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Xanthohumol does not affect the composition of rat intestinal microbiota

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Xanthohumol (XN), a prenylated chalcone, has been proposed to have beneficial effects on human health, including antimicrobial activity. To clarify whether the exposure to XN has an impact on the composition of the intestinal microbiota, 100 mg XN/kg body weight was given daily to rats for 4 wk. Diversity of the fecal microbial community was analyzed using PCR-DGGE. Although intact XN was detected in the feces of the rats at a concentration of up to 2.3 mg/g fecal dry weight, major shifts in the PCR-DGGE patterns in response to this flavonoid were not observed. The similarity index decreased slightly from 70 to 62% for the XN-treated rats and from 71 to 63% for the untreated animals. Thus, changes in the rat fecal microbiota observed in the course of the XN application are most likely due to intraindividual variability. However, the water content of the feces increased significantly during the XN treatment period.

Keywords: Chalcone / Intestinal microbiota / Microbial diversity / PCR-DGGE / Xanthohumol

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1 Introduction

The prenylated chalcone xanthohumol (XN) is the main flavonoid found in hop (*Humulus lupulus* L.) inflorescence (cone) extracts [1] and to a smaller extent in beer [2]. XN is likely to exert various beneficial effects on human health. Effects on cellular activities considered as relevant for the purported anticarcinogenic properties of XN have been described. XN inhibits the growth of cancer cells *in vitro*, which is probably due to the inhibition of DNA synthesis [3, 4]. *In vitro* effects of XN on xenobiotic-metabolizing enzymes have been observed [4 – 7]. XN has been characterized as an effective antioxidant [3, 4]. Contrary to its previously characterized antiestrogenic property [4, 8], XN has been shown to inhibit bone resorption [9]. XN influences the triacylglycerol formation *in vitro* by inhibition of diacylglycerol acyltransferase [10]. In addition, XN exhibits anti-inflammatory effects [4] and inhibits the production of nitric oxide (NO) by suppressing the expression of the inducible NO synthase [4, 11]. Antifungal [12] and antiviral [13] activities of XN have also been reported. Since hop resins are known to have antimicrobial activity [14, 15], the antibacterial properties of XN have been investigated as well. *Staphylococcus aureus* and *Streptococcus mutans* were inhibited by XN [12, 16]. The growth of other oral streptococci, such as *Streptococcus salivarius* and *Streptococcus sanguis* was also repressed by XN [16].

To date, there are no data on the absorption, metabolism, and excretion in humans of XN and its metabolites, which are probably formed from this compound following ingestion. After intake, nonabsorbed XN may reach the colon and undergo transformation by intestinal bacteria. Investigations in rats have shown that XN is poorly absorbed after oral administration [17] and only a minor fraction of XN is metabolized in the gastrointestinal tract [18]. Therefore, a high intake of XN results in considerable concentrations of intact XN in the gut, which in turn may influence the growth of intestinal bacteria by its antibacterial activity. The aim of the present study was to clarify whether the exposure of rats to XN has an impact on the composition of the intestinal microbiota.

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Abbreviations: C_s, Sorenson's pairwise similarity coefficient; **XN**, xanthohumol

2 Materials and methods

2.1 Chemicals

All chemicals and solvents were of analytical grade. Reagents for PCR-DGGE were of molecular biology grade.

XN was provided by Professor Hans Becker, University of the Saarland, Institute for Pharmacognosy and Analytical Phytochemistry, Saarbrücken, Germany.

2.2 Animals and treatment

Sprague Dawley rats (aged 10 – 11 wk) weighing 250 – 350 g were obtained from Charles River Wiga, Sulzfeld, Germany. The animals were housed in polycarbonate cages on wood chips at 20°C on a 12 h light-dark cycle. They had unrestricted access to a standard rodent diet (Altromin, Lage, Germany) and drinking water. XN (100 mg/kg body weight) was given daily to each 12 male and 12 female rats for 4 wk in drinking water (containing 1.5% Tween 20 and 0.75% ethanol). Fresh fecal samples were collected from the rats before, once a week during, and 1 wk after XN administration. For comparison, feces were collected from a control group without XN supplementation that consisted of 12 male and 12 female rats. The samples were frozen and stored at −80°C until analysis. Ethical approval for the animal experiments was obtained by the Karlsruhe government agency.

2.3 DNA fingerprinting of bacterial populations

Fecal samples were homogenized in sterile PBS (pH 7.4) and centrifuged for 1 min at 300 × *g*. The supernatant, which contained the bacteria, was removed and centrifuged for 5 min at 14 000 × *g*. The supernatant was discarded, and the pellet was washed twice with sterile PBS (pH 7.4). After treatment of the fecal samples with glass beads and 150 µL TE buffered phenol in the UNIPREP gyrator (UniEquip, Munich, Germany), DNA was extracted for PCR-DGGE analysis, using phenol, phenol/chloroform/isoamyl alcohol (25 : 24 : 1, v/v/v) and chloroform/isoamyl alcohol (24 : 1, v/v), followed by ethanol precipitation in sodium acetate (968 mM final concentration) over night [19 – 21]. After centrifugation and washing of the pellets, DNA was resuspended in 100 µL TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0).

The DNA was subsequently used as a template to amplify the variable regions V6 – V8 of the bacterial 16S rRNA genes with primers U968-GC-f (5′-CGC CCG GGG CGC GCC CCG GGC GGG GCG GGG GCA CGG GGG GAA CGC GAA GAA CCT TAC-3′) and L1401-r (5′-CGG TGT GTA CAA GAC CC-3′) [22]. The 40-nucleotide GC-rich sequence (G + C clamp) at the 5′-end of the primer U-968-GC-f is underlined. PCR amplification was performed with a *Taq* DNA polymerase kit (Invitrogen, Karlsruhe, Germany) as follows: Each reaction mixture contained each deoxynucleoside triphosphate at a concentration of 0.25 mM, 2.5 U of *Taq* polymerase, Tris-HCl buffer (10 mM Tris-HCl, 120 mM NaCl, pH 8.0), 65 mM $MgCl_2$, 10 pM of each primer, and 1 µL of DNA solution obtained from the fecal DNA extractions. The final volume of the reaction mixture was adjusted to 50 µL with water. PCR amplification was performed using a PCR thermal cycler (ThermoHybaid, Ashford, UK) under the following conditions: initial denaturation at 94°C for 5 min, denaturation at 94°C for 30 s, and touchdown primer annealing temperature decreasing linearly from 66 to 59°C within 21 cycles for 20 s, followed by 14 cycles with an annealing temperature of 59°C for 20 s, and primer extension at 72°C for 40 s. The PCR amplification was run for a total of 35 cycles followed by a final extension for 10 min at 72°C. Amplification of PCR products of the proper size was confirmed by agarose gel electrophoresis. For DGGE analysis, the amount of PCR products was estimated by comparison with a low DNA mass ladder (Invitrogen).

Approximately 100 ng of PCR products was analyzed by DGGE with the CBS Scientific DGGE system (CBS Scientific, Del Mar, CA, USA) on a denaturing polyacrylamide gel (dimensions, 175 × 200 × 1 mm), consisting of 8% v/v acrylamide (acrylamide/bisacrylamide, 37.5 : 1) and TAE buffer (20 mM Tris, 10 mM acetic acid, 5 mM EDTA, pH 8.3), with a 40 – 55% gradient of 7 M urea and 40% v/v formamide that increased in the direction of the electrophoresis. The denaturing gel was overlaid by a stacking gel without denaturing agents. Gels were polymerized on GelBond PAG gel support films (Cambrex Bio Science Verviers, Apen, Germany). Electrophoresis was conducted at a constant temperature of 60°C at 212 V for 10 min followed by 100 V for 22 h in TAE buffer (40 mM Tris, 20 mM acetic acid, 10 mM EDTA, pH 8.3). After electrophoresis, gels were silver stained as described by Sanguinetti and coworkers [23] with the following modifications. Gels were incubated in fixing solution for 60 min. The developing solution contained 1.5% w/v NaOH and 0.3% v/v formaldehyde. The gels were stained for 10 min in 11.8 mM $AgNO_3$ solution and washed twice with water for 5 min. After fixation, the gels were placed for 7 min in a storage solution containing 25% v/v ethanol and 10% v/v glycerol. Gels were scanned using GS-800 calibrated imaging densitometer (BioRad, Munich, Germany) and analyzed with the Quantity One software version 4.3.1 (BioRad). Comparison of the DGGE band patterns of individual samples was performed by Sorensen's pairwise similarity coefficient (C_s) [24] using the similarity matrix function of the software. C_s is defined as

$$C_s = \left[\frac{2j}{a+b}\right] \times 100$$

where a is the number of bands in lane one, b is the number of bands in lane two, and j is the number of common bands in both lanes. Two identical profiles result in a C_s value of

100%, whereas completely different profiles (no common band) result in a C_s value of 0%. Reproducibility of PCR-DGGE was tested by running several DGGE gels with the PCR products from identical randomly selected samples. The variation coefficient determined from similarity analyses of these samples was below 10%.

2.4 Extraction of fecal samples

One volume of each fecal sample was acidified with 0.25 vol. v/w of 5 M formic acid and lyophilized. To determine the fecal water content, the difference between the wet and the dry weight of the feces was calculated.

Eight volumes v/w of methanol (relating to wet weight of the original fecal sample) were added, the samples were sonicated for 10 min, shaken for 10 min, and centrifuged at $7500 \times g$ for 10 min at room temperature. Extraction of the dried fecal material was repeated five times. The pooled supernatants were dried by vacuum centrifugation (Jouan, RC 10.22, Saint Herblain, France). Each residue was dissolved in 1 vol. v/w of methanol (relating to wet weight of the original fecal sample) and centrifuged for 5 min at $18\,200 \times g$. The supernatant was analyzed by HPLC. The experimentally determined recovery rate of XN was 102.1%.

2.5 HPLC analysis

To detect XN in feces, the methanol-extracted samples 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), and a diode-array-detector (UVD 340U PDA). A 20 µL aliquot of the samples was injected and chromatographed on a C18 LiChroSpher RP column (5 µm, 4.6×250 mm), 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% TFA (solvent A) in methanol (solvent B) applied in a gradient elution mode. The solvent was delivered at 0.8 ml/min with a linear gradient of 0–100% B for 25 min. Detection was at 370 nm. Calibration curves were used for quantitation. The LOD was 1.77 ng/ml and the LOQ was 35.44 ng/ml. Relative recoveries were taken into consideration when calculating the XN content of the sample.

2.6 Statistical analysis

Values were tested for normal distribution using the Kolmogorov–Smirnov test. Differences were checked for significance by the *t*-test for nonpaired samples and for non-normal distributed data by the Mann–Whitney test using the software SPSS 11.5 (SPSS Inc., Chicago, IL, USA).

3 Results

The aim of the present study was to find out whether the daily oral exposure of rats to XN for 4 wk has an impact on the intestinal microbiota of rats. For that purpose, 100 mg XN/kg body weight was applied daily to male and female rats for 4 wk. A control group remained untreated. The 16S rRNA gene variety in fecal samples from both male and female rats was analyzed by PCR-DGGE. The rRNA gene sequences comprise highly conserved sequence domains interspersed with more variable regions. The highly conserved nature of rRNA enables to amplify rRNA genes from all bacteria using universal 16S rDNA bacteria-domain primers. Due to a denaturing gradient, established in a polyacrylamide gel with urea and formamide, individual PCR products could be separated based on their sequence to generate a specific band pattern, representing the different microbial species present in the sample. PCR-DGGE profiles of the fecal samples of the rats were determined prior, weekly during, and after the application of XN. In addition, the fecal XN content was determined.

3.1 Impact of XN on the composition of rat fecal microbiota

To monitor intraindividual changes in microbiota composition in response to XN, the DGGE profiles obtained from the fecal samples of a given animal collected before the application of XN were compared with those collected at various time points during and after XN application. In the course of the study, intraindividual C_s values slightly decreased from 70 to 62% for the XN group and from 71 to 63% for the control group (Fig. 1). No conspicuous differences attributable to the application of XN could be observed in the PCR-DGGE band patterns. Gender-related effects were not observed.

Interindividual differences were determined by comparing the PCR-DGGE profiles of either untreated or XN-treated animals for a given time point. PCR-DGGE profiles of female rats showed a similarity of 61–69%. Fecal microbiota of male animals had a slightly higher similarity coefficient of 67–74%. During the observation period, interindividual C_s values increased slightly in both genders. Interindividual similarities of the fecal microbiota ranged from 66 to 71% in the XN group and from 61 to 71% in the control group. In addition, PCR-DGGE band patterns of selected female rats were compared to those of selected male rats of both the treated and the control groups. The gender-specific similarity was 50–61% and unrelated to the time point of the study.

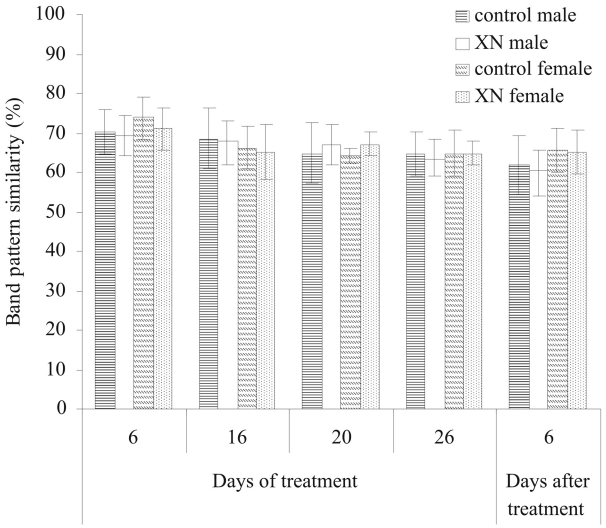

Figure 1. PCR-DGGE band pattern similarity (C_s values) of rat fecal samples after different periods of XN treatment. Fecal samples of a given animal collected before the application of XN were compared with those collected at various time points during and after application of XN. Values are given as the mean ± SD.

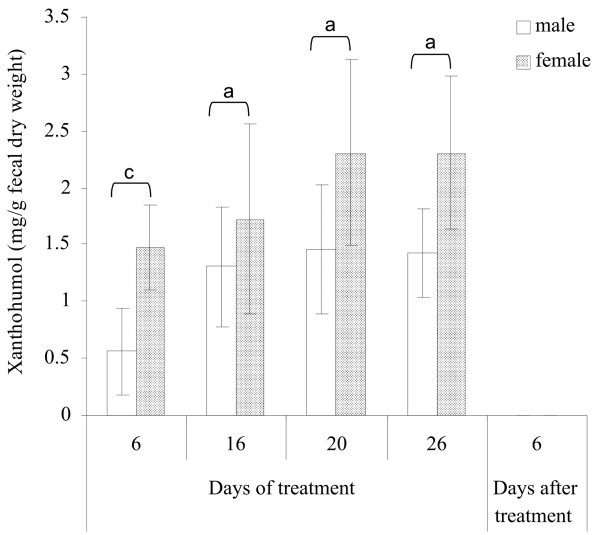

Figure 2. XN content of fecal samples of rats to which XN was administered. Values are given as mean ± SD. (a) Significant ($p < 0.05$), (b) highly significant ($p < 0.01$), (c) very highly significant ($p < 0.001$).

3.2 Detection of XN in rat fecal samples

XN was recovered in concentrations of up to 2.3 mg/g fecal dry weight from fecal samples of rats treated with this compound, whereas XN was neither detected in the feces of untreated animals nor in samples collected before the application of XN in the XN-treated group. Daily XN application resulted in an increased fecal XN content, which was maximal after 20 days of treatment (Fig. 2). The XN content in fecal samples after 20 days of treatment remained stable until the end of the treatment period. Changes in fecal XN content in the course of the study were found to be similar in male and female rats. However, gender-specific significant differences were observed in the amount of fecal XN ($p < 0.05$). While the fecal XN content of male rats increased during the first 3 wk of the treatment period to approximately 1.4 mg/g fecal dry weight at day 16 of XN treatment, the fecal XN content of female rats reached a concentration of 1.5 mg/g fecal dry weight already after 6 days of daily XN application and increased further to 2.3 mg/g fecal dry weight after 20 days (Fig. 2). Six days after finishing the treatment, XN was still detectable in fecal samples. There were no significant differences in the fecal XN content of male and female rats after discontinuation of the daily XN application.

3.3 Impact of XN on the fecal water content in rat fecal samples

XN application led to a significant increase in the fecal water content during the treatment period ($p < 0.05$)

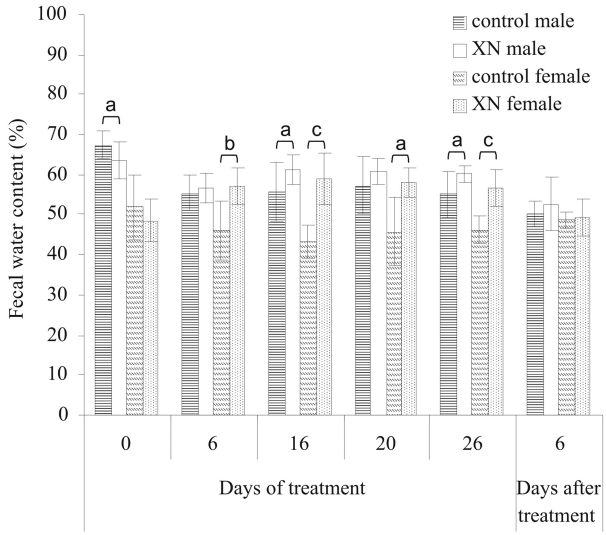

Figure 3. Water content of rat fecal samples after different periods of XN treatment. Values are given as the mean ± SD. (a) Significant ($p < 0.05$), (b) highly significant ($p < 0.01$), (c) very highly significant ($p < 0.001$).

(Fig. 3). The samples collected before and after the treatment and also the samples collected from the untreated rats did not show this difference. The fecal water content of female rats was 50% at the beginning and at the end of the study in both the treatment and the control groups. During the study the fecal water content of female rats increased by up to 16% after 16 days of XN application (Fig. 3). At the beginning of the study the fecal water content of male rats differed significantly between the control group and the treatment group. The fecal water content of male rats was significantly increased by 5% after 16 and 26 days by XN

treatment ($p < 0.05$). After discontinuation of the XN treatment the fecal water content of male rats was 50% in both the XN and the control group (Fig. 3).

The fecal water content differed significantly between male and female rats (data not shown). Independent of XN application, the fecal water content of female rats was significantly lower than that of male rats ($p < 0.05$). Significant differences in the fecal water content of male and female rats were observed at days 0, 6, 16, and 26 ($p < 0.05$). While the fecal water content of males decreased from 65% at the beginning to 50% at the end of the study, it remained more or less constant (54–49%) in the female rats.

4 Discussion

Polyphenolic plant compounds such as condensed tannins have been described to influence intestinal bacteria populations in rats [25]. Using PCR-DGGE, changes in the composition of rat fecal bacteria in response to the exposition to tannins have been observed [25]. This may be due to the fact that certain human intestinal bacteria were reported to be inhibited by tannins [26]. Earlier investigations using cultivation-based techniques also indicated an impact of polyphenols on intestinal bacteria. The supplementation of pigs with tea polyphenols led to a decrease in the number of total bacteria and *Bacteroides* spp. and to an increase in lactobacilli [27]. Intake of green tea polyphenols for 4 wk was reported to result in a decrease of *Clostridium* spp. in humans [28].

The study presented herein should provide information about the impact of XN on the fecal bacteria composition of rats. This is of interest because antibacterial properties of XN against opportunistic pathogens have already been described [12, 16].

Investigating changes in the intestinal microbiota composition is challenging because of the complexity of bacterial communities. Only a small proportion of bacterial species from mammalian gastrointestinal tract are represented by cultivation-based techniques [29, 30]. Molecular fingerprinting analysis, such as PCR-DGGE [31], is a useful technique to achieve a rapid cultivation-independent view of the diversity of intestinal microbiota [32]. This method was used to investigate the impact of XN on the composition of the rat fecal microbiota. The similarity of bacterial composition of rat fecal samples decreased slightly in both the XN-treated and the control groups. Alterations in the PCR-DGGE patterns of fecal samples of rats as a result of the application of XN were not observed. After the application of XN to the rats, intact XN was detected in considerable amounts in the rats feces. Thus, excreted XN apparently had no effect on the fecal microbiota composition of the rats. Changes in the dominant intestinal bacteria composition in animals of both groups observed during the treatment period can most likely be attributed to intraindividual temporal variability. The small temporal variations observed in humans do not affect the overall stability of the gut microbiota. Similarity indices of human fecal samples in a 4 month period were reported to be in a range of 65–88% [33].

In spite of our results it cannot be excluded with certainty that the intestinal bacteria composition was yet affected but that possible changes were not detected because of the limitations of PCR-DGGE. It has to be kept in mind that only dominant groups in the ecosystem will result in a noticeable band in the DGGE gel. Moreover, bacterial diversity may be underestimated by PCR-DGGE, because heterologous sequences may comigrate and denature at the same position in the gel. It is also not really possible to quantitate the bacteria in a given sample by PCR-DGGE. Sorensen's similarity index, which was chosen to analyze the presented data, only considers the presence or absence of bands, but not their intensity. It can therefore not be excluded that XN caused changes in the size of the microbial populations underlying the DGGE bands. Although hundreds of different bacterial species exist in the gastrointestinal tract [34], only a few species predominate, while most of the bacterial species occur in small numbers. It has been estimated that 90–99% of the bacterial community are detectable with PCR-DGGE [19, 31]. However, only dominant fractions of the population can be visualized [19].

The interindividual similarities of the fecal PCR-DGGE profiles of rats are relatively high, showing similarity indices of 61–74% in both the XN-treated and the control groups. This is approximately in the same range as reported for the interindividual variations of 50–70% in genetically identical mice [35]. Although an outbred strain was used in our study, feeding and housing of the rats were done under identical conditions.

The fecal XN contents of male rats reached 1.3 mg/g fecal dry weight after 16 days of daily XN application. The application of 100 mg XN/kg body weight as a single dose to male rats, described in a similar study, resulted in a fecal XN content of approximately 2 mg/g after 24 h [17]. XN might be metabolized by certain bacteria, which in turn may have an impact on the fecal XN content. Fecal XN was detectable in the XN-treated rats 6 days after discontinuation of the treatment in the present study. In contrast, only trace amounts of XN were detected in rat feces 3 days after the application of a single dose of XN [17]. The fecal excretion of XN following the end of the daily XN treatment for several days could be indicative of a transient accumulation of XN in body compartments and excretion into the intestine. Female rats exhibited a higher fecal XN content than

male rats. This could be due to a higher total fecal mass in male rats. However, this hypothesis could not be proven, because total fecal mass was not determined in the presented study.

The fecal water content in the rats increased in response to XN treatment. This effect was particularly pronounced in female rats. Thus, a laxative effect of XN in rats may be suggested. An increased water content of feces has also been observed in rats treated with the phenolic compound tannic acid [36]. The diminished dry weight content in fecal samples may be caused by a lower gut transit time. This would result in a higher proportion of nonfermented carbohydrates, many of which have a considerable water retention capacity.

In conclusion, although XN was present in the large intestine, no impact on the composition of the rat fecal microbiota by XN could be observed using PCR-DGGE. It may be concluded that the oral intake of XN-containing herbal preparations is likely to have no major impacts on the microbial community composition in the intestine. Based on this finding, the reported antibacterial properties of XN appear to be selectively effective against opportunistic pathogens.

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