The Journal of Nutrition
Nutrient Physiology, Metabolism, and Nutrient-Nutrient Interactions

Eubacterium limosum Activates Isoxanthohumol from Hops (*Humulus lupulus* L.) into the Potent Phytoestrogen 8-Prenylnaringenin In Vitro and in Rat Intestine[1–3]

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Abstract

Recently, it was shown that the exposure to the potent hop phytoestrogen 8-prenylnaringenin (8-PN) depends on intestinal bacterial activation of isoxanthohumol (IX), but this occurs in only one-third of tested individuals. As the butyrate-producing *Eubacterium limosum* can produce 8-PN from IX, a probiotic strategy was applied to investigate whether 8-PN production could be increased in low 8-PN producers, thus balancing phytoestrogen exposure. Using fecal samples from high (Hop +) and low (Hop −) 8-PN–producing individuals, a Hop + and Hop − dynamic intestinal model was developed. In parallel, Hop + and Hop − human microbiota-associated rats were developed, germ-free (GF) rats acting as negative controls. IX and then IX + *E. limosum* were administered in the intestinal model and to the rats, and changes in 8-PN production and exposure were assessed. After dosing IX, 80% was converted into 8-PN in the Hop + model and highest 8-PN production, plasma concentrations, and urinary and fecal excretion occurred in the Hop + rats. Administration of the bacterium triggered 8-PN production in the GF rats and increased 8-PN production in the Hop − model and Hop − rats. 8-PN excretion was similar in the feces (294.1 ± 132.2 nmol/d) and urine (8.5 ± 1.1 nmol/d) of all rats ($n = 18$). In addition, butyrate production increased in all rats. In conclusion, intestinal microbiota determined 8-PN production and exposure after IX intake. Moreover, *E. limosum* administration increased 8-PN production in low producers, resulting in similar 8-PN production in all rats. J. Nutr. 138: 1310–1316, 2008.

Introduction

Hops have been identified as a source of the potent phytoestrogen 8-prenylnaringenin (8-PN)[8] (1) and hop extracts are used to relieve menopausal complaints (2). Previously, we have shown that the final level of 8-PN does not depend on the presence of 8-PN itself in hop products but rather on the combined presence in the plant of a more abundant precursor, isoxanthohumol (IX), and the metabolic potential of the intestinal microbiota (3,4). Intestinal bacteria were shown to demethylate IX into 8-PN, thereby increasing the 8-PN exposure 10-fold. As IX is the main prenylflavonoid present in both beer and hop extracts (5), prenylflavonoids are now considered as a 3rd important group of phytoestrogens relevant to human nutrition, in addition to isoflavones and lignans (6).

However, due to interindividual intestinal variability, only about one-third of the individuals were found to be capable of efficiently performing this transformation, leading to large differences in 8-PN exposure after consumption of hops (7). *Eubacterium limosum* is an anaerobic Gram-positive rod and is present in the colon of most humans (8). Over the last few years, this bacterium has gained increased attention because of its beneficial effects in inflammatory bowel disease, possibly attributed to its butyrate-producing capacity (9,10). Butyrate is known to modulate epithelial cell proliferation and has protective effects on inflammatory bowel disease (11), but oral butyrate

[1] Supported by a post-doctoral grant from the Fund for Scientific Research-Flanders (Fonds Wetenschappelijk Onderzoek-Vlaanderen; to S. Possemiers). A. González-Sarrías is holder of a fellowship from the Ministerio de Educación y Ciencia (Spain). This research was conducted in cooperation with Metagenics Europe.

[2] Author disclosures: S. Possemiers, S. Rabot, J. C. Espín, A. Bruneau, C. Philippe, A. González-Sarrías, A. Heyerick, F. A. Tomás-Barberán, D. De Keukeleire, and W. Verstraete, no conflicts of interest.

[3] Supplemental Table 1 and Supplemental Figure 1 are available with the online posting of this paper at jn.nutrition.org.

[8] Abbreviations used: DGGE, denaturing gradient gel electrophoresis; GF, germ-free rats; HMA, human microbiota-associated; Hop +, high 8-prenylnaringenin–producing; Hop −, low 8-prenylnaringenin–producing; IX, isoxanthohumol; PCA, principal components analysis; 8-PN, 8-prenylnaringenin; SHIME, simulator of the human intestinal microbial ecosystem.

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Manuscript received 14 January 2008. Initial review completed 12 February 2008. Revision accepted 7 May 2008.

treatment did not prove efficient because of the difficulty of delivering butyrate to the colon (12). Therefore, a good alternative would be to administer butyrate-producing probiotics, such as *E. limosum* (13).

Moreover, this bacterium was recently shown to efficiently activate IX into 8-PN (4). Because of this ability and its probiotic potential, we hypothesized that the combined uptake of *E. limosum* and hop extracts could lead to increased health effects by improving the colonic environment (e.g. bacterial butyrate production) and by the production of 8-PN in people who lack the appropriate intestinal metabolic potential. This hypothesis was investigated in vitro in a dynamic model of the intestine, followed by an in vivo trial with germ-free (GF) and human microbiota-associated (HMA) rats.

Materials and Methods

Chemicals. The isolation of xanthohumol, isomerization into IX, and chemical 8-PN synthesis were performed as described earlier by Possemiers et al. (4). Stock solutions of IX and 8-PN were prepared in ethanol (5 g/L).

Preparation of the E. limosum inocula. *E. limosum* strain LMG P-23546 (4) was grown anaerobically at 37°C in brain heart infusion broth (Oxoid) with 0.5 g/L L-cysteine HCl, 5 g/L yeast extract, and 5 mg/L haemin. After growth to a concentration of approximately log 12 colony-forming units/L, 15 mL of the growth medium were centrifuged (15,000 × *g*; 15 min) and resuspended in 15 mL saline (0.9 g/L NaCl). This suspension was immediately used for administration of the bacterium to the simulator of the human intestinal microbial ecosystem (SHIME) or to the rats.

Identification of high and low 8-PN–producing human fecal microbiota. Twelve volunteers delivered a fecal sample for incubation purposes. The samples were prepared immediately and incubated with 25 mg/L IX (4). Based on these results, high (Hop +) and low (Hop −) 8-PN–producing individuals were selected. Their 8-PN production status was confirmed by administering a capsule containing 5.59 ± 0.97 mg IX for 4 consecutive days and by quantifying the relative 8-PN excretion on d 4 in a 24-h urine sample. To avoid background prenylflavonoids, subjects were asked to refrain from consuming hop-containing products from 4 d before to the end of the intervention. The high and low 8-PN producers were asked to deliver fecal samples for inoculating the SHIME and GF rats.

SHIME experiments. The reactor setup was adapted from the SHIME (14), consisting of 5 successive reactors, simulating the stomach, small intestine, and ascending, transverse, and descending colon. The microbial community in the last 3 reactors is derived from a selected fresh fecal sample. Reactor setup, inoculum preparation, and reactor feed composition have been previously described (15). For this experiment, a TWINSHIME setup was developed by operating 2 systems in parallel. The systems were inoculated with a fecal sample from the identified high and low 8-PN producers and designated respectively as Hop + and Hop – compartments. After the reactor start up and a 3-wk stabilization period (15), 25 mg/L IX was administered for 4 wk to the SHIME feed entering both systems (Expt. 1). After 2 wk, log 9 *E. limosum* was administered daily to the ascending colon part of both systems for 2 wk (Expt. 2).

Rat experiments. GF 5- to 7-wk-old male and female F344 rats were obtained from our GF rodent breeding facilities. They were randomly separated into 3 groups of 12 rats (6 males and 6 females), housed in 3 sterile isolators (Ingenia) that were maintained in controlled conditions of light (0700–1900 h), temperature (20–22°C), and humidity (45–55%). Within each isolator, male and female rats were kept separately in groups of 3 in standard macrolon cages containing a bed of wood shavings. Throughout the study, they were given free access to autoclaved tap water and a pelleted semipurified diet (**Table 1**) (16) commercially prepared by SAFE and sterilized by γ-irradiation at 45 kGy (IBA Mediris). One group of rats remained GF and rats from the 2 other groups were colonized with the fecal microbiota of either the Hop + or Hop −, using gavage with freshly prepared fecal suspensions (16). The experiment started after a 3-wk acclimatization phase to permit establishment of the microbiota and adaptation of the rats. All procedures were carried out according to European guidelines for the care and use of laboratory animals and with permission 78–58 of the French Veterinary Services.

TABLE 1 Composition of the diet[1]

Ingredient	
	g/kg diet
Mashed potato	290.0
Cornstarch	289.85
Sucrose	50.0
Casein	50.0
Soy isolate[2]	120.0
Corn oil	30.0
Lard	30.0
Cholesterol	0.15
Cellulose	60.0
Mineral mix[3]	70.0
Vitamin mix[4]	10.0

[1] Analytical compounds of dry matter were: crude proteins, 18%; crude fat, 8%; ash, 6%; carbohydrates, 68% (energy 19.33 MJ/kg; Eurofins Scientific Analytics).
[2] Nurish 1500 from DuPont Protein Technologies.
[3] Supplied the following (to provide g/kg diet): Ca, 2.11; P, 5.46; Na, 2.74; K, 3.67; Mg, 1.02; Fe, 0.10; Cu, 0.09; Mn, 0.55; Zn, 0.31; I, 0.0043; Co, 0.0007.
[4] Supplied the following (to provide mg/kg diet, except as noted): all-*trans* retinol acetate, 6.88; cholecalciferol, 62.5 μg; all-*rac*-α-tocopheryl acetate, 175; menadione sodium bisulfite, 35.2; thiamin hydrochloride, 22.4; riboflavin, 15; nicotinic acid, 100; calcium DL-pantothenate, 7.5; pyridoxine hydrochloride, 12.15; folic acid, 5; D-biotin, 0.3; cyanocobalamin, 0.05; ascorbic acid, 0.8; choline chloride, 1.56 g; *myo*-inositol, 150.

Two separate experiments were designed. The purpose of Expt. 1 was to assess the formation of 8-PN from IX in GF, Hop –, and Hop + rats. Therefore, all rats were gavaged every morning for 4 d with IX (2 mg/kg body weight) dissolved in ethanol:propylene glycol (50:50, v:v). Rats were housed in metabolism cages during the last 2 d of the dosing period to collect 24-h urine and fecal outputs (17) for IX and 8-PN analysis. Fresh feces were also collected before and at the end of the dosing period for microbiota analysis. On the day following the last gavage, one-half of the rats of each group ($n = 6$, 3 males and 3 females) were anesthetized with isoflurane (Aerrane, Baxter) and blood was collected from the inferior vena cava in heparinized tubes to prepare plasma for IX and 8-PN analysis. Rats were killed by section of the abdominal aorta and liver, kidneys, brain, and uterus in females were collected for IX and 8-PN analysis. Cecal and colonic contents were collected for incubation experiments with IX and a cecal content sample was stored for butyrate analysis. All samples were stored at −80°C until analyses.

After a 3-wk washout period, the remaining 6 rats of each group entered into Expt. 2. This was designed to assess the formation of 8-PN from IX in GF, Hop –, and Hop + rats supplemented with *E. limosum*. Therefore, all rats were gavaged every day for 4 d with the IX solution and for 6 d with *E. limosum* (10^9 colony-forming units per rat). *E. limosum* administration started 2 d prior to IX dosage to ensure a steady-state presence of the bacterium in the rat intestine. Furthermore, IX was given in the morning and *E. limosum* in the afternoon to avoid a possible direct interaction during the bolus transit. Biological fluids and organs were collected as in Expt. 1.

Incubation experiments with the rat intestinal contents. The cecal and colonic contents were diluted to 10% (v:v) in supplemented brain heart infusion broth, homogenized with an Ultra-Turrax blender, and incubated anaerobically at 37°C for 72 h.

Processing of SHIME samples and rat biological fluids and tissues. Sampling and prenylflavonoid extraction from the colon compartments of the SHIME systems were carried out as described previously (3).

Urine was centrifuged (8000 × *g*; 5 min at 4°C) to remove particulates and fecal samples were lyophilized and ground. To 1 mL urine supernatant, 1 mL sodium acetate buffer (0.1 mol/L, pH 5.0) and 50 μL β-glucuronidase/arylsulfatase (10/0.33 MU/L, Sigma-Aldrich) were added. Similarly, to 500 mg feces, 9.5 mL sodium acetate buffer and 250 μL β-glucuronidase/arylsulfatase were added and to 300 μL plasma, 1.7 mL sodium acetate buffer and 50 μL β-glucuronidase/arylsulfatase were added. After overnight incubation at 37°C, 4-hydroxybenzophenone was added as internal standard. The prenylflavonoids from urine and feces samples were extracted in triplicate with ethyl acetate (4). Plasma samples were extracted with ethyl acetate and hexane. To a 2-mL sample, 3 mL water (pH 2), 5 mL ethyl acetate, and 1 mL hexane were added. After vortexing and centrifugation (8000 × *g*; 10 min), 4 mL supernatant was collected. A second extraction was performed and 5 mL supernatant was collected and pooled with the first extract. The extracts were dried under nitrogen, then dissolved in 250 μL water/methanol (1:1, v:v) with formic acid (0.025%, v:v).

Organs (2 g liver, 0.7 g kidney, 0.6 g brain, and 0.15 g uterus) collected from each rat and extra organ samples from control rats without IX supplementation were processed as reported elsewhere (18). Using this protocol, ~85% of IX and 8-PN standards were recovered when 1 μmol/L of each compound were added to the initial mixture.

Chemical analysis of SHIME samples and rat biological fluids and tissues. Quantification of butyrate in the SHIME samples and rat cecal contents was done as previously described (19). SHIME samples and rat urine, feces, and plasma were analyzed for prenylflavonoids by HPLC (4).

Organ samples were analyzed by liquid chromatography-tandem MS (Agilent Technologies) using a reverse phase C_{18} LiChrospher column (25 × 0.4 cm, 5 μm, Merck) according to Espín et al. (18) (**Supplemental Fig. 1**). IX, 8-PN, and their corresponding metabolites were identified according to their UV and MS spectra as well as tandem MS fragments. The quantitation of the peaks was carried out by the selected ion-monitoring mode. Due to the lack of both IX- and 8-PN-glucuronide standards, the values for these metabolites were scored as high (+++), medium (++), low (+), and not detected (−). 'Not detected' were those that either did not permit the MS-MS fragmentation of the ion or the ion intensity was below the background noise; low values were those that permitted proper compound identification (mean integrated areas ~1×10^6); medium and high values exceeded the ion intensity of low values by ~2- to 5-fold (mean integrated areas ~3×10^6) and 10- to 30-fold (mean integrated areas ~2×10^7), respectively. For ion intensity comparison, the ionization of the mass spectrometer was checked daily using IX and 8-PN standards.

PCR-denaturing gradient gel electrophoresis fingerprinting of fecal and cecal bacterial communities. DNA extraction of the fecal and cecal samples was performed as previously described (15). The 16S ribosomal RNA genes for all bacteria were amplified by PCR using the primers PRBA338f and P518r (20), and a 40-bp GC clamp was added to the forward primer. Denaturing gradient gel electrophoresis (DGGE) was performed using the Bio-Rad D gene system with 8% (w:v) polyacrylamide gels and denaturing gradients from 45 to 60% (15). Principal components analysis (PCA) of the DGGE profiles was performed using the BioNumerics software v2.0 (Applied Maths) and PCA ordinations were calculated using the Pearson product-moment correlation coefficient. Within each character set, this coefficient subtracts each character from the mean and divides it by the variance of the character set.

Real-time PCR quantification of E. limosum. A real-time PCR protocol specific for *E. limosum* was developed based on the method of Kageyama et al. (21). Amplification was performed with an ABI Prism SDS 7000 instrument with the PCR Master Mix (2×) SYBR Green kit (Applied Biosystems). A total of 300 nmol/L of the primers LimoF (TGG-ATC-CTT-CGG-GTG-ACA-TT) and LimoR (CTC-ATT-GGG-TAC-CGT-CAT-TC) were used and the protocol consisted of 2 min at 50°C; 10 min at 95°C; 40 cycles of 30 s at 94°C, 30 s at 63°C and 30 s at 72°C. Standard curves were constructed with cloned 16S ribosomal RNA genes from *E. limosum* in a concentration range from log 16 to log 8 DNA copies per liter and DNA from at least 10 nontarget species was used to test amplification specificity.

Statistical analyses. Values are expressed as means ± SEM. Expt. 1 and Expt. 2 were analyzed as separate factorial studies, with bacterial status (Hop − and Hop + in SHIME assays; GF, Hop –, and Hop + in rat assays), intestinal segment (ascending, transverse, and descending colon in SHIME assays; cecum and colon in in vitro incubations with rat intestinal contents), and gender (male and female in rat assays) as treatments. The effects of bacterial status and intestinal segment (SHIME assays and in vitro incubations) or bacterial status and gender (rat assays) were analyzed with 2-way ANOVA. Following significant ANOVA, the Newman-Keuls test was used for pairwise comparisons. In Expt. 2, *E. limosum* was administered to the same SHIME Hop – and Hop + compartments as those used in Expt. 1. Similarly, in the rat assays, one-half of the rats used in Expt. 1 for IX and 8-PN analysis in urine and feces were further used in Expt. 2 for the same purpose. Therefore, 8-PN production in the SHIME and IX and 8-PN excretion in the rats with (Expt. 2) and without (Expt. 1) *E. limosum* were compared with ANOVA for repeated measurements. When data were not normally distributed, nonparametric tests were used. The significance level was set at $P < 0.05$. Calculations were performed with Statview software (version 5.0, SAS Institute).

Results

Activation of IX into 8-PN by E. limosum in the Hop + and Hop – SHIME model. Using the in vitro TWINSHIME setup, the steady-state 8-PN production in each colon reactor in the absence (Expt. 1) or presence (Expt. 2) of *E. limosum* was quantified (**Table 2**). When only IX was dosed, no 8-PN production occurred in the Hop – compartment inoculated with intestinal microbiota derived from the Hop – individual. In contrast, IX was partially converted in both the transverse and descending colon reactors of the Hop + compartment, inoculated with intestinal microbiota derived from the Hop + individual. When *E. limosum* was administered with IX, the 8-PN production did not significantly increase in the Hop + compartment compared with Expt. 1, whereas 8-PN production reached 40% in the descending colon reactor of the Hop – compartment. In parallel, butyrate production increased compared with Expt. 1 in the ascending colon from both the Hop + and Hop – compartments (data not shown), respectively, from 5.7 and 2.3 mmol/L to 10.8 and 4.5 mmol/L ($P < 0.01$).

Activation of IX into 8-PN by E. limosum in incubations of the rats' cecal and colonic contents. We assessed the ability of the intestinal contents of the rats to produce 8-PN after in vitro incubation of the cecal and colonic contents with IX (Table 2). In Expt. 1, the 8-PN production was highest for all the rats in the contents derived from the Hop + group. Only very low 8-PN production was noted in the contents derived from the Hop – rats and no 8-PN was produced in the incubation of the GF rat contents. *E. limosum* administration to the rats (Expt. 2) significantly increased 8-PN production in the cecal and colonic contents derived from the Hop – group compared with Expt. 1, but not in those from the Hop + group. The intestine of the GF rats now contained only *E. limosum*, leading to 100% IX conversion into 8-PN. Cecum and colon incubations did not differ in any of the rat groups.

Excretion of IX and 8-PN in rat feces and urine. IX and 8-PN were analyzed in the fecal and urinary outputs collected in the

TABLE 2 Conversion of IX into 8-PN and concentration of *E. limosum* in the colon compartments of the SHIME and in in vitro incubations of rat cecum and colon contents[1]

		Expt. 1 (IX)		Expt. 2 (IX + *E. limosum*)	
		8-PN ratio[2]	*E. limosum*	8-PN ratio	*E. limosum*
SHIME		*%*	*log (DNA copies/g)*	*%*	*log (DNA copies/g)*
Ascending colon	Hop +	0.0 ± 0.0	n.d.[3]	0.0 ± 0.0	7.77 ± 3.95
	Hop −	0.0 ± 0.0	n.d.	0.0 ± 0.0	7.82 ± 4.51
Transverse colon	Hop +	38.6 ± 8.5^{a*}	n.d.	53.7 ± 11.2^{a*}	7.56 ± 4.36
	Hop −	0.0 ± 0.0^{b}	n.d.	0.0 ± 0.0^{b}	8.06 ± 4.12
Descending colon	Hop +	78.4 ± 5.7^{a+}	n.d.	90.1 ± 6.9^{a+}	7.84 ± 4.28
	Hop −	0.0 ± 0.0^{b}	n.d.	40.2 ± 2.4$^{b*\#}$	7.52 ± 4.27
Rats					
Cecum	Hop +	32.7 ± 15.2^{a}	n.d.	54.6 ± 10.7^{a}	5.58 ± 2.32^{a}
	Hop −	2.7 ± 1.2^{b}	n.d.	9.0 ± 1.5^{b}	6.48 ± 2.65^{a}
	GF	0.0 ± 0.0^{c}	n.d.	100.0 ± 0.0^{c}	8.95 ± 3.65^{b}
Colon	Hop +	38.4 ± 16.1^{a}	/	44.3 ± 9.9^{a}	/
	Hop −	1.6 ± 0.9^{b}	/	28.9 ± 15.3^{a}	/
	GF	0.0 ± 0.0^{c}	/	100.0 ± 0.0^{b}	/

[1] Values are means ± SEM, $n = 6$. Within each experiment and intestinal segment, means with superscripts without a common letter differ; $^{*,+}$, within each experiment and bacterial status, means with different symbols differ; $^{\#}$different from Expt. 1, $P < 0.05$.
[2] 8-PN ratio = 8-PN/(IX+8-PN) × 100.
[3] n.d., Not detected; /, not performed.

last 2 d of the dosing period of Expt. 1 and Expt. 2. Because the daily excretion values were correlated ($0.62 < r < 0.82$; $P < 0.001$), the mean excretion of the 2 d was used as the variable (**Fig. 1**).

Without taking bacterial status into account, excretion of IX in feces reached 263.5 ± 40.2 nmol/d in Expt. 1 and decreased to 99.8 ± 19.2 nmol/d in Expt. 2. In Expt. 1, 8-PN excretion was negligible in GF rats and it was significantly higher in the urine of the Hop + rats than in the Hop – rats. Consumption of *E. limosum* (Expt. 2) increased the 8-PN excretion in the Hop – and GF rats compared with Expt. 1 but did not influence the Hop + rats, resulting in similar excretions in all rats in the feces (294.1 ± 132.2 nmol/d) and urine (8.5 ± 1.1 nmol/d). No consistent gender effects were observed (**Supplemental Table 1**).

The importance of liver phase II metabolism was assessed by quantifying the amounts of IX and 8-PN recovered in the urine and feces of the GF rats with or without β-glucuronidase/arylsulfatase treatment of the samples prior to analysis (**Table 3**).

When the rats consumed *E. limosum*, a 35% increase ($P < 0.05$) in butyrate was detected in the ceca of both Hop + and Hop – rats compared with Expt. 1, increasing to levels of 3.6 and 3.7 μmol/g (data not shown). The ceca of GF rats contained 1.8 ± 0.6 μmol butyrate/g after *E. limosum* treatment.

Concentrations of IX and 8-PN in rat plasma and different organs. Plasma IX concentrations were similar for all rats and reached, respectively, 0.63 ± 0.05 and 0.54 ± 0.05 μmol/L in Expt. 1 and Expt. 2. In Expt. 1, no 8-PN was detected in the GF rats' plasma and 8-PN concentrations were significantly higher in the Hop + (2.46 ± 1.02 μmol/L) than the Hop – rats (0.77 ± 0.04 μmol/L). However, the ratio of 8-PN concentration to the combined IX and 8-PN concentration was not significantly higher in Hop + rats than in Hop – rats (Fig. 1). After treatment with *E. limosum* (Expt. 2), plasma 8-PN concentrations were similar in all rats (1.71 ± 0.23 μmol/L) and 8-PN ratios in the GF rats' plasma were even higher than in the HMA rats.

Plasma samples of the GF rats from Expt. 2 were investigated for the presence of free aglycones and phase I and II liver metabolites. Whereas low concentrations of aglycones were detected, IX and 8-PN mainly circulated as phase II glucuronides. Moreover, very low quantities of phase I metabolites with the same molecular mass as IX- and 8-PN-alcohols were detected.

No unconjugated IX or 8-PN was detected in any tissue. The IX- and 8-PN-glucuronides were the only metabolites detected (**Table 4**). Glucuronidase treatment of the tissue removed the glucuronides, but the corresponding aglycones could not be detected because these showed lower ionization than their corresponding glucuronides. IX-Glucuronide was detected in 97% 60, and 50% of liver, kidney, and uterus tissues, respectively. In Expt. 1, 8-PN-glucuronides were detected in neither GF nor Hop – rats, whereas they were detected in 85% of the Hop + rats. After *E. limosum* administration, 8-PN metabolites were detected in all rats but with differences depending on the tissue: 89, 33, and 28% of liver, kidney, and uterus tissues, respectively. No IX or 8-PN metabolites were detected in brain tissues.

E. limosum concentrations and bacterial community composition. When only IX was dosed, the bacterium could not be detected in either the SHIME or the rat cecal community (Table 2). After administration to the TWINSHIME, *E. limosum* was present in similar concentrations in all colon reactors of both the Hop + and Hop – compartments. In Expt. 2 of the rat trial, the cecal contents of the GF rats contained 2 to 3 log higher concentrations than those of their Hop + and Hop – counterparts.

PCA analysis of the microbial community profiles in the feces of the Hop + and Hop – rats showed that the profiles grouped based on their bacterial status (**Fig. 2**). Within 1 bacterial status, the profiles grouped per 3, relating to the fact that the rats were housed per 3. Whereas the DGGE profiles of the GF rats contained no bands in Expt.1, 1 single band was present at the end of Expt. 2, further confirming the presence of *E. limosum*.

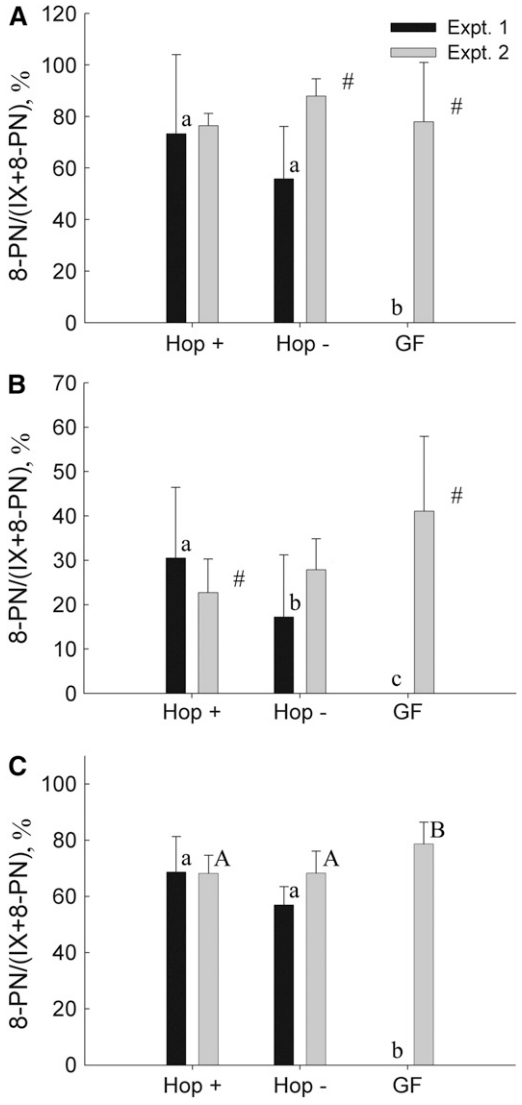

FIGURE 1 Recovery of 8-PN in the feces (*A*), urine (*B*), and plasma (*C*) of rats administered IX in the absence (Expt. 1, $n = 12$) or presence (Expt. 2, $n = 6$) of *E. limosum*. Values are means ± SEM, expressed as the ratio of 8-PN concentration to the combined IX and 8-PN concentration. Within each experiment, means without a common letter differ; # different from Expt. 1, $P < 0.05$.

Discussion

Until now, the application of probiotics has mainly been limited to strains belonging to a few genera, such as bifidobacteria and lactobacilli (22). However, the human gut is colonized by hundreds of species and many perform important metabolic reactions or exert biological activities that might translate into clinical benefits (23). In this research, *E. limosum* is presented as an important candidate probiotic because of its butyrate production (13) and its specific metabolic potential to reduce interindividual variability in hop phytoestrogen metabolism and exposure.

Interindividual variation in phytoestrogen metabolism has been reported by several authors (24,25) and was recently also shown for hop phytoestrogens (3,4). As noted for isoflavones and lignans (26), the results from this study also definitively show the crucial role of the intestinal microbiota in the production of 8-PN from IX. Whereas 8-PN was not detected in any

TABLE 3 Recovery of IX and 8-PN aglycones in urine and feces of GF rats administered IX in the absence (Expt. 1) or presence (Expt. 2) of *E. limosum*[1]

	Urine		Feces	
	IX	8-PN	IX	8-PN
		%		
Expt. 1 (IX)	30.6 ± 6.9	n.d.[2]	47.2 ± 13.1	n.d.
Expt. 2 (IX + *E. limosum*)	32.9 ± 7.2	45.8 ± 7.2*	37.4 ± 7.8*	68.8 ± 9.7*

[1] Values are means ± SEM, $n = 6$. *Different from Expt. 1, $P < 0.05$.
[2] n.d., Not detected.

of the biological samples from the GF rats after dosing IX, colonization of the rats with fecal microbiota from subjects with a high (Hop +) or a low (Hop −) 8-PN production status, resulted in rats with different 8-PN production and excretion. Similarly, inoculation of the TWINSHIME with these samples resulted in an 8-PN–producing (Hop +) and nonproducing (Hop −) compartment.

Moreover, a relation was found between the ability of the rat intestinal contents to produce 8-PN from IX in vitro and the 8-PN recovery in rat excreta. Also, 8-PN was only detected in organs of the Hop + rats after IX administration. This can be considered as definitive evidence that differences in the intestinal metabolic potential not only determine 8-PN production but also 8-PN exposure after hop consumption.

As differences in phytoestrogen exposure due to differential intestinal metabolism may hamper therapeutic applicability, *E. limosum* was used as probiotic to equilibrate the 8-PN exposure. When only IX was administered to the TWINSHIME, 8-PN production was observed in only the Hop + SHIME, with up to 80% conversion. After dosing the probiotic in the ascending colon of both SHIME compartments, high bacterial concentrations were detected in the colon regions, coinciding with 40% 8-PN production in the Hop – compartment. Moreover, the butyrate production in the ascending colon doubled, also leading to increased butyrate concentrations in the rest of the intestine. The survival of probiotics in the SHIME (19,27) has been tested several times and recently the efficacy of a probiotic microbial consortium to increase equol production was shown (28).

Similarly, the Hop + rats produced much more 8-PN than did the Hop – rats. Probiotic administration increased the IX activation in the rat large intestine, as shown by the in vitro incubations of the rat cecal and colonic contents. Molecular analysis confirmed the efficacy of the treatment. *E. limosum* could be recovered in all cecal samples, with the highest titers in the GF rats, due to the lack of competition with indigenous bacteria in these rats. After probiotic treatment, the 8-PN excretion in both feces and urine was similar for all rats. Coincidentally, butyrate concentrations also increased in the cecum of all rats. This shows that the probiotic can survive the stomach and small intestinal environment and equilibrates 8-PN exposure by increasing the IX activation in low 8-PN producers to the levels found in high producers. This is further confirmed by the fact that the phytoestrogen was now detected in the organs of all rats.

Without taking bacterial status into account, the first 24-h total prenylflavonoid excretion relative to the daily dose (2 mg/kg body weight) reached 35.0 ± 6.6% in the feces and 2.4 ± 0.6% in the urine. Although no pharmacokinetic data of IX are available, these data correspond with the few available data on 8-PN excretion. After single oral administration of 50 mg to postmenopausal women, 8-PN was excreted within 48 h in feces

TABLE 4 Comparison of ion intensities of IX-glucuronides and 8-PN-glucuronides in liver, kidney, and uterus of rats administered IX in the absence (Expt. 1) or presence (Expt. 2) of *E. limosum*[1]

	Expt. 1 (IX)				Expt. 2 (IX + *E. limosum*)			
	IX-Glucuronide		8-PN-glucuronide		IX-Glucuronide		8-PN-glucuronide	
	Male	Female	Male	Female	Male	Female	Male	Female
Liver								
Hop +	++	++	++	++	++	++	++	++
Hop −	++	++	—	—	++	++	+++	+++
GF	+++	+++	—	—	+++	++	++	++
Kidney								
Hop +	+	+	—	+	++	++	—	++
Hop −	+	+	—	—	+	++	+	++
GF	+	+	—	—	+	—	+	—
Uterus								
Hop +		—		+		—		+
Hop −		—		—		—		—
GF		++		—		+		+

[1] Ion intensities are scored as follows: high, +++; medium, ++; low, +; not detected, −.

and urine to levels of 23 and 8%, respectively (29). Plasma concentrations were also well within the range of plasma 8-PN concentrations (14.5 μmol/L) detected after 3-mo oral treatment of 6.8 mg 8-PN/kg to rats (30).

GF rats are a good model to study enterohepatic circulation of ingested compounds due to the absence of deconjugating bacteria. The assessment of liver metabolites in the GF rat samples showed that there is enterohepatic circulation of prenylflavonoids. Also, 53% IX-glucuronides were recovered from the GF rat feces. This shows that a major part of IX is first absorbed and then excreted back into the intestine by the liver, similar to what happens with other flavonoids (31). However, in contrast to the low urinary recovery of aglycones for most flavonoids (32), 30% of IX excreted in urine in this research was composed of aglycones and this increased to 46% for 8-PN. Whereas some researchers have shown that the administration of flavonoids through oral gavage may decrease phase II metabolism (32), an alternative explanation is that the presence of the prenyl chain limits phase II metabolism.

In conclusion, this work further indicates the important role of intestinal bacteria in determining the final exposure to 8-PN after consumption of IX-containing hop products. Whereas no 8-PN production was noted in the GF rats, higher 8-PN production occurred in the Hop + intestinal model and rats compared with the Hop – model and rats. In addition, orally administered *E. limosum* reached the colon, increased the butyrate production in the SHIME and rats, and increased the 8-PN production in the Hop – model and Hop – and GF rats. In this way, the final exposure to hop phytoestrogens was modulated by the probiotic, with similar 8-PN production in all HMA rats.

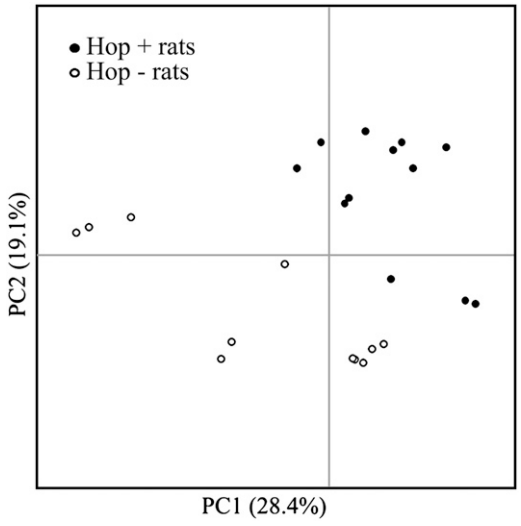

FIGURE 2 PCA score plots from the rat study. Bacterial DNA was extracted from fecal samples taken from Hop + (black circles) and Hop − rats (white circles) ($n = 12$) prior to the start of the experiments, general bacterial DNA was amplified by PCR, and microbial community fingerprints were obtained by DGGE. Clustering of samples indicates similarity in the microbial fingerprints. Percentages of the total variation explained by each principal component are indicated on the axes.

Acknowledgments

We thank Tom Van de Wiele, Bram Pauwels, and Selin Bolca for critically reviewing this manuscript.

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