RESEARCH ARTICLE

Recovery and metabolism of xanthohumol in germ-free and human microbiota-associated rats

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The impact of human intestinal bacteria on the bioavailability of the prenylflavonoid xanthohumol (XN) was studied by comparing germ-free (GF) and human microbiota-associated (HMA) rats. After XN application, XN, XN conjugates, and isoxanthohumol (IX) conjugates occurred in blood samples of GF and HMA rats, whereas IX was detected only in the blood of HMA rats. Overall excretion of XN and its metabolites within 48 h was only 4.6% of the ingested dose in GF rats and 4.2% in HMA rats, feces being the major route of excretion. While both GF and HMA rats excreted XN, IX, and their conjugates with urine and feces, 8-prenylnaringenin and its corresponding conjugates were exclusively observed in the feces of HMA rats. The microbial formation of 8-prenylnaringenin was confirmed by incubation of XN and IX with human fecal slurries. The amount of conjugates excreted in urine and feces was lower in HMA rats compared to GF rats indicating their hydrolysis by human intestinal microbiota. Thus, the impact of bacteria on the XN metabolism in the gut may affect the *in vivo* effects of ingested XN.

Keywords:
8-Prenylnaringenin / Bioavailability / Human gut microbiota / Isoxanthohumol / Xanthohumol

1 Introduction

The prenylflavonoid xanthohumol (XN) (Fig. 1) is the main flavonoid found in hop (*Humulus lupulus* L.) inflorescences (1 g/100 g) [1] and to a smaller extent in beer (up to 0.07 mg/100 mL) [2]. XN isomerizes during wort boiling to isoxanthohumol (IX) [3] (Fig. 1). Owing to proposed health benefits of XN, variations of the brewing process have been developed resulting in XN contents in beer of up to 2 mg/100 mL [4]. Dietary intake of XN is up to 102 μg/day, largely depending on beer consumption [5]. Furthermore, XN-containing dietary supplements marketed for reduction of metabolic stress and natural breast enhancement may contribute to the daily XN intake with up to 4830 μg [6].

XN shows antioxidative and anti-inflammatory properties *in vitro* [7]. The antiproliferative and proapoptotic effects, as observed, for example, with colon cancer cells [8, 9], indicate anticarcinogenic properties of XN. Antimicrobial activities of XN have also been reported [10–15]. In addition to isoflavones and lignans, prenylflavonoids are considered a main group of estrogen-like non-steroidal plant compounds (phytoestrogens) relevant to human nutrition. Also XN shows (anti)estrogenic properties *in vitro* [6, 16–18] and in animal experiments [19]. However, other studies did not find estrogen-like effects of XN [20–22].

There is a general need to understand and consider bioavailability of XN prior to establishment of dose-effect health claims. So far, the bioavailability of XN or other prenylflavonoids including their conversion has been studied sparsely. However, its low recovery following intake of XN by rats or hop extract by humans indicate a limited availability of the intact compound [5, 23, 24], which could

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Abbreviations: GF, germ-free; **HMA,** human microbiota-associated; **i.g.,** intragastric; **IX,** isoxanthohumol; **8-PN,** 8-prenylnaringenin; **XN,** xanthohumol

be due to low absorption, rapid excretion, and/or extensive metabolization of XN. Besides undergoing conversion by phase I/II enzymes, flavonoids reaching the intestine may be transformed by gut microbiota [25, 26]. The application of XN to rats led to the recovery in feces of XN and 22 XN-derived metabolites formed by rat and/or microbial enzymes [27]. However, knowledge on the metabolism of XN in particular by the human gut microbiota is still limited [28].

The aim of this study was to elucidate the impact of human gut bacteria on the bioavailability of XN. Rats associated with a complex human intestinal microbiota (human microbiota-associated, HMA, rats) compared with their germ-free (GF) counterparts (GF rats) were used as a model.

2 Materials and methods

2.1 Chemicals

XN was isolated and kindly provided by Professor Hans Becker (University of the Saarland, Institute for Pharmacognosy and Analytical Phytochemistry, Saarbrücken, Germany) [7]. 8-Prenylnaringenin (8-PN) was obtained from Sigma-Aldrich (Munich, Germany) and IX was from Phytolab (Vestenbergsgreuth, Germany).

2.2 Stability in simulated gastric and intestinal fluids

XN was incubated with sterilized simulated gastric and intestinal fluids [29] under continuous shaking for 2 h at 37°C. XN was added from a 10 mmol/L stock solution in methanol to a final concentration of 100 μmol/L. At different time points, aliquots were taken and stored at −80°C until analysis by HPLC. The incubations were carried out in triplicate. Values are mean ± SD.

2.3 Incubation experiments with human fecal slurries

Fecal suspensions were prepared in an anoxic workstation (MAKS MG, Meintrup dw Scientific, Germany) with a gas phase of N_2:CO_2:H_2 (80:10:10, v/v/v). Human feces from a female healthy volunteer, aged 45 years, who had not taken antibiotics for at least 6 months prior to the study, was diluted to 1% w/v in BHI medium (Roth, Karlsruhe, Germany) supplemented with 0.5 g of L-cystein-HCl and 2.0 mg of resazurin *per* liter. XN and IX were added from a 10 mmol/L stock solution in methanol to a final concentration of *ca.* 100 μmol/L. Cultures (10 mL) were incubated under a gas phase of N_2:CO_2 (80:20, v/v) in 16-mL tubes fitted with butyl rubber stoppers and screw caps for 100 h at 37°C in a water bath equipped with a rotary shaker (120 rpm/min). XN, IX, and fecal suspensions incubated separately in medium served as controls. At different time points, aliquots were taken and were immediately lyophilized. Residues were dissolved in an equivalent volume of methanol and sonicated (45 kHz) for 5 min. After homogenization, the samples were centrifuged (18 200 × *g*, 5 min) and analyzed by HPLC/DAD. All incubations were carried out in triplicate. Values are mean ± SD.

2.4 Animals and treatment

Twenty-five GF male Sprague–Dawley rats bred in our animal facility from stock obtained from Charles River (Sulzfeld, Germany) were used. The rats were kept in positive-pressure isolators (Metall und Plastik, Radolfzell, Germany) and housed in polycarbonate cages on wood chips at 20°C on a 12-h light/dark cycle. They had unrestricted access to an irradiated standard rodent diet (Altromin, Lage, Germany) and autoclaved drinking water. While 14 rats were maintained GF, 11 rats were associated at 10 wk of age with human intestinal microbiota *via* intragastric (i.g.) application of 1 mL of a fecal suspension prepared under anoxic conditions (1:50, w/v, in 0.85% NaCl, 2.7–5.5 × 10^9 cells). Human feces were collected from the volunteer, whose microbiota was tested for XN conversion *in vitro* (2.3). The stability of the intestinal microbiota composition in rats was monitored throughout the study by PCR-coupled denaturing gradient gel electrophoresis using fecal material and calculation of the Sorensen's pairwise similarity coefficient (C_S) as described previously [30]. Throughout the study, the intestinal microbiota of the HMA rats had 55.8–64.5% similarity compared with the human fecal sample used to associate the rats. Likewise, within the time frame of the study, the fecal microbiota of the human donor had 57.4–78.6% similarity to that present at the time of association. The inter-individual similarity of the intestinal microbiota composition of rats ranged from 63.3 to 75.8%. Similarities did not differ significantly between time points. These data correspond to those reported in a previous long-term study with rats [31]. The GF status of the GF rats was confirmed throughout the study by microscopic examination of fresh fecal samples after Gram-staining and by testing bacterial growth in a complex medium inoculated with rat fecal samples as described previously [32]. Fourteen days after inoculation with bacteria, the diet of both, GF and HMA rats, was changed from the standard rodent diet (Altromin) to a semi-purified flavonoid-free diet containing 23.7% casein, 6% mineral mixture (Altromin), 3.3% vitamin mixture (Altromin), 0.2% ascorbic acid, 4.8% corn oil, 3.8% cellulose powder, and the remainder carbohydrate (sucrose:starch, 1:2, w/w). At 13 wk of age, 1 mL of distilled water was i.g. administered to each rat as a control. Urine and feces were collected using metabolic cages (Tecniplast, Hohenpeißenberg, Germany) for 24 h after application of water and pooled for every 12 h. Blood samples were taken from the retrobulbary venous plexus using heparinized

micropipettes (Brand, Wertheim, Germany) at 0.5 and 1 h after application of water. XN was suspended in water and a mean dose ± SD of 48.4 ± 9.4 μmol/kg body weight was i.g. applied twice to each rat, at 18 and 24 wk of age. Following each XN application, urine and feces were collected for 48 h, pooled for every 12 h. Blood samples were taken before and at 0.5, 1, 2, 4, 8, 10, 24, and 48 h after XN administration.

In this animal study, we also investigated the impact of human intestinal microbiota on the bioavailability of two other flavonoids, apigenin-7-glucoside [29] and cyanidin-3-glucoside (our unpublished data), which were applied to the same rats preceding and following the application of XN at intervals of 2 wk each. The results obtained for the first and second XN application predominantly revealed no significant differences. Thus, the intermediate administration of cyanidin-3-glucoside and apigenin-7-glucoside appeared not to influence the metabolism and excretion of XN and the two data sets resulting from the two XN applications were combined for each rat.

After the study was completed, rats were killed by CO_2 inhalation and pathohistological examinations of liver, lung, kidney, myocardium, and spleen were conducted. Furthermore, differential blood counts were determined (Vet-Med-Labor, Ludwigsburg, Germany). According to these examinations, the weight gain, and the overall behavior, the rats' health status was not affected by bacterial colonization or XN application. The Office for Agriculture, Ecology and Regional Planning of the State Brandenburg approved the animal experiments according to § 8.I Animal Welfare Act (approval no. 32-44456).

2.5 Sample preparation

Urine was filtered sterile immediately after sampling. Both urine and whole blood were acidified with 0.1 volumes of acetic acid (1.23 mol/L). Until analysis, urine, feces, and blood were stored at −80°C. XN and its metabolites were extracted from urine by adding methanol and sonication according to a method described by Avula *et al.* [23]. Whole blood and fecal samples were processed as described previously [29]. Briefly, blood samples were repeatedly extracted using acetone, whereas the acidified and lyophilized fecal samples were repeatedly extracted with methanol supported by sonication. The respective pooled supernatants were dried by vacuum centrifugation and dissolved in methanol. Urine and feces were extracted in duplicate. Extraction of blood was carried out once, because of the low sample amount. Glucurono- and sulpho-conjugates were determined indirectly by comparative HPLC/DAD analysis of samples with and without enzymatic hydrolysis of the conjugated forms by β-glucuronidase/sulfatase from *Helix pomatia* (Sigma-Aldrich) as described previously [29]. To determine the recovery of XN, one-fifth of the samples collected during the study were extracted and analyzed a second time after spiking with defined amounts of XN (100 μmol/L). The XN recovery was 66% for blood, 63% for urine, and 95% for feces. The values of the content of XN, IX, and 8-PN in the samples obtained by HPLC analysis were corrected for the relative recovery of XN.

2.6 HPLC analysis

The samples (20-μL aliquots) were analyzed by a Summit HPLC system (Dionex, Idstein, Germany) consisting of a pump (P 680A LPG), an autosampler (ASI-100T), a column oven (TCC-100), a diode-array detector (UVD 340U PDA), and a C18 LiChroSpher reversed-phase column (5 μm, 4.6 × 250 mm; Merck, Darmstadt, Germany) equipped with a C18 LiChroSpher guard column (5 μm). The column temperature was maintained at 35°C. The mobile phase was a mixture of aqueous 0.1% trifluoroacetic acid (solvent A) and methanol (solvent B) and delivered at 0.8 mL/min with a linear gradient of 0–100% B for 25 min. Detection was at 290 and 370 nm. Calibration curves were used for quantification. The limit of detection was 0.05 μmol/L for XN, 0.01 μmol/L for IX, and 1.0 μmol/L for 8-PN, whereas the limit of quantitation was 0.1, 0.05, and 5.0 μmol/L, respectively.

2.7 Data evaluation and statistical analysis of the animal study

The excretion of XN and its metabolites with urine and feces of rats was calculated for each animal and application as percentage of the ingested XN dose based on molar amounts of substance. Statistical analysis was carried out using the software SPSS 11.5 (SPSS, Chicago, IL, USA). Values were tested for normal distribution using the Shapiro–Wilks test [33]. Time effects were tested by analyzing differences between the two XN applications in GF and in HMA rats using a paired *t*-test or, when data were non-normally distributed, by the Wilcoxon's test [34]. Independent data were tested for homogeneity of variance by the Levene test and differences were checked for significance by using an unpaired *t*-test when data were normally distributed [35]. Differences of non-normally distributed data were checked for significance by the Mann–Whitney test [36]. Differences between GF and HMA rats were considered significant at $p < 0.05$ and a trend was defined as $p < 0.1$. Data are presented as their median.

3 Results

3.1 Stability of XN in simulated gastric and intestinal fluids

The stability of XN was tested *in vitro* by incubating XN for 2 h with simulated gastric and intestinal fluids, respectively. The treatment with simulated gastric fluid decreased the XN concentration to 83 ± 26% of its initial value. The incubation with simulated intestinal fluid led to an XN recovery of

80 ± 27% after 1 h and 47 ± 18% after 2 h. IX was not formed under these conditions.

3.2 Incubation of XN and IX with human fecal slurries

During incubation of XN for 100 h with fecal slurries of the volunteer, who provided the sample to generate the HMA rats, 43 ± 8% of XN were degraded. Without bacteria, the XN concentration was 84 ± 1% of the initial concentration after 100 h. IX was detected after incubation of XN with bacteria (average molar percentage of recovery after 100 h, 4.4 ± 1.0%) and without bacteria (8.7 ± 0.8%). In contrast, 8-PN was formed in small amounts only in the presence of bacteria (2.6 ± 1.7%).

Since IX occurred as a product of XN incubation, its transformation was also studied with human fecal slurries. IX concentration remained stable during incubation in the presence and in the absence of bacteria (98 ± 15 and 90 ± 24% of the initial concentration after 100 h, respectively). During incubation of IX with human fecal slurries, small amounts of 8-PN were formed (average molar percentage of recovery after 100 h, 3.9 ± 0.9%).

3.3 Metabolization and excretion of XN in GF and HMA rats

After application of XN (48 μmol/kg body weight), XN, XN conjugates, and IX conjugates occurred in the blood of both GF and HMA rats at low micromolar concentrations. Average concentrations did not differ between GF and HMA rats with one exception. The median concentration of IX conjugates was significantly higher in GF rats 4 h after XN application ($p < 0.05$). IX occurred only in the blood of HMA rats. Maximal concentrations of the compounds detected in the blood of individual rats are listed in Table 1. In GF rats, those concentrations were observed 1 h after XN application, whereas maximal concentrations in HMA rats occurred at 8 h (IX conjugates) or later (10 h: XN and IX, 24 h: XN conjugates).

Table 1. Maximal concentrations of XN and its main metabolites in blood (μmol/L) and feces (μmol/kg wet weight) of individual GF and HMA rats[a)]

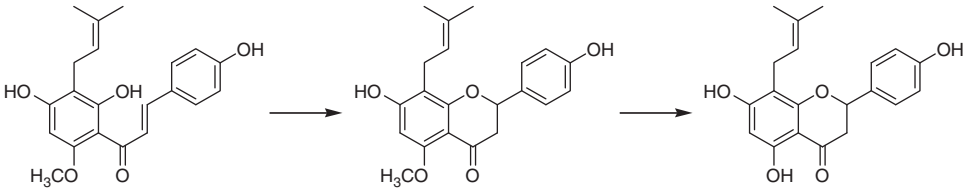
		Blood		Feces	
		GF	HMA	GF	HMA
XN	Free	0.65	0.65	706	958
	Conjugated	0.17	0.11	583	226
IX	Free	ND[b)]	1.04	524	613
	Conjugated	2.24	4.87	201	38.0
8-PN	Free	ND	ND	ND	836
	Conjugated	ND	ND	ND	62.5

a) Values are means of duplicates, $n = 1$.
b) ND, not detected.

In the urine, both GF and HMA rats excreted small amounts of XN and IX in their free and conjugated forms throughout the observation period of 48 h after XN application, resulting in a recovery of less than 0.1% of the ingested XN dose (Figs. 2 and 3). HMA rats had a lower urinary excretion of XN ($p < 0.05$), XN conjugates ($p < 0.001$), and IX ($p < 0.001$) than GF animals (Fig. 2). In both GF and HMA rats, urinary excretion of XN and its metabolites was maximal between 12 and 24 h after XN application (Fig. 3).

The recovery of XN and its metabolites in the feces was similar for GF and HMA rats, whereas the excretion of conjugates was higher in GF rats ($p < 0.01$) (Fig. 4). Conjugates contributed to 21 and 22% of the sum of fecal free and conjugated XN and IX, respectively, excreted by GF rats. The XN and IX conjugates recovered in the feces of HMA rats accounted for only 4.5 and 3.8% of the sum of free and conjugated XN and IX, respectively. The compounds were recovered in the feces of the HMA rats mainly within the first 36 h of collection, whereas the excretion by GF rats took place over the entire observation period of 48 h after XN application (Fig. 5). In addition to XN, IX, and their conjugates, HMA rats excreted 0.85% of the applied XN dose in the form of 8-PN in their feces (Fig. 4). Maximal excretion of the HMA-rat specific 8-PN and its conjugates occurred predominantly 12 to 24 h after XN application (Fig. 5C). Several, not yet identified, potential metabolites were observed in blood, urine, and feces of HMA rats following XN application. The corresponding peaks were not observed in samples of GF animals.

The overall recovery of XN and its identified metabolites in urine and feces was low in both GF and HMA rats, namely 4.6 and 4.2%, respectively, of the applied dose. XN and its metabolites were preferentially excreted with feces, corresponding to 98 and 99% of total excretion in GF and HMA rats, respectively.



Xanthohumol (XN) Isoxanthohumol (IX) 8-Prenylnaringenin (8-PN)

Figure 1. Structures of XN and its derivatives IX and 8-PN.

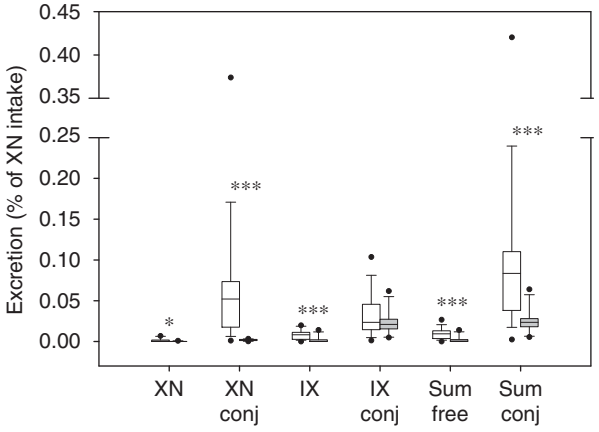


Figure 2. Excretion of XN and its metabolites in their free and conjugated (conj) forms in urine of GF rats ($n = 14$, white bars) and HMA rats ($n = 11$, grey bars) within 48 h after application of XN. In the box-and-whisker plots, the middle line shows the median, the bottom and top of the box represent the 25th and 75th percentiles, and the whiskers indicate the 10th and 90th percentiles. The dots identify outliers. Differences between GF and HMA rats: $^{*}p \leq 0.05$; $^{**}p \leq 0.01$; $^{***}p \leq 0.001$.

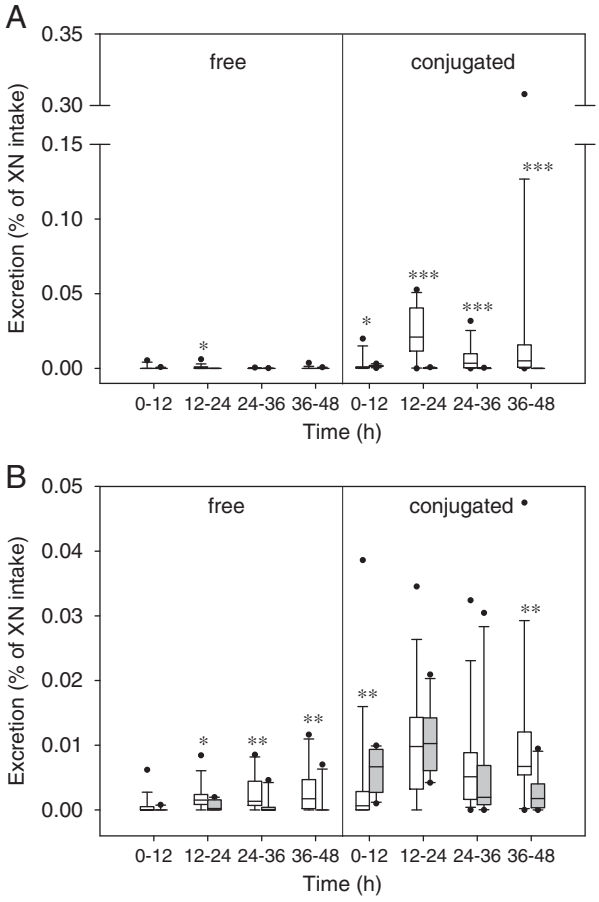


Figure 3. Time-dependent excretion of XN (A) and IX (B) in their free and conjugated forms in urine of GF rats ($n = 14$, white bars) and HMA rats ($n = 11$, grey bars) within 48 h after XN application. Urine of 12-h collection periods was pooled. In the box-and-whisker plots, the middle line shows the median, the bottom and top of the box represent the 25th and 75th percentiles, and the whiskers indicate the 10th and 90th percentiles. The dots identify outliers. Differences between GF and HMA rats: $^{*}p \leq 0.05$; $^{**}p \leq 0.01$; $^{***}p \leq 0.001$.

4 Discussion

Data on the bioavailability of XN and other bioactive prenylflavonoids are needed to judge their efficacy. Hitherto, XN has mainly been investigated regarding its modification by phase I/II enzymes in the liver and the gastrointestinal tract [37, 38]. Thus, our study was focused on the impact of gut bacteria on the recovery of XN. We investigated the conversion of XN by human intestinal microbiota in HMA rats in comparison to GF animals. Beside XN and its conjugates, IX in its free and conjugated forms occurred in both HMA and GF rats. The formation of some IX was also observed during incubation of XN in the absence of bacteria. XN is known to cyclize spontaneously to IX under various conditions [28, 37]. The subsequent conjugation of both XN and IX was previously demonstrated by liver microsomes of rats and humans [39].

Following the application of XN, the *O*-demethylation product of IX, 8-PN, was detected in feces of HMA rats but not in GF rats. This indicates that the conversion of IX to 8-PN is catalyzed by intestinal bacteria but not by rat enzymes. However, liver microsomes and cytochrome P450 enzymes of humans do catalyze the *O*-demethylation of IX [37, 40]. The conversion of IX to 8-PN by the human fecal microbiota was described previously [28, 41]. Moreover, the formation of 8-PN is negatively correlated with the use of antibiotics in humans [5]. *Eubacterium limosum,* a common inhabitant of the human gut, forms 8-PN from IX [28]. Administration of *E. limosum* to GF rats enabled the formation of 8-PN [42]. In our study, 8-PN was formed *in vitro* by incubating XN or IX with fecal slurries of the donor, who provided the sample to generate the HMA rats. The rate of 8-PN formation was similar to that described previously for active human fecal slurries incubated with XN [28]. The *O*-demethylation and cyclization of XN to yield 6-prenylnaringenin is catalyzed neither by bacterial nor human enzymes [28, 37].

Although both urine and feces were analyzed in this study, the overall recovery of the applied XN dose was low, independent of the microbial status of the rats. Total excretion of XN and its metabolites after application of 48 μmol XN *per* kilogram body weight (17 mg/kg body weight) was only 4.2% of the ingested XN dose in HMA rats and 4.6% in GF rats. XN and its metabolites were mainly recovered in the feces; urinary excretion was less than 0.1% of the ingested XN dose in both GF and HMA rats. XN and its metabolites were recovered in the feces of HMA rats preferentially within 36 h after XN application, whereas XN and its metabolites were recovered in the feces of GF animals throughout the entire observation period of 48 h.

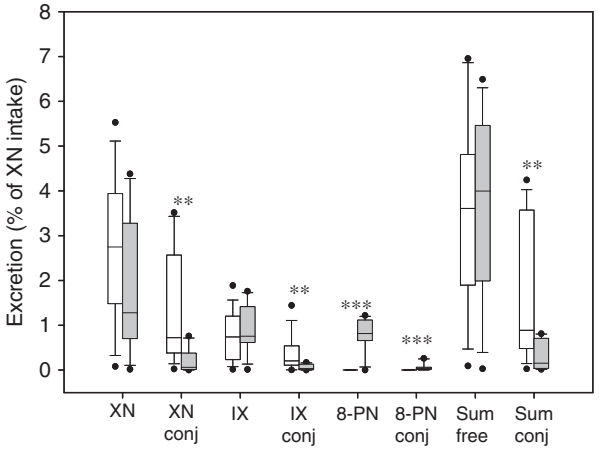


Figure 4. Recovery of XN and its metabolites in their free and conjugated (conj) forms in feces of GF rats ($n = 14$, white bars) and HMA rats ($n = 11$, grey bars) within 48 h after application of XN. In the box-and-whisker plots, the middle line shows the median, the bottom and top of the box represent the 25th and 75th percentiles, and the whiskers indicate the 10th and 90th percentiles. The dots identify outliers. Differences between GF and HMA rats, $^{*}p \leq 0.05$; $^{**}p \leq 0.01$; $^{***}p \leq 0.001$.

This acceleration of excretion with feces may have been caused by a reduced transit time mediated by colonization of the intestine [43]. Recovery of XN and IX conjugates in the feces was tenfold higher in GF compared to HMA rats. In colonized rats, conjugates that are secreted *via* bile or formed by the intestinal epithelium may undergo hydrolysis by bacteria and subsequent enterohepatic circulation. This is in accordance with the fact that the maximal concentration of free XN in blood of HMA rats was observed later than of GF rats. The low recovery of XN has already been observed in previous studies. Conventional rats excreted 0.002 and 1.7% of the ingested XN *via* urine and feces, respectively, within 72 h after application of 50 mg XN *per* kilogram body weight [23]. In the same study, the application of 300 mg XN *per* kilogram body weight resulted in the recovery of 0.02 and 3.6% of the ingested XN dose, in urine and feces, respectively [23]. Excretion of XN and XN glucuronides *via* urine of conventional rats within 24 h after application of 50 mg XN *per* kilogram body weight accounted for 0.2 and 0.35% of the ingested XN dose, respectively [24]. In humans, on average 0.3% of the ingested XN dose was recovered from urine after ingestion of hop extracts [5]. In our study, XN and its metabolites were excreted until the end of the observation period of 48 h after XN application to GF and HMA rats. Possibly, XN excretion was not complete within 48 h owing to its accumulation in tissues of the rats. Conventional rats excreted XN over a period of 72 h [23]. The excretion of XN and its metabolites could have also been delayed by coprophagy, which was not prevented by the experimental design of our animal study. The reduced recovery may to some extent be due to the decomposition of XN to unknown products during intestinal passage. Such

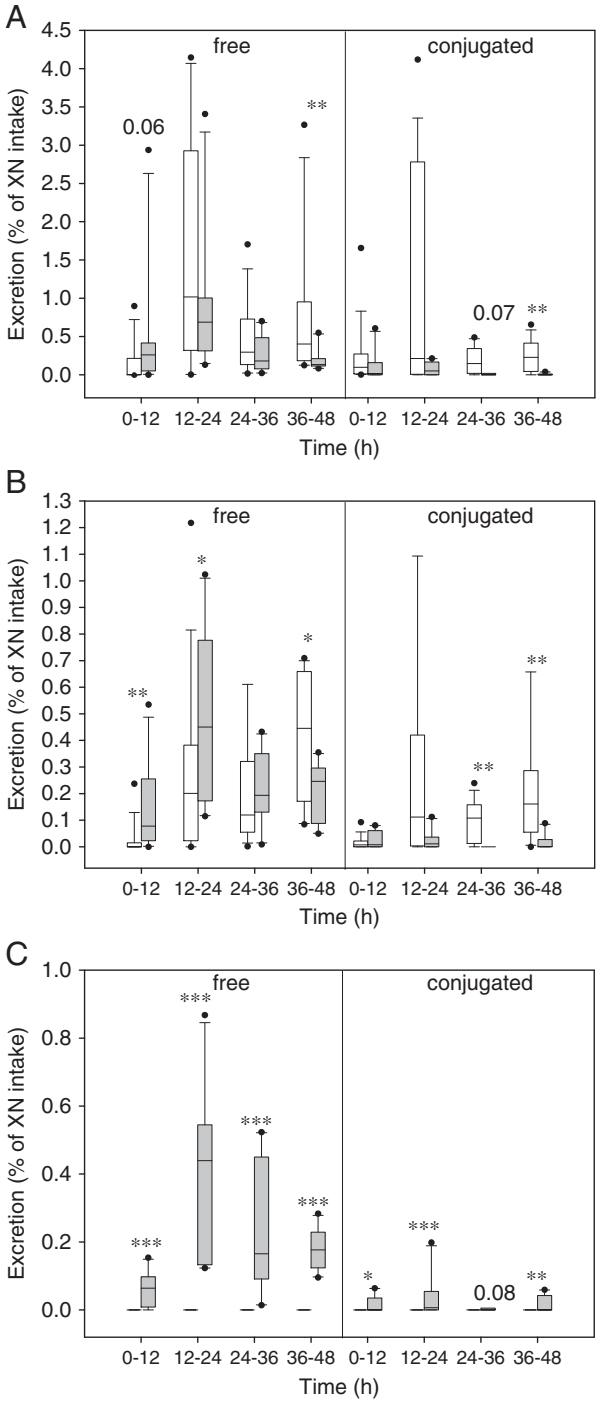


Figure 5. Time-dependent recovery of XN (A), IX (B), and 8-PN (C) in their free and conjugated forms in feces of GF rats ($n = 14$, white bars) and HMA rats ($n = 11$, grey bars) within 48 h after XN application. Feces of 12-h collection periods were pooled. In the box-and-whisker plots, the middle line shows the median, the bottom and top of the box represent the 25th and 75th percentiles, and the whiskers indicate the 10th and 90th percentiles. The dots identify outliers. Differences between GF and HMA rats, $^{*}p \leq 0.05$; $^{**}p \leq 0.01$; $^{***}p \leq 0.001$.

decomposition was observed *in vitro* following the incubation of XN with simulated gastric and intestinal fluids. The detection of not yet identified compounds in blood, urine, and feces of HMA rats following XN application suggests that further microbial metabolites were formed from XN. In feces of conventional rats, a number of minor metabolites were observed following XN application [27].

Maximal concentrations of XN, IX, and 8-PN observed in their free form in blood and feces of HMA rats in this study (Table 1) were compared with concentrations reported to be effective in *in vitro* studies. XN shows chemo-preventive properties, such as modulation of xenobiotic-metabolizing enzymes or inhibition of proliferation, at concentrations up to 30-fold lower than those observed in blood of the HMA rats [7, 9]. At the concentrations measured in blood of the HMA rats, IX has antiangiogenic and antiestrogenic properties [18, 44]. Thirty-fold lower IX concentrations than those detected in blood of the HMA rats are effective in modulating the expression of xenobiotic-metabolizing enzymes [7]. The maximal concentration of IX conjugates observed in the blood of the HMA rats was 4.7-fold higher than that of free IX. However, to date there are no studies on the effects of glucurono- or sulfo-conjugates of IX. On the other hand, these conjugates could be cleaved in target tissues thereby releasing the bioactive IX. 8-PN, which was only detected in the feces of the HMA rats, shows (anti)estrogenic effects. The observed fecal 8-PN concentrations exceed concentrations reported to be effective by four orders of magnitude [17, 45–47]. At 10- to 100-fold lower XN concentrations than those observed in feces of the HMA rats XN has antimutagenic [48], anti-inflammatory [7, 49, 50], and antioxidative effects [7], modulates xenobiotic-metabolizing enzymes, and inhibits proliferation of colon cancer cells [8, 51]. Furthermore, at a 30-fold lower concentration than measured in feces of the HMA rats XN is active against bacterial pathogens [15], whereas the intestinal microbiota of conventional rats is not affected [30]. Antimutagenic [51], anti-inflammatory [7], and anticarcinogenic properties, such as modulation of xenobiotic-metabolizing enzymes or inhibition of proliferation of colon cancer cells [7, 9, 44, 52], have been described for IX at up to 1000-fold lower concentrations than those observed in feces of the HMA rats. However, tenfold lower concentrations of XN and IX than those observed in the feces of the HMA rats possess pro-oxidative properties [53]. Taken together, the maximal concentrations of XN, IX, and 8-PN reached in blood and feces of HMA rats in our study indicate efficacy of these compounds in the systemic circulation and in the colon. The effects reported after intake of XN, *e.g.* reduced tumor growth and angiogenesis in mice [54], may be due to either XN or its metabolites. Since the XN dose used in our animal study largely exceeds the amounts ingested with normal foodstuffs by far, conclusions regarding effects in humans have to be drawn with caution.

In summary, colonization of the rats' gut by human intestinal bacteria did not affect overall recovery of the applied XN dose. However, the excretion of XN and IX conjugates was lower in HMA rats indicating their hydrolysis by human intestinal microbiota. In addition, 8-PN was formed by bacterial *O*-demethylation of IX exclusively in HMA rats. Thus, the microbial impact on metabolism of XN in the gut needs to be considered for evaluation of *in vivo* effects of XN.

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The authors have declared no conflict of interest.

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