

## Activation of Proestrogens from Hops (*Humulus lupulus* L.) by Intestinal Microbiota; Conversion of Isoxanthohumol into 8-Prenylnaringenin

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Hop, an essential ingredient in most beers, contains a number of prenylflavonoids, among which 8-prenylnaringenin (8-PN) would be the most potent phytoestrogen currently known. Although a number of health effects are attributed to these compounds, only a few reports are available about the bioavailability of prenylflavonoids and the transformation potency of the intestinal microbial community. To test these transformations, four fecal samples were incubated with xanthohumol, isoxanthohumol (IX), and 8-PN. Upon incubation with IX, present in strong ales up to 4 mg/L, 36% was converted into 8-PN in one fecal sample and the estrogenic properties of the sample drastically increased. In an experiment with 12 fecal cultures, this conversion was observed in one-third of the samples, indicating the importance of interindividual variability in the intestinal microbial community. *Eubacterium limosum* was identified to be capable of this conversion (*O*-demethylation) of IX into 8-PN, and after strain selection, a conversion efficiency of 90% was achieved. Finally, strain supplementation to a nonconverting fecal sample led to rapid and high 8-PN production at only 1% (v/v) addition. Up to now, the concentration of 8-PN in beer was considered too low to affect human health. However, these results show that the activity of the intestinal microbial community could more than 10-fold increase the exposure concentration. Because prenylflavonoids are present in many beers with IX being the major constituent, the results raise the question whether moderate beer consumption might contribute to increased in vivo levels of 8-PN and even influence human health.

**KEYWORDS:** Hop; isoxanthohumol; 8-prenylnaringenin; prenylflavonoids; intestine; bacteria; *Eubacterium limosum*; beer; phytoestrogens; *O*-demethylation

### INTRODUCTION

Hops (*Humulus lupulus* L.) have been used for centuries as an essential raw material in beer brewing providing bitterness and flavor to beer. In the past few years, the plant has gained increasing attention as a source of prenylflavonoids, a subclass of polyphenols. These are present in the lupulin glands, found in the female hop cones. In this group, two prenylchalcones, xanthohumol (X) and desmethylxanthohumol (DMX), and three prenylflavanones, isoxanthohumol (IX), 8-prenylnaringenin (8-PN), and 6-prenylnaringenin (6-PN) (**Figure 1**), now receive much attention because of their possible health-promoting properties. X has been identified as a strong cancer chemopreventive agent (*1*), while 8-PN has been shown to be one of the most potent phytoestrogens identified so far, with a considerably

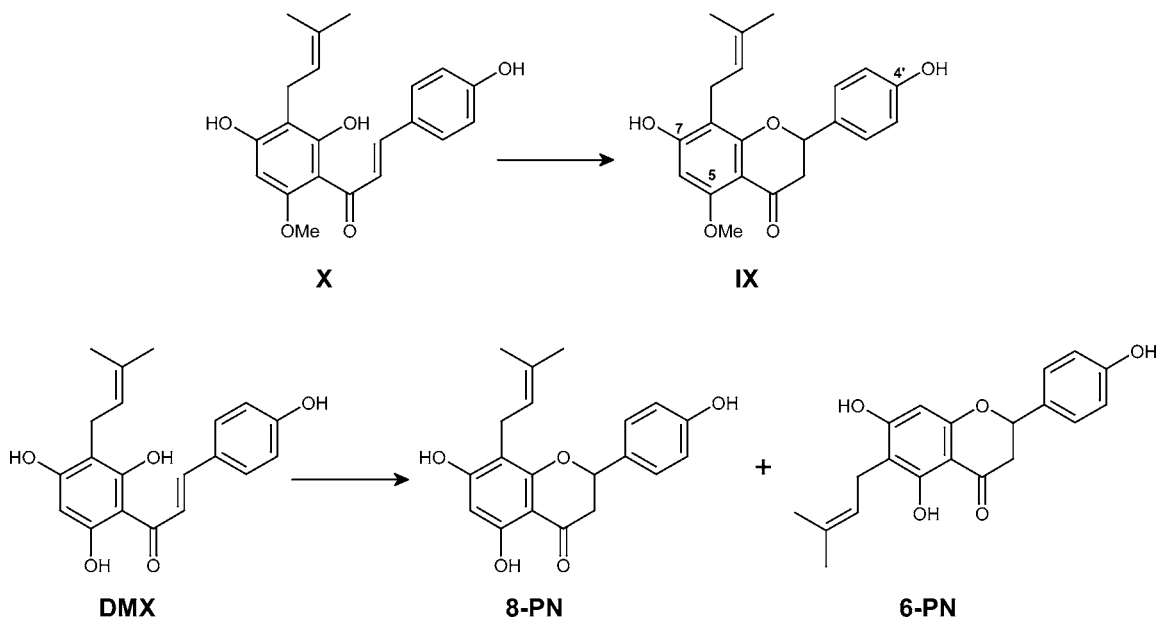
higher activity than the well-known soy phytoestrogens (*2, 3*). These interesting properties have led to intense research aiming at deciphering various bioactivities. 8-PN showed in vivo estrogenic activity (*4, 5*), prevented bone loss in rats (*6*), inhibited angiogenesis (*7*) and metastasis (*8*), and exhibited antiandrogenic activity (*9*).

There is considerable interest in whether human exposure to phytoestrogens has either health risks or benefits (*10–12*). In the case of hop prenylflavonoids, beer is the main dietary source, with an estimated daily intake of about 0.14 mg prenylflavonoids (*13*). However, the concentrations detected in beer (and, therefore, the average intake) significantly depend on the brewing process, as strong ales contain up to 4 mg/L. Although X is present as a predominant prenylchalcone [up to 1% (m/m)] (*14*), most of it is transformed into IX by thermal isomerization during wort boiling. As a result, IX is the major prenylflavonoid found in beer in concentrations from 500 µg/L (lager/pilsner) up to 4 mg/L (strong ale) (*13, 15*). DMX is

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**Figure 1.** Structures of hop prenylflavonoids. By thermal isomerization, the chalcones X and DMX are converted into the respective flavanones IX (from X) and 8-PN and 6-PN (from DMX).

equally converted into 8-PN giving final concentrations in beer up to 100  $\mu\text{g}$  8-PN/L and IX vs 8-PN ratios between 20 and 40. However, despite the high activity of 8-PN, the total estrogenicity of beers is still 500–1000-fold lower than the concentration needed for harmful *in vivo* activity ( $\sim 100$  mg/L) in rat experiments (4). Moreover, many beers are now made using hop extracts instead of whole hops, giving lower concentrations of 8-PN or even no 8-PN. Therefore, it is generally agreed that with the current knowledge, no detrimental health effects due to estrogens in beer are to be expected through moderate beer consumption (2, 4, 16). Besides beer, a number of hop-based dietary supplements are marketed, but contents may vary (17).

To exert *in vivo* effects claimed *in vitro*, dietary flavonoids need to be absorbed from the gut and reach their targets unchanged. However, up to now, no peer-reviewed data with regard to the bioavailability of prenylflavonoids are available. In general, monomeric flavonoids reach the small intestine unchanged (18), where absorption from the gut in the mesenteric circulation can take place. *In vitro* studies indicated extensive liver biotransformation of X (19, 20), IX (21), and 8-PN (22) upon absorption. However, the extent of dietary polyphenol absorption in the small intestine is rather limited (10–20%) (23, 24), thereby implying that a large proportion reaches the colon. Naringenin, the nonprenylated analogue of 8-PN, showed intensive microbial biotransformation in the intestine, including ring cleavage and dehydroxylation (25), followed by absorption and urinary excretion (26). The extent of degradation strongly depended on compound concentration and individual composition of the gut microbiota of the different human subjects. On the other hand, when X was fed to rats, it was mainly recovered in unchanged form from the feces (89%) (27).

As a source of 8-PN, IX, and X, moderate beer consumption leads to the intake of bioactive compounds, which could either act beneficially or be harmful depending on the final concentration reaching the target organs in the human body. In many cases, the bioavailability of phytoestrogens is strongly influenced by the composition and activity of the gut microbial community (28–30). As the microbiota can activate as well as degrade these components, the focus of the present study is to investigate the role of the microbial community toward the *in vivo* estrogenic

properties of beer or food supplements containing hops by investigating the biotransformation or the breakdown of X, IX, and 8-PN in fecal cultures and to evaluate interindividual differences. Understanding these processes is a crucial step toward the estimation of the final bioactivity of dietary flavonoids derived from hops and the evaluation of possible effects associated with such intake.

## MATERIALS AND METHODS

**Chemicals.** X was isolated from spent hops (i.e., the residue that remains after liquid or supercritical carbon dioxide extraction of hop cones, NateCO<sub>2</sub>, Wolnzach, Germany) via methanol/ethyl acetate (9/1, v/v) extraction, flash chromatography fractionation, and purification by semipreparative chromatography on a Varian Omnisphere C-18 column (250 mm  $\times$  21.4 mm, 10  $\mu\text{m}$ , Varian, St.-Katelijne-Waver, Belgium) using a Gilson 322 Pump (Gilson, Middleton, United States) with a Gilson UV-vis 156 detector and a Gilson 206 Fraction Collector. IX was prepared from X by isomerization under reflux in a 5% ethanolic KOH solution. IX was purified from the reaction mixture by semipreparative high-performance liquid chromatography (HPLC). 8-PN was prepared by prenylation of naringenin with 2-methylbut-3-en-2-ol in dioxane in the presence of boron trifluoride (31). All chemicals were obtained from Sigma-Aldrich (Bornem, Belgium). Using semipreparative HPLC, 8-PN was purified from a fraction resulting from flash chromatography containing both 8-PN and 6-PN and a B ring prenylated naringenin. The identities of X, IX, and 8-PN were confirmed by comparison of <sup>1</sup>H NMR and <sup>13</sup>C NMR data (Varian 300 MHz) with literature values (32), and the purities were assessed to be more than 99% by HPLC.

**Fermentation Conditions. Bacterial Cultures.** Fecal samples were obtained from 12 healthy subjects between the ages of 20 and 35 and designated A–L. None of the subjects had a history of gastrointestinal disease and had not taken antibiotics during 3 months prior to sample delivery. Fecal slurries of 20% (w/v) fresh fecal samples were prepared by homogenizing the feces with phosphate-buffered saline (0.1 M, pH 7) containing 1 g/L sodiumthioglycolate as a reducing agent. The particulate material was removed by centrifugation (1 min, 500g). *Eubacterium limosum* DSM 20543 was obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany).

**Culture Medium.** All fermentation experiments were performed in brain heart infusion broth (37 g/L, Oxoid, Hampshire, United Kingdom) with 0.5 g/L L-cystein HCl. Fecal bacteria as well as *E. limosum* require

**Table 1.** Microbial Transformation of X, IX, and 8-PN after Incubation with Fecal Samples A–D<sup>a</sup>

start	% recovery								
	X			IX			8-PN		
	X	IX	8-PN	X	IX	8-PN	X	IX	8-PN
A	74.9 (10.7)	5.9 (0.8)	ND <sup>b</sup>	ND	90.1 (6.3)	ND	ND	ND	11.2 (0.5)
B	80.5 (2.5)	9.1 (2.4)	ND	ND	83.0 (5.1)	ND	ND	ND	67.2 (13.1)
C	73.6 (3.1)	2.2 (0.1)	5.3 (0.2)	ND	19.0 (2.9)	36.4 (7.4)	ND	ND	54.6 (2.7)
D	65.4 (4.2)	11.7 (1.6)	ND	ND	86.0 (4.5)	ND	ND	ND	7.6 (1.3)

<sup>a</sup> The compounds were incubated for 8 days at a concentration of 25 mg/L, and results are presented as average (+ SD) molar percentage recovery of X, IX, or 8-PN relative to the dosed amount of flavonoid. <sup>b</sup> ND, not detected.

anaerobic conditions (low redox potential) for growth. Therefore, resazurin (2 mg/L) was added as a redox indicator. A pink color indicated a high redox potential (>−80 mV), and a colorless solution showed a low redox potential (<−80 mV), i.e., anaerobic. The redox potential in the large intestine typically ranged between −150 and −280 mV. The medium was autoclaved at 121 °C for 15 min.

Prior to addition to the autoclaved growth medium in the fermentation vessels, stock solutions of the different prenylflavonoids (X, IX, and 8-PN) were prepared in dimethyl sulfoxide (DMSO) in a concentration of 5 g/L. Changing the solvent from DMSO to ethanol did not influence the outcome of the experiments (data not shown).

**Fermentation.** The fermentation volume was either 25 or 50 mL. In the case of the fecal fermentation experiments, each batch culture consisted of 90% brain heart infusion medium, 10% fecal slurry, and 25 mg/L of X, IX, or 8-PN (5  $\mu$ L stock solution/mL batch culture). Culture experiments with *E. limosum* were performed by adding 100  $\mu$ L of bacterial stock to the brain heart infusion medium and 25 mg/L of X, IX, or 8-PN (5  $\mu$ L stock solution/mL batch culture). Each batch was sealed with rubber tops, and anaerobiosis was obtained by flushing the flasks with N<sub>2</sub> during 20 cycles of 2 min at 700 mbar overpressure and 900 mbar underpressure. Cultures were incubated at 37 °C on a shaker for the duration of the experiment. At given time points, samples were taken using syringes. All experiments were performed in triplicate. Prior to the start of the experiment, all fecal samples were tested for background concentrations of prenylflavonoids that could be present in the sample itself, but none were detected.

**Chemical Analysis. Extraction Protocol.** Two extraction procedures were compared to reach the best recovery of prenylflavonoids from fecal cultures: solid phase extraction using Bond Elut C18 silica columns (3 mL, 200 mg, Varian) and liquid/liquid extraction with ethyl acetate. The latter protocol gave the best results with recoveries of >90% for X, IX, and 8-PN, and it was further optimized. Samples originating from fecal cultures were diluted 5-fold in acidified H<sub>2</sub>O (pH 2) prior to extraction. Samples from *E. limosum* incubations were used as such. A 1 mL sample was added to 4 mL of acidified H<sub>2</sub>O (pH 2) and 5 mL of ethyl acetate in test tubes. After they were rigorously vortexed, the samples were centrifuged at 3000g for 10 min. Three milliliters of the ethyl-acetate phase was transferred to a new test tube and dried under N<sub>2</sub> stream. Finally, the extracts were dissolved in exactly 500  $\mu$ L of methanol, transferred into HPLC vials, and stored at −20 °C prior to analysis.

**High-Performance Liquid Chromatography (HPLC) Analysis.** Quantitative analyses were effected by HPLC using a Waters 2695 Alliance Separations Module (Waters, Milford, United States) equipped with a Waters 996 Photodiode Array Detector and Waters Millennium Software v3.20. A C18 reversed phase column (Varian, Omnisphere, 250 mm  $\times$  4.6 mm, 5  $\mu$ m) was used in combination with a gradient composed of solvent A [water acidified with 0.025% (v/v) formic acid] and solvent B [methanol acidified with 0.025% (v/v) formic acid]. Gradient profile: 0–3 min, 45% B in A; 3–32 min, from 45% B in A to 95% B in A; 32–37 min, 95% B in A; 37–45 min, 95% B in A to 45% B in A; 45–47 min, 45% B in A. The sample size was 20  $\mu$ L, the flow rate was 1 mL/min, and the column temperature was 35 °C. Detection was done simultaneously at 295 nm (for IX, 8-PN, and 6-PN) and at 370 nm (for X) using diode array detection. Peaks were identified by comparison of the retention times with those of authentic isolated reference compounds, as well as by inspection of the respective UV

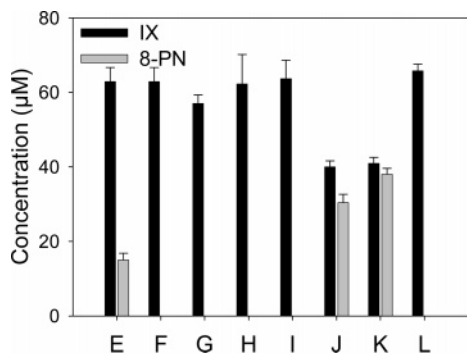
spectra. External five-point calibration curves were established for X, IX, 8-PN, and 6-PN ( $R^2 > 0.999$ ). Concentrations were calculated based on peak area integration. An extra confirmation of the identity of the compounds in the samples was achieved based on the typical fragmentation patterns in liquid chromatography–mass spectrometry/mass spectrometry (LC-MS/MS) analysis using a Waters Quattro micro with electrospray ionization (32).

**In Vitro Estrogenicity Testing.** To test the estrogenic activity of the prenylflavonoids and their metabolites, we used a modified protocol from De Boever et al. (33) that was based on the protocol developed by Routledge and Sumpter (34) for the yeast estrogen bioassay. In brief, *Saccharomyces cerevisiae* was transformed with the human estrogen receptor (ER $\alpha$ ) gene, together with expression plasmids containing responsive elements and the *lacZ* reporter gene (encoding the enzyme  $\beta$ -galactosidase). The estrogenic activity of the samples was expressed as percentage equivalence to 10 nM 17 $\beta$ -estradiol (E2), which elicited a 100% response in the estrogen receptor bioassay. The bioassays were performed in 96 well plates in which 10  $\mu$ L of the test compounds was incubated with 240  $\mu$ L of the genetically modified yeast (absorbance of 0.25 at 610 nm). Serial dilutions of the test compounds were made in DMSO, which allowed generating dose–response curves for dose (ordinate) vs activity (abscissa). The data were fitted by a four-parametric logistic model using the Marquardt–Levenberg algorithm (Sigmaplot 4.0, SPSS, Chicago, IL) (33).

## RESULTS

**Biotransformation of Hop Prenylflavonoids. Incubation of X, IX, and 8-PN with Human Fecal Samples.** The capacity of the cultures obtained from fecal samples A, B, C, and D (further referred to as cultures A–D) to degrade or transform the hop prenylflavonoids X, IX, and 8-PN was tested by incubating the cultures with 25 mg/L of each individual compound for a period of 8 days. Results are presented as molar % recovery of X, IX, or 8-PN, relative to the dosed amount (Table 1). X proved to be relatively stable with a recovery of above 65%. In all cultures incubated with X, IX was detected (few %). However, as this was also observed after incubation of autoclaved fecal material with X (data not shown), no bacterial mechanisms are involved in the isomerization of X into IX. In culture C, also 5% of 8-PN was recovered. In cultures A, B, and D, IX was very resistant to transformation as more than 80% was recovered. In culture C, however, the bacterial community was able to transform IX into 8-PN, as IX strongly disappeared and about 35% of 8-PN was recovered. This transformation did not occur when testing autoclaved fecal material. Remarkably, 90% of the initial amount of 8-PN was degraded in cultures A and D, while more than 50% of 8-PN was still recovered in cultures B and C. These results indicate that different fecal cultures are characterized by varying capacities to transform the hop prenylflavonoids.

**Microbial Conversion of IX into 8-PN by Fecal Cultures.** The most interesting data obtained from Table 1 are the results from culture C. A considerable amount of IX was transformed into 8-PN, showing the role of IX as a pro-estrogen, which can



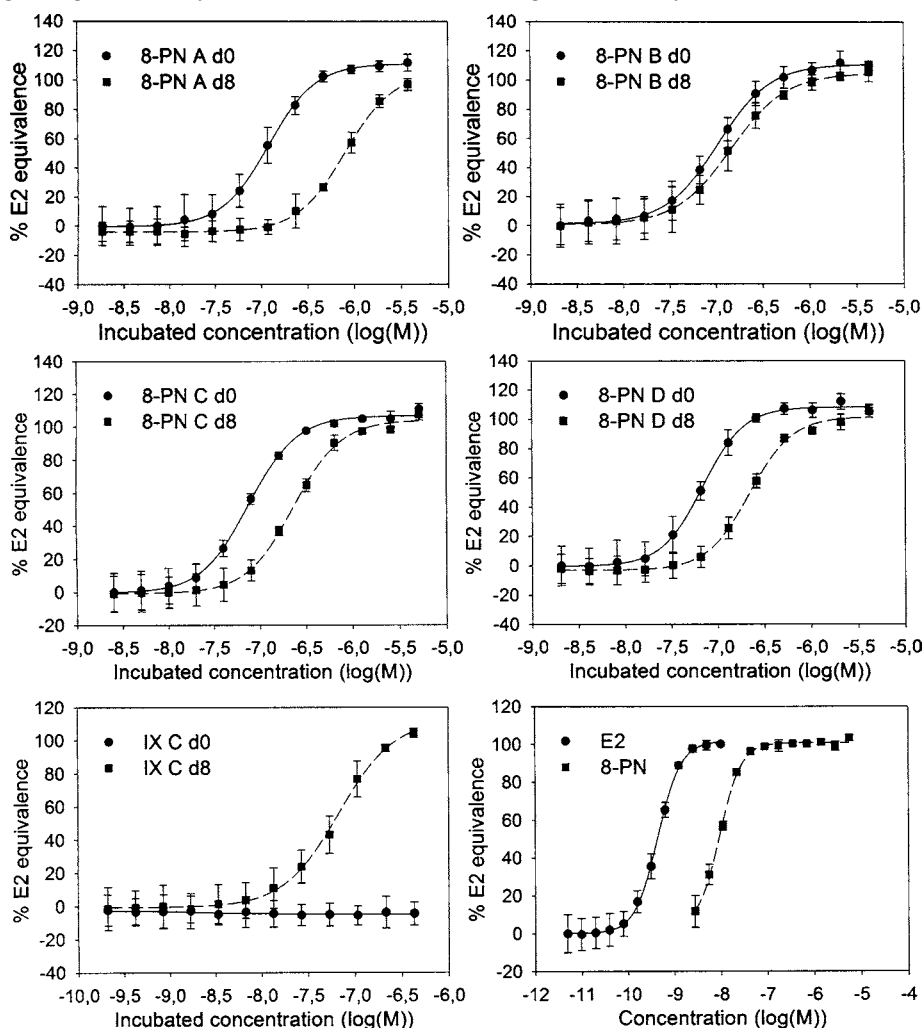
**Figure 2.** Transformation of IX by human fecal cultures. Samples E–L were incubated with 25 mg/L IX for 3 days. Results are presented as average (+ SD) concentrations of IX and 8-PN recovered ( $n = 3$ ). When no bars are displayed, no 8-PN was detected.

be activated by the intestinal microbial community. To further investigate these transformations, cultures E–L were incubated with 25 mg/L IX and samples were taken after 72 h (Figure 2). In cultures E, J, and K, varying amounts of 8-PN were recovered, and especially in cultures J and K, a lower recovery of IX was found in comparison to the non-8-PN-producing cultures. These results stress the significance of the difference in microbial community composition and activity, since conversion of IX into 8-PN was evident in only four of 12 cultures.

*In Vitro Estrogenic Response of the Incubations.* As 8-PN is known to have strong estrogenic activity, the conversion of IX

into 8-PN and the degradation of 8-PN were monitored by measuring the estrogenic response of the incubations of cultures A–D with IX and 8-PN at time zero and after 8 days (Figure 3). As no increase of the estrogenic response was detected for the cultures A, B, and D upon incubation with IX, data are not shown. In contrast, the high estrogenic activity was determined in culture C, strongly indicating transformation of IX into an estrogenically active reaction product. The estrogenic response of all cultures incubated with 8-PN decreased after the incubation because the dose–response curve shifted to higher concentrations. This suggests the degradation of 8-PN into non-estrogenic derivatives. The strongest decrease was seen in culture A as the  $EC_{50}$  value rose from 120 to 800 nM. The lowest decrease was seen for culture B, while for cultures C and D a shift of 0.5 log units of the  $EC_{50}$  values was detected. As HPLC analysis showed that more than 90% of the initially dosed 8-PN disappeared in culture D, a more pronounced shift in estrogenic activity was expected for this sample. Therefore, the higher remaining activity could be due to other degradation products with estrogenic activity. This will be investigated in further research. In this experiment, 8-PN was 25-fold less active than E2 based on their  $EC_{50}$  values.

**Incubation of X, IX, and 8-PN with *E. limosum*.** As *E. limosum* is an intestinal bacterium capable of *O*-demethylation and degradation of flavonoids, this species was tested for its capacity to transform or degrade X, IX, or 8-PN. To investigate long-term stability, the bacterium was incubated with 25 mg/L



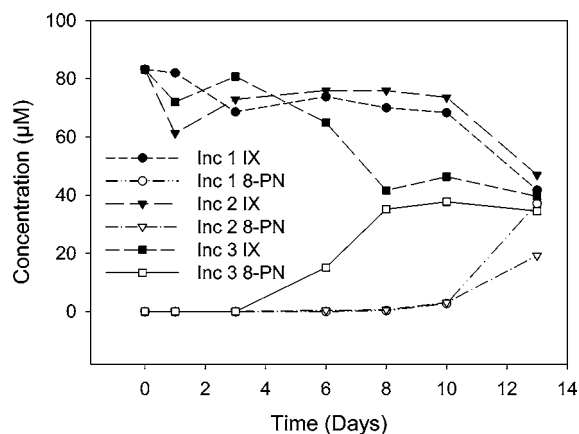
**Figure 3.** Average (+ SD) estrogen response of 8-PN-incubated fecal samples A, B, C, and D and of IX-incubated fecal sample C, as measured at day 0 and after 8 days of incubation. The estrogen responses of 8-PN and E2 are also shown ( $n = 3$ ).



**Table 2.** Transformation of X, IX, and 8-PN by *E. limosum*<sup>a</sup>

start	<i>E. limosum</i>			
	% recovery			
	X	IX	8-PN	6-PN
X	85.0 (2.6)	16.9 (3.5)	ND <sup>b</sup>	ND
IX	ND	51.4 (4.6)	36.4 (11.6)	ND
8-PN	ND	ND	98.3 (1.0)	0.4 (0.1)

<sup>a</sup> The compounds were incubated for 13 days at a concentration of 25 mg/L, and results are presented as average (+ SD) molar percentage recovery of X, IX, 8-PN, or 6-PN. <sup>b</sup> ND, not detected.

**Figure 4.** Three parallel incubations of *E. limosum* with 25 mg/L IX. The disappearance of IX (black symbols) and production of 8-PN (white symbols) were monitored over a period of 13 days.

of each compound for a period of 13 days. Results are presented as molar % recovery of X, IX, or 8-PN, relative to the dosed amount of individual compound (Table 2). Again, conversion of X into IX was a spontaneous process, as similar results were obtained with autoclaved bacterial cultures. *E. limosum* was able to transform IX into 8-PN, but because incubated 8-PN could almost fully be recovered after 13 days, further degradation did not occur. The small proportion of 6-PN recovered is probably due to isomerization of 8-PN via DMX as an intermediate.

**Production of 8-PN by *E. limosum*. Transformation Kinetics.** To investigate the transformation kinetics of the conversion of IX into 8-PN, the same *E. limosum* was incubated with IX in three parallel experiments and the concentrations of IX and 8-PN were monitored for a period of 13 days (Figure 4). Although incubation conditions were nearly identical, the 8-PN production already started after 3 days in incubation 3, while a very long lag phase (10 days) was noted in incubations 1 and 2. Moreover, the transformation rate was higher in incubation 1 than in incubation 2. This shows that transformation kinetics differed depending on the grown *E. limosum* culture.

**Strain Selection.** Because of the differences in transformation rate, it was attempted to select the most efficient IX-converting strain performing three successive selection rounds. This consisted of six parallel incubations of *E. limosum* with 25 mg/L IX and incubation for 8 days. Next, the culture that produced the highest amount of 8-PN was selected and used as inoculum for the next round of six parallel incubations (Table 3). While in the first selection round, the lowest production was only 2%, an increase of up to 82% was apparent after three selection steps, and the most efficient culture transformed all of the dosed IX into 8-PN. The mean production of all six incubations in each round increased from 22.5 up to 90.5%, and the standard deviation (SD) decreased from 20 to 7% after the selection

**Table 3.** Selection of 8-PN Producing *E. limosum* by Three Repeated Incubations<sup>a</sup>

	molar % IX $\Rightarrow$ 8-PN conversion		
	I	II	III
lowest	2.1	24.3	82.1
highest	46.5	79.4	102.5
mean	22.5	57.9	90.5
SD	19.3	19.6	6.9

<sup>a</sup> Six strains were incubated for 8 days with 25 mg/L IX, and the best IX converting strain was used for the next selection round. Lowest and highest 8-PN production as well as mean (+ SD) for each selection round are listed.

**Table 4.** Supplementation of *E. limosum* to Culture B in Different Proportions Ranging from 0% (Solely Fecal Sample) up to 100% (Axenic *E. limosum* Culture) and Incubation with 90 µM IX ( $n = 3$ )<sup>a</sup>

% suppl.	time (days)				
	0	1	3	5	7
	8-PN production (µM)				
0	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)
1	0.0 (0.0)	49.0 (3.1)	36.8 (8.3)	48.1 (14.0)	46.4 (8.9)
5	0.0 (0.0)	41.1 (12.6)	38.9 (14.7)	40.1 (10.2)	37.3 (10.6)
25	0.0 (0.0)	38.7 (7.4)	36.8 (4.3)	38.5 (20.6)	31.6 (10.7)
50	0.0 (0.0)	76.2 (20.2)	61.2 (9.5)	59.2 (21.6)	50.1 (16.9)
75	0.0 (0.0)	74.0 (18.6)	63.2 (15.5)	60.4 (14.1)	60.4 (13.8)
100	0.0 (0.0)	88.5 (3.8)	74.7 (6.8)	83.7 (18.4)	75.7 (2.7)

<sup>a</sup> Results are presented as average (+ SD) 8-PN production. As culture B did not convert IX into 8-PN, the 8-PN production was linked to the supplemented strain. The nonconverted IX remained present as unchanged component throughout the incubation period.

procedure. This means that, after only three rounds, a strain was selected, which converted almost all IX (high mean) and was also stable (low SD).

***E. limosum* Supplementation to Fecal Cultures.** From Table 1 and Figure 2, it was concluded that interindividual differences ultimately determined the capacity to transform IX into 8-PN. Therefore, we supplemented the most efficient *E. limosum* strain from the selection experiments to the nonproducing culture B to examine the capacity of this strain to start up production of 8-PN in the complex environment of an intestinal suspension. The strain was added to the culture in proportions ranging from 0 up to 100%. The mixture was incubated with IX for a period of 7 days (Table 4), and the concentration of 8-PN was monitored every other day. The results show that, with increasing supplementation of *E. limosum*, the production of 8-PN increased. The pure *E. limosum* culture gave a 100% conversion of IX into 8-PN, but even at 1% supplementation, half of the dosed IX was already transformed into 8-PN after only 1 day. Remarkably, a maximum concentration of 8-PN was reached for all incubations on the first day, which indicates that all of the available IX was immediately converted. No further transformation of 8-PN was detected as the concentrations of 8-PN at days 1 and 7 were not significantly different (Student's *T*-test,  $p > 0.05$ ).

## DISCUSSION

In this study, the conversion of hop prenylflavonoids by human intestinal microbiota was investigated. These plant-derived compounds are considered as one of the four principal groups of phytoestrogens in food and are mainly ingested through beer consumption. Because 8-PN concentrations in beer are generally very low, possible health effects due to exposure

to these substances are usually considered negligible. However, this view could change when considering that IX, which is quantitatively the most important prenylflavonoid in beer, may act as a pro-estrogen in food due to microbial *O*-demethylation. This could lead to enhanced concentrations of 8-PN and to a possibly important exposure after beer consumption. However, there seem to be important interindividual differences, as only one-third of the tested human fecal samples showed IX activation. The intestinal bacterium *E. limosum* was able to perform this conversion, and after strain selection, the bacterium rapidly converted all IX into 8-PN. Strain supplementation to nonconverting fecal samples led to rapid and efficient 8-PN production, even at low ratios.

The possibility that IX would act as a pro-estrogen was recently detected in vivo by Schaefer et al. (35). After dosing 10 mg of IX to two men, increased 8-PN concentrations, corresponding to up to 5% conversion, were detected in urinary samples. According to the authors, this in vivo activation would be an oxidative demethylation process in the liver, based on the findings of Nikolic et al. (21). Although the prenyl chain of IX was the primary target of liver modifications, the latter researchers also recovered small amounts of 8-PN after the incubation of IX with liver microsomes. This contradicts with the findings of Coldham et al. (36) who could not find IX activation by the liver. However, the liver is not the only transformation site inside the human body. The human colon contains  $\sim 10^{12}$  microorganisms/cm<sup>3</sup>, with an enormous metabolic potential. Bacterial enzymes may catalyze many reactions including hydrolysis, dehydroxylation, demethylation, ring cleavage, and decarboxylation. The importance of this microbial community in the metabolism of phytoestrogens has been clearly established. Decroos et al. (37) recently isolated a microbial consortium capable of transforming the soy phytoestrogen daidzein into equol, and Wang et al. (38) found two bacteria responsible for the conversion of lignans. Moreover, several intestinal bacteria possess  $\beta$ -glucosidases, which are necessary for the hydrolysis of phytoestrogen glucosides (30). Therefore, the gut microbiota are now considered as an important factor influencing phytoestrogen bioavailability (29).

Our results confirm a similar activity related to the group of prenylflavonoids, since IX can be converted into the potent phytoestrogen 8-PN. These transformation products can subsequently be absorbed from the colon, as it is established for soy isoflavones (39), to exert biological activity inside the body. While 8-PN was only one out of a large number of liver transformation products of IX (21), the human intestinal microbiota selectively converted IX into 8-PN with high conversion efficiencies (>35%). These results show that the impact of microbial activities on in vivo conversion of IX will most likely be more pronounced than possible liver transformations. However, as only one-third of the samples converted IX, interindividual variability in microbial community activity could strongly influence this in vivo conversion. Finally, Schaefer et al. (35) noted that urinary 8-PN excretion after beer consumption was slower than expected (up to several days), which could very well be due to delayed conversion of IX into 8-PN in the intestine, followed by absorption and renal excretion. Enterohepatic circulation is another important factor determining the retention of IX and 8-PN.

Up to now, little is known about intestinal transformations of prenylflavonoids. Nookandeh et al. (27) dosed 1000 mg X/kg body weight to rats and isolated 22 metabolites from the feces. The majority (89%) of the recovered flavonoids was unchanged X, which is in agreement with our data, as we detected only

very minor degradation of X. The rest was composed of small amounts of different metabolites including some IX. Avula et al. (40) performed a similar experiment with rats and detected mainly unchanged X next to a number of unidentified metabolites. However, no published data were available with regard to the availability of IX and 8-PN to gut microbiota. In our experiments with fecal samples, IX was either partially transformed into 8-PN or remained very stable during the incubations. 8-PN, on the other hand, was more sensitive to degradation as, in some experiments, 90% of the dosed amount disappeared. One possible explanation for this difference in stability between IX and 8-PN is the protective effect of the methoxy group on the A ring of IX, as this is the only structural difference with 8-PN. In general, the first step in the degradation of flavonoids is the opening and scission of the heterocyclic ring (41) and the absence of a free hydroxyl in positions 5, 7, or 4' would protect the compound from this cleavage (42, 43). An important consequence regarding optimum bioavailability could be a slow release of 8-PN from IX in the colon and absorption through the gut epithelium, taking into account that 8-PN is prone to undergo decomposition.

To identify possible microbial degradation pathways of prenylflavonoids, a comparison can be made with naringenin, lacking the prenyl side chain. This flavanone has been extensively studied and is mainly present in citrus fruits in a glycosidic form (44) and in tomatoes as aglycone (45). Degradation of naringenin by intestinal bacteria starts with heterocyclic ring cleavage, followed by dehydroxylation yielding phloroglucinol and 3-phenylpropionic acid (25). The extent of this degradation strongly depended on the nature of the fecal sample, which resembles the results obtained after the incubation of the fecal samples with 8-PN. This again shows that interindividual differences in the microbial community of the intestine are decisive on the fate of 8-PN and related flavonoids in the human body.

Microbial *O*-demethylation (8-PN from IX) is known for a number of anaerobic bacteria that can even use methyl ethers for respiratory growth. These bacteria either use fumarate (*Desulfotobacterium*) as an electron acceptor (46) or are so-called homoacetogens converting CO<sub>2</sub> into acetate (47). *E. limosum* is a homoacetogenic bacterium, which is frequently isolated from human feces (48). It is known to be involved in the biotransformation of specific isoflavonoids (49), and a related *Eubacterium* strain was identified as a producer of the lignan enterodiol (38). Instead of trying to isolate a bacterium with the ability to demethylate IX into 8-PN, we investigated the activity of this *E. limosum*. The strain proved to be able to produce 8-PN and did not further degrade it, as this compound could be completely recovered following the incubation of *E. limosum* with 8-PN. After strain selection, the bacterium even quantitatively converted IX into 8-PN after only 1 day of incubation. Because IX can easily be produced from X, a microbial fermentation process of IX could facilitate efficient large scale production of 8-PN. On the other hand, strain supplementation to a nonconverting fecal sample led to rapid and high IX conversion. This could imply that cosupplementation of IX and a transforming bacterium leads to a "slow release" 8-PN production in situ in the intestine, after which the produced 8-PN can be absorbed and transported to its targets.

Several reports now correlate possible health benefits with intentional phytoestrogen uptake (10). Yet, at the same time, questions rise whether environmental exposure to phytoestrogens could impose health risks such as endocrine disruption. Moreover, overall health effects may result from a combination of

phytochemicals with multiple and perhaps additive or interfering activities. Up to now, only isoflavones and lignans are considered relevant phytoestrogens in the human diet (10), especially because 8-PN concentrations in beer are considered to be too low for positive or negative health effects (4, 16). However, the intestinal microbial community may have a crucial role in the in vivo conversion of IX into 8-PN. Ultralow dose estrogen therapy at a dose of only 0.25 mg E2 per day over a period of 3 years significantly improved bone density as compared with placebo without significant effects on endometrial thickness (50). According to our results, in some humans, at least 35% of the ingested IX may be converted into 8-PN. Taking into account an accumulation factor of 2 based on slow elimination of 8-PN (35), a daily consumption of 1 L of beer containing 2 mg IX/L would lead to a daily 8-PN burden of 1.4 mg. As 8-PN is only about 10 times less active as compared to E2, the daily exposure of about 0.14 mg E2 equivalents is well within the range of positive biological activity (e.g., protective effect on bone metabolism) without negative side effects (e.g., proliferation of the endometrium). Although this example is very simplified and does not account for differences in bioavailability, it clearly indicates the intriguing role of the gut microbial community in the exposure to biologically active compounds. As microbial O-demethylation of IX could readily lead to a 10-fold increase of the ingested 8-PN, supposing an IX vs 8-PN ratio in beer of 30 (13), large population studies are warranted in order to acquire insight into the in vivo bioavailability of hop prenylflavonoids throughout the intestine and the extent of microbial transformations in the colon. Such studies are essential to validate our findings on the capacity of individuals to produce 8-PN from IX, giving rise to possible health effects of IX consumption.

#### ABBREVIATIONS

8-PN, 8-prenylnaringenin; IX, isoxanthohumol; X, xanthohumol; DMX, desmethylxanthohumol; HPLC, high-performance liquid chromatography; E2, 17 $\beta$ -estradiol; LC-MS/MS, liquid chromatography–mass spectrometry/mass spectrometry.

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