis of xanthohumol and related prenylflavonoids in hops and beer by liquid chromatography tandem mass spectrometry. *J Chromatogr A.* 1999;832:97–107.
19. Schaefer O, Bohlmann R, Schleuning WD, Schulze-Forster K, Humpel M. Development of a radioimmunoassay for the quantitative determination of 8-prenylnaringenin in biological matrices. *J Agric Food Chem.* 2005;53:2881–9.
20. Possemiers S, Heyerick A, Robbens V, De Keukeleire D, Verstraete W. Activation of proestrogens from hops (*Humulus lupulus* L.) by intestinal microbiota; conversion of isoxanthohumol into 8-prenylnaringenin. *J Agric Food Chem.* 2005;53:6281–8.
21. Prestwood KM, Kenny AM, Kleppinger A, Kulldorff M. Ultralow-dose micronized 17 beta-estradiol and bone density and bone metabolism in older women—a randomized controlled trial. *JAMA.* 2003;290:1042–8.
22. Molly K, Woestyne MV, Verstraete W. Development of a 5-step multichamber reactor as a simulation of the human intestinal microbial ecosystem. *Appl Microbiol Biotechnol.* 1993;39:254–8.
23. Possemiers S, Verthé K, Uyttendaele S, Verstraete W. PCR-DGGE-based quantification of stability of the microbial community in a simulator of the human intestinal microbial ecosystem. *FEMS Microbiol Ecol.* 2004;49:495–507.
24. Stevens JF, Ivancic M, Hsu VL, Deinzer ML. Prenylflavonoids from *Humulus lupulus*. *Phytochemistry.* 1997;44:1575–85.
25. Routledge EJ, Sumpter JP. Estrogenic activity of surfactants and some of their degradation products assessed using a recombinant yeast screen. *Environ Toxicol Chem.* 1996;15:241–8.
26. De Boever P, Demare W, Vanderperren E, Cooreman K, Bossier P, Verstraete W. Optimization of a yeast estrogen screen and its applicability to study the release of estrogenic isoflavones from a soygerm powder. *Environ Health Perspect.* 2001;109:691–7.
27. Nollet L, VandeVelde I, Verstraete W. Effect of the addition of *Peptostreptococcus productus* ATCC35244 on the gastro-intestinal microbiota and its activity, as simulated in an in vitro simulator of the human gastro-intestinal tract. *Appl Microbiol Biotechnol.* 1997;48:99–104.
28. Boon N, Top EM, Verstraete W, Siciliano SD. Bioaugmentation as a tool to protect the structure and function of an activated-sludge microbial community against a 3-chloroaniline shock load. *Appl Environ Microbiol.* 2003;69:1511–20.
29. Rinttila T, Kassinen A, Malinen E, Krogus L, Palva A. Development of an extensive set of 16S rDNA-targeted primers for quantification of pathogenic and indigenous bacteria in faecal samples by real-time PCR. *J Appl Microbiol.* 2004;97:1166–77.
30. Spencer JPE. Metabolism of tea flavonoids in the gastrointestinal tract. *J Nutr.* 2003;133:3255S–61.
31. Nikolic D, Li Y, Chadwick LR, Pauli GF, van Breemen RB. Metabolism of xanthohumol and isoxanthohumol, prenylated flavonoids from hops (*Humulus lupulus* L.), by human liver microsomes. *J Mass Spectrom.* 2005;40:289–99.
32. De Boever P, Deplancke B, Verstraete W. Fermentation by gut microbiota cultured in a simulator of the human intestinal microbial ecosystem is improved by supplementing a soygerm powder. *J Nutr.* 2000;130:2599–606.
33. Cummings JH. The large intestine in health and disease. Brussels: Institut Danone; 1997.
34. Diekert G, Wohlfarth G. Metabolism of homoacetogens. *Antonie Van Leeuwenhoek.* 1994;66:209–21.