A 14-Day Double-Blind, Randomized, Controlled Cross-Over Intervention Study With Anti-bacterial Benzyl Isothiocyanate From Nasturtium (Tropaeolum majus) on Human Gut Microbiome and Host Defense

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Article

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Abstract: Despite substantial heterogeneity of studies, there is evidence that antibiotics commonly used in primary care influence the composition of the gastrointestinal microbiota in terms of changing the composition and/or diversity. Benzyl isothiocyanate (BITC) from the food and medicinal plant nasturtium (*Tropaeolum majus*) is known for its antimicrobial activity and is used for the treatment against infections of the draining urinary tract and upper respiratory tract. Against this background, we raised the question whether 14 d nasturtium intervention (3 g daily, N=30 healthy females) could also impact the normal gut microbiota composition. Spot urinary BITC excretion highly correlated with a weak, but significant antibacterial effect against *Escherichia coli*. A significant increase in human beta defensin 1 as parameter for host defense was seen in urine and exhaled breath condensate (EBC) upon *verum* intervention. Pre-to-post analysis revealed that mean gut microbiome composition did not significantly differ between groups, nor did circulating serum metabolome. On an individual level, partly large changes were observed between sampling points, though. Explorative Spearman rank correlation analysis in subgroups revealed associations between gut microbiota and the circulating metabolome, as well as between changes in blood markers and bacterial gut species.

Keywords: nasturtium; BITC; gut microbiome; antimicrobial; metabolome; *Escherichia coli*; human beta defensin 1

1. Introduction

Antibiotics are commonly used in the treatment of bacterial infections. Even though these medications are saving millions of lives, the use of antibiotics is also increasingly leading to multidrug resistant microorganisms. New antibiotics are lacking and thus, there has been great interest in plant antimicrobial properties [1–3].

Nasturtium (Indian cress, *Tropaeolum majus*) is a medicinal plant from the order Brassicales, family *Tropaeolaceae*, which meets the German "Commission E" standards of herbal medicines in 1978 [4]. It is known for its anti-bacterial, anti-fungal, and anti-viral potential and is used for the treatment against infections of the draining urinary tract and upper respiratory tract [5,6]. In combination with horseradish root, nasturtium is also recommended for the prevention of recurrent urinary tract infections (UTI) according to the S3 guideline [5]. Among the most important phytochemicals produced by Brassicales plants are the biologically inactive pro-drugs, named glucosinolates (GLS). The common structure of GLS comprises a β-D-thioglucose group, a sulfonated oxime moiety and a variable side-chain derived from amino acids [7]. Upon cell injury, GLS get into contact with the plants' enzyme myrosinase—a heat sensitive thioglucosidase—which cleaves the sugar moiety and leads to the formation of isothiocyanates (ITC), thiocyanates, or nitriles [8]. ITC are very reactive products and have been intensively investigated for their health promoting and therapeutic activity [9–11]. The degradation product of glucotropaeolin, benzyl isothiocyanate (BITC), is thought to be mainly responsible for the antimicrobial activity observed from nasturtium [9]. As BITC was found to inhibit the growth of different Gram-positive and Gram-negative bacteria, isolated from human faecal material [8], it might be useful in controlling human pathogens through the diet. One mechanistic explanation of the antimicrobial activity is based on the reaction of ITC with thiol groups to form dithiocarbamates, as well as with amino groups to form thioureases [12,13]. These reactions could lead to increased oxidation and inhibition of vital proteins and/or enzymes, resulting in bacterial cell death. In addition, using the Gram-negative *Campylobacter jejuni* NCTC1118i68, transcriptomic analysis suggested that BITC triggers bacterial signaling pathways inducing heat shock and oxidative stress response, protein aggregation and the dysfunction of energy metabolism, leading to bacterial death [14]. On the other hand, this antimicrobial capacity of BITC/nasturtium might also imply the potential for adverse consequences to the homeostasis of the healthy gut microbiome.

Today, various antibiotics have been recognized as major disruptors of the gut microbiota, leading to reduced diversity and dysbiosis, which are associated in consequence with adverse health effects [15–18]. Even a single antibiotic treatment was found to trigger considerable microbial shifts and antibiotic resistance enrichment in faeces of healthy humans [16]. Thus, the aim of the present study was to investigate the effect of BITC-containing nasturtium on the bacterial microbiome in the human gut of healthy subjects. This was investigated as an exploratory endpoint in the context of a two-week randomized, double-blind, controlled crossover nasturtium food intervention.

2. Materials and Methods

2.1. Study Design

This study is a follow-up to a previously conducted trial registered on the German Clinical Trials Register (DRKS) with the ID DRKS00016548. Details of the design and results of the primary study, which assessed the impact of BITC-containing nasturtium on the lipid regulator prostaglandin E2 in human blood samples, have been already reported in [19]. In the present study, we focus on the analysis of secondary parameters. Briefly, the study was conducted as a double-blind, monocentric, controlled, randomized crossover intervention. Subjects were all female, between 20 and 45 years of age, healthy, and non-smokers. Exclusion criteria were acute and chronic diseases, acute gastric/intestinal ulcers, acute kidney inflammation, pregnancy, lactation, intake of antibiotics (acute, during the last three months), known allergy to cruciferous vegetables, or a BMI >25 and <18.5. Following the protocol, 1.5 g nasturtium plant powder was freshly mixed with water and consumed by subjects two times per day, which over the 14-day intervention period corresponded to a total

intake of 42 g. *Verum* contained nasturtium with preformed BITC at the amount of 22.05 ± 8.48 μmol, while the amount of BITC in nasturtium used as control was almost negligible (0.11 ± 0.15 μmol). The subjects were asked to follow their usual dietary habits but excluding foods rich in GLS. In addition to other tests, stool samples were collected from N = 34 subjects at the beginning and end of each intervention phase. No DNA could be isolated from stool samples of 4 subjects due to low amount. High-throughput 16S ribosomal RNA (rRNA) gene sequencing was performed on N = 30. In the first intervention phase, subjects were randomly assigned to either *verum* or control, each of which was administered for two weeks. Following a crossover procedure, in the second intervention phase the subjects who had started with the *verum* sample received the control and vice versa. In between, a wash-out phase of at least two weeks was used to exclude possible carry-over effects. The treatment order was randomized by dividing participants using a random-number generator. Participants documented their diet, medications, unusual exercise, and general well-being on a daily basis.

2.2. Collection of Stool, Urine and Serum Sample

Subjects were asked to collect stool samples before the first consumption of nasturtium on day 1 and on the last intake on day 14 using the OMNIgene-GUT Microbiome kit (DNA Genotek, Ottawa, ON, Canada). Aliquots of stool samples were stored at -80 °C until analysis. Blood samples were collected in the morning before the first intake of nasturtium and 4 h after the last intake on day 14 in coagulant vacutainers by venipuncture. The tubes were gently shaken after blood collection and centrifuged at 2,000 x g for 10 minutes at 4 °C. The upper layer (serum) was collected in 0.6 mL frozen tubes, overlayed with nitrogen and stored at -80 °C until further analysis. On day 1 before taking nasturtium, and on day 14 before the last nasturtium intake, the subjects were asked to collect a spontaneous urine sample and store them in the fridge. When subjects arrived at the study center on day 1 or 14, they provided us with the urine samples, which were then aliquoted and stored at -80 °C until analysis.

2.3. Collection of Exhaled Breath Condensate (EBC)

Exhaled breath condensate (EBC) was collected in the morning before the first intake of nasturtium and 4 h after the last intake on day 14. Participants were asked to breathe uniformly for 10 minutes through the mouth into a Teflon-lined tube (diameter 12 mm, Carl Roth GmbH & Co. KG, Karlsruhe, Germany), cooled with dry ice as described in [20]. The collected EBC was transferred to a 5 mL reaction tube, snap frozen, and stored at -80 °C. For analysis, the samples were freeze-dried (Alpha 2-4 LD, Martin Christ, Osterode am Harz, Germany), and the lyophilized samples were resuspended in 210 μL PBS w/o Ca^{2+} and Mg^{2+} (Life Technologies GmbH, Darmstadt, Germany). Protein quantity was assessed using a NanoDrop ND-1000 spectrophotometer at 230 nm (Thermo Scientific, Freiburg, Germany).

2.4. Faecal Microbial DNA Extraction

The faecal microbial DNA from stool samples was extracted using the QIAamp DNA Stool Mini Kit (Qiagen, Hilden, Germany), according to the manufacturer's protocols. The DNA was quantified using a NanoDrop ND-1000 spectrophotometer (Thermo Scientific, Freiburg, Germany).

2.5. High-Throughput 16S Ribosomal RNA (rRNA) Gene Sequencing

The variable region 3 (V3) and 4 (V4) of the 16S rRNA gene was amplified using the forward primer 5' TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG and reverse primer 5' GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC and sequenced using the 16S sequencing library protocol provided by Ilumina on an Illumina MiSeq System (Illumina, USA) as described elsewhere [21]. In addition, the ZymoBIOMICS Microbial Community DNA Standard from Zymo Research (Freiburg, Germany) was co-sequenced. One of the 30 data sets had to be excluded due to data incompleteness.

2.6. Processing of Microbiome Sequence Data

Sequence data were processed with the mothur-software (v1.39.5) [22] implemented in the public workflow management server Galaxy (usegalaxy.eu, [23]). The analysis was carried out according to the MiSeq SOP as proposed by Kozich et al. [24]. Operational Taxonomic Units (OTUs) were picked against the Greengenes database (v13_8_99) [25] with at least 97% similarity. Very rare OTUs (less than three total counts) were excluded. The sequences were rarefied to a sampling depth of 34,894 sequences per sample. The associated taxonomy was visualized using the Phinch data visualization framework [25].

2.7. Metagenomic Prediction

To estimate the functional-gene profiles across genera, the PICRUSt package integrated in the mother software was applied [27]. Subsequently, high-level phenotypes represented by KEGG Pathways [28] were determined using the BugBase web application [29] with default settings (Sep-2021).

2.8. Metabolome Analysis

Targeted short chain fatty acid (SCFA) analysis was performed using a QTRAP 5500 triple quadrupole mass spectrometer (Sciex, Darmstadt, Germany) coupled to an ExionLC AD (Sciex, Darmstadt, Germany) ultrahigh performance liquid chromatography system, equipped with two ExionLC AD pump systems, an ExionLC degasser, an ExionLC AD autosampler, an ExionLC AD column oven, and an ExionLC controller. A multiple reaction monitoring (MRM) method was used for the detection and quantification of SCFA. The electrospray voltage was set to -4500 V, and the following MS parameters were used: curtain gas (35 psi), collision gas (medium), ion source gas 1 (55 psi), ion source gas 2 (65 psi), and temperature (500 °C). The parameters of the MRM were optimized using commercially available SCFA standards. Chromatographic separation was achieved on a 100 × 2.1 mm, 100 Å, 1.7 μm, Kinetex C18 column (Phenomenex, Aschaffenburg, Germany) using 0.1% formic acid as eluent A and 0.1% formic acid in acetonitrile as eluent B. An injection volume of 1 μL and a flow rate of 0.4 mL/min were used. The gradient elution started at 23% B, which was held for 3 minutes; afterwards, the concentration was increased to 30% B within 1 minute, and to 40% B within 2.5 minutes, and, finally, to 100% B within 1.5 minutes, which was held for an additional 1.5 minutes, followed by equilibration at starting conditions for 2.5 minutes. The column oven was set to 40 °C, and the autosampler to 15 °C. Data acquisition and instrumental control were performed with Analyst 1.7 software (Sciex, Darmstadt, Germany). Data evaluation was performed using MultiQuant (Sciex, Darmstadt, Germany). Serum samples (30 μL) were homogenized in methanol (270 μL) for 20 minutes at 10 °C using a Thermomix (Eppendorf, Hamburg, Germany). For SCFA analysis the derivatization method of [30] was adapted, as previously reported [31]. Specifically, 40 μL of the sample extract was spiked with 15 μL of isotopically labeled standards (ca. 50 μM) and mixed with 20 μL 120 mM EDC HCl-6% pyridine-solution and 20 μL of 200 mM 3-NPH HCl solution. After shaking at 1,000 rpm for 30 minutes at 40 °C using an Eppendorf Thermomix (Eppendorf, Hamburg, Germany), 900 μL acetonitrile/water (50/50, v/v) was added. After centrifugation at 11,337 x g for 2 minutes, the clear supernatant was used for LC-MS/MS analysis.

Targeted analysis of amino acids and amino acid derivates was carried out as previously reported [32] utilizing a QTRAP 6500+ mass spectrometer (Sciex, Darmstadt, Germany) operated in positive ionization mode (ESI+), with an ion spray voltage of +5500 V. Nitrogen served as the curtain gas at 35 psi, while gas 1 and gas 2 were set at 55 psi and 65 psi, respectively. The source temperature was maintained at 450 °C. Multiple reaction monitoring (MRM) mode was utilized, with scheduled detection windows tailored to each metabolite. The UHPLC system (Sciex, Darmstadt, Germany) employed for chromatographic separation consisted of an ExionLC column oven AC, two ExionLC binary gradient pumps, an ExionLC system controller, and an ExionLC autosampler AD. The separation was carried out on an ACQUITY UPLC BEH Amide column (2.1 mm × 100 mm, 130 Å, 1.7 μm, Waters Corporation, Milford, Massachusetts). The column was maintained at a temperature of

40 °C. The mobile phase consisted of water (solvent A) and acetonitrile/water (95/5, v/v, solvent B), both containing 5 mM ammonium acetate buffer (pH 3, adjusted with acetic acid). The following gradient profile was used at a flow rate of 0.4 mL/min: 100% B for 0–1.5 minutes, decreasing to 92% at 3.5 minutes, decreasing to 90% at 7 minutes, decreasing to 78% at 10 minutes, decreasing to 65% at 11.5 minutes, decreasing to 2% B at 12 minutes, holding for 2 minutes, increasing to 100% at 15.5 minutes, and finally holding for 4.5 minutes to re-equilibrate the column. A mixture of 25 µL of serum and 15 µL of isotopically labeled standards (ca. 100 µM) was prepared and equilibrated on a shaker at room temperature for 30 minutes. Protein precipitation and analyte extraction were achieved by adding 95 µL of methanol to the samples, followed by shaking for 3 minutes and centrifugation (15 minutes, 4 °C, 11,337 x g; centrifuge 5424 R, Eppendorf AG, Hamburg, Germany). The resulting supernatant was transferred in an autosampler vial and subjected to UHPLC-MS/MS analysis (injection volume 1 µL).

2.9. Quantification of hBD-1 Release by ELISA Assay

The photometric quantification of human beta-defensin-1 (hBD-1) in urine, serum and EBC samples was performed using the BD-1 Mini ABTS ELISA Development Kits from PrepoTech (Hamburg, Germany) as recommended by the manufacturer. The absorbance was measured after 7–25 minutes of incubation by a microplate reader set at 405 nm and with 650 nm as wavelength correction.

2.10. Broth Microdilution Assay

The well-established, benign laboratory strain *Escherichia coli* (*E. coli*) K12 and the mesophilic human pathogen *E. coli* CFT073, originally isolated from blood and urine of a woman with acute pyelonephritis, were purchased from Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures GmbH (Braunschweig, Germany). *E. coli* AM, isolated from a urine sample of an ambulant patient suffering from UTI, was kindly provided by the Dept. of Hygiene and Microbiology, University Medical Center Freiburg, Germany. Stock cultures for each strain were prepared from a 10 mL overnight culture in LB broth containing 10 g/L tryptone, 5 g/L yeast extract, 10 g/L sodium chloride (18 h, 37 °C, 150 rpm) with 25% glycerol and stored as aliquots at -80 °C. The microdilution method was adapted from the Clinical Laboratory Standards Institute (CLSI) to test the antibacterial capacity of the urine samples (CLSI, 2015). A bacteria suspension of 1×10^8 colony forming units (CFU)/mL was diluted in freshly prepared double concentrated Mueller Hinton (Sigma-Aldrich, Taufkirchen, Germany) cation adjusted broth to reach a test inoculum of approximately 1×10^5 CFU/mL, which is defined as a diagnostic criterion for urinary tract infection. 100 µL of the bacterial suspension were added into the wells of a 96-well U-shaped bottom plate. As positive control, ampicillin (20–2.5 µg/mL, Sigma-Aldrich, Taufkirchen, Germany) was used. 100 µL of the antimicrobial agent or 100 µL of the centrifuged (4500 x g, 10 min) urine samples were added in duplicates to the bacterial suspension in the wells. The plate was sealed and incubated at 37 °C for 7 h without shaking. Absorbance was measured at 600 nm immediately after resuspension and the values were subtracted from a negative control (water and medium). The minimal inhibitory concentration (MIC) for the antibiotics was determined as the lowest concentration, which could still inhibit the visible and spectrophotometric detectable growth of the bacteria.

2.11. Statistical Analysis

The significance of the difference in OTU abundances between *verum* and control treatment was determined using a Wilcoxon signed-rank test with the resulting p-values being controlled for multiple testing by the Benjamini-Hochberg procedure.

To describe the α-diversity of each sample, Berger-Parker, Bootstrap, Chao, inverse-Simpson, and Shannon indices were calculated based on the OTU abundances of each sample. For measuring β-diversity, principal coordinates analysis (PCoA) and non-metric multidimensional scaling (NMDS) of the thetaYC distance matrix among all samples calculated based on their 97% OTU composition

and abundances were conducted. To determine significant differences in β-diversity, analysis of molecular variance (AMOVA) and homogeneity of molecular variance (HOMOVA) tests were applied.

To evaluate the change in hBD-1 expression or antibacterial activity upon intervention, a paired Student's t-test was conducted using the GraphPad Prism 6.0 software (LaJolla, California, USA). Here, p-values   0.05 (*) and   0.01 (**) were considered statistically significant and highly statistically significant, respectively.

For the Spearman correlation analysis the OTU and metabolite abundances were log2-transformed. Species OTU counts were aggregated to genus level counts using the tax_glom function of the phyloseq R package. To determine the fold changes of the metabolites and OTUs caused by the *verum* intervention the differences of the log2-transformed abundances before and after intervention were calculated for all metabolites and OTUs. The Spearman correlation was then calculated between the difference of metabolites and the difference of OTUs. Only those OTUs were included in this analysis which were present for both intervention and control. Also only those subjects were included for which both metabolite and microbiome information was available. For the subgroup of individuals with a >25% upregulated serum PGE_2 this resulted in information on 61 genera, 35 hydrophilic metabolites, and 8 SCFAs acquired from the same 10 subjects. For the subgroup of individuals with a >25% downregulated serum PGE_2 this resulted in information on 56 genera, and 35 hydrophilic metabolites for 5 subjects, which could be used for the correlation analysis between hydrophilic metabolites and the microbiome. Also for this subgroup this resulted in information on 54 genera and 8 SCFAs for 4 subjects, which could be used for the correlation analysis between SCFAs and the microbiome. For this subgroup, all subjects and all OTUs included for the correlation analysis between SCFAs and the microbiome were also present for the correlation analysis between hydrophilic metabolites and the microbiome.

3. Results

3.1. Microbiome Sequence Data

The final dataset after quality control consisted of 10,654,860 sequences from 116 samples (four stool samples each from 29 subjects) with an average length of approximately 450 base pairs, which corresponded to the length of the V3-V4 region (approximately 430 base pairs) [24]. From the initial dataset, a total of 5,922,871 reads were removed in quality filtering, mainly because sequence length was not sufficient. In total, this corresponded to approximately 50,000 reads filtered out per sample.

The mean ± SD bacterial OTU was 91,852 ± 38,244, with a maximum of 220,543 counts and a minimum of 34,894 counts per sample.

3.2. Microbial Composition

Firmicutes and *Bacteroidetes* were the two major phyla of bacteria found in the stool samples of the subjects (Figure 1**A**). 55 families were identified. Seven families (*Ruminococcaceae, Bacteroidaceae, Lachnospiraceae, Prevotellaceae, Rikenellaceae, Porphyromonadaceae, Alcaligenaceae*) comprised >90% of sequences. The core set of typical bacterial families found in the intestines of healthy humans (*Bacteroidaceae, Clostridiaceae, Prevotellaceae, Eubacteriaceae, Ruminococcaceae, Bifidobacteriaceae, Lactobacillaceae, Enterobacteriaceae*) [33] was present in all samples.

On the genus level, it could be seen that all four groups were dominated by *Bacteroides* (23.06–24.46%), followed by *Faecalibacteria* (1.41–18.04%) and *Prevotella* (8.02–10.73%) (Figure 1**B**). 101 genera were classified, with 12 representing >90% of total sequences (*Bacteroides, Faecalibacterium, Prevotella, Oscillospira, Roseburia, Ruminococcus, Parabacteroides, Coprococcus, Alistipes, Sutterella, Clostridium, Akkermansia*) and 70 representing <1%. No significant differences were found in the means of bacteria phyla and genera in the pre-to-post analysis for *verum* and control intervention. In some individuals profound changes in microbiome composition could be seen during the study, though.
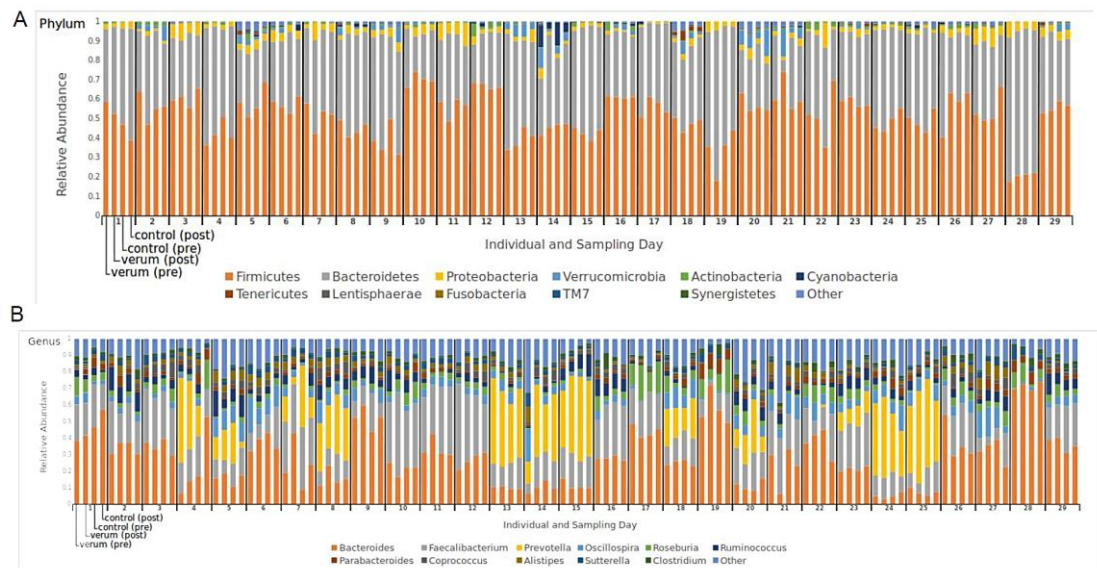
Figure 1. Microbial composition. Fractions of phyla (**A**) and genera (**B**) for each sample; n=29 test subjects.

Using PCA, we found that samples were clustered by individuals rather than by time of sampling, which could suggest stability of the individual microbiome throughout intervention and high inter-individual variation in microbiome composition (Figure 2**A**). Walker et al. [32] also observed this in a clinical trial of 14 overweight men on different controlled diets containing indigestible starch or non-starch polysaccharides. They suggested that this could be associated with inter-individual different responses of the gut microbiota to dietary changes [32]. There were also no significant differences between baseline samples, which could be indicative of the absence of a carry-over effect between the two intervention phases.
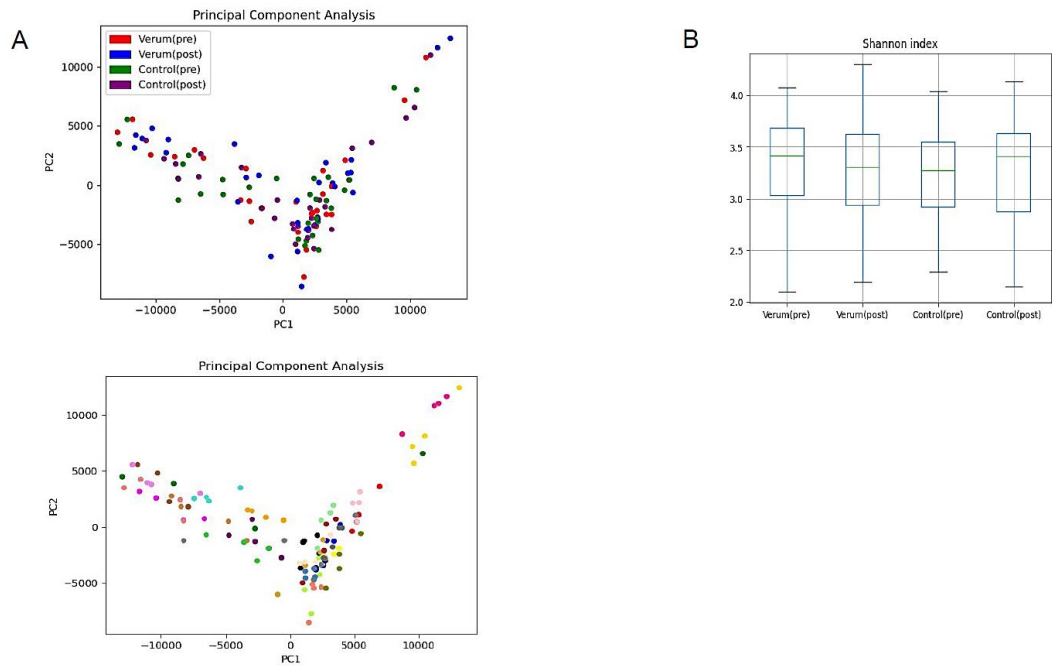

Figure 2. Microbial diversity analysis. Principal Component Analysis (**A**) using the k-means clustering algorithm (above colored by sampling day, below colored by individual), and Boxplot of Shannon index as alpha diversity metric (**B**); n=29 test subjects.

By further inspecting potentially uropathogenic bacteria such as members from the class of Bacilli and the order of Enterobacterales, we found partly big differences in absolute abundance on an individual level, especially in the genera *Streptococcus*, *Turicibacter* (Figure 3**A**) and *Escherichia* (Figure 4**A**). Grouped by sampling day there were again no significant changes in pre-to-post analysis for *verum* and control intervention in these genera, mainly because of high SD as seen in Figures 3**B** and 4**B**.
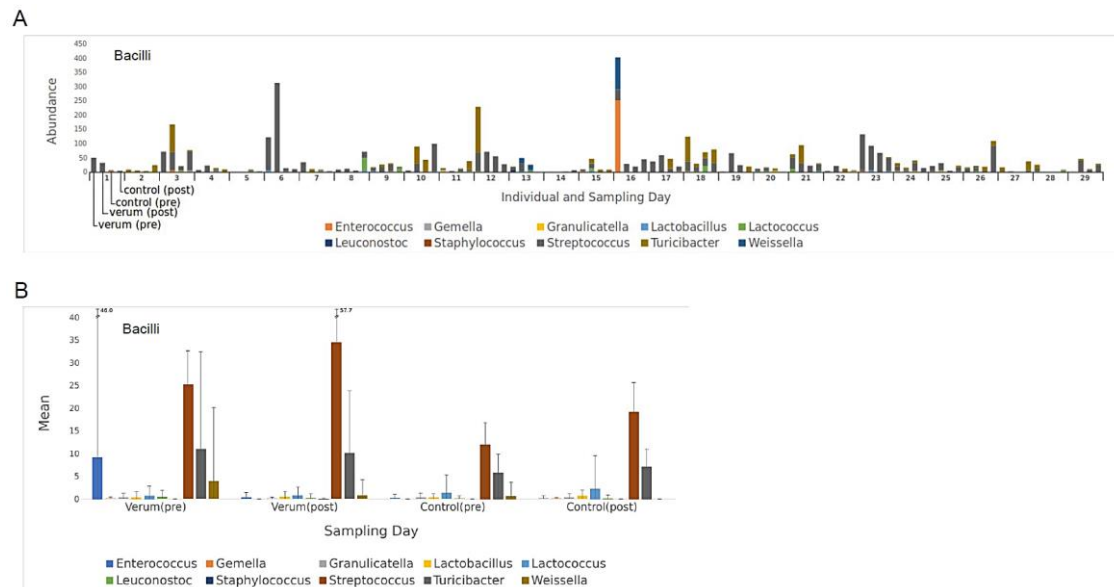

Figure 3. Abundance of potentially uropathogenic bacteria. Absolute abundance per individual and sampling day of bacterial genera from the class Bacilli (**A**), and for each genus the mean and standard deviation per sampling day (**B**).
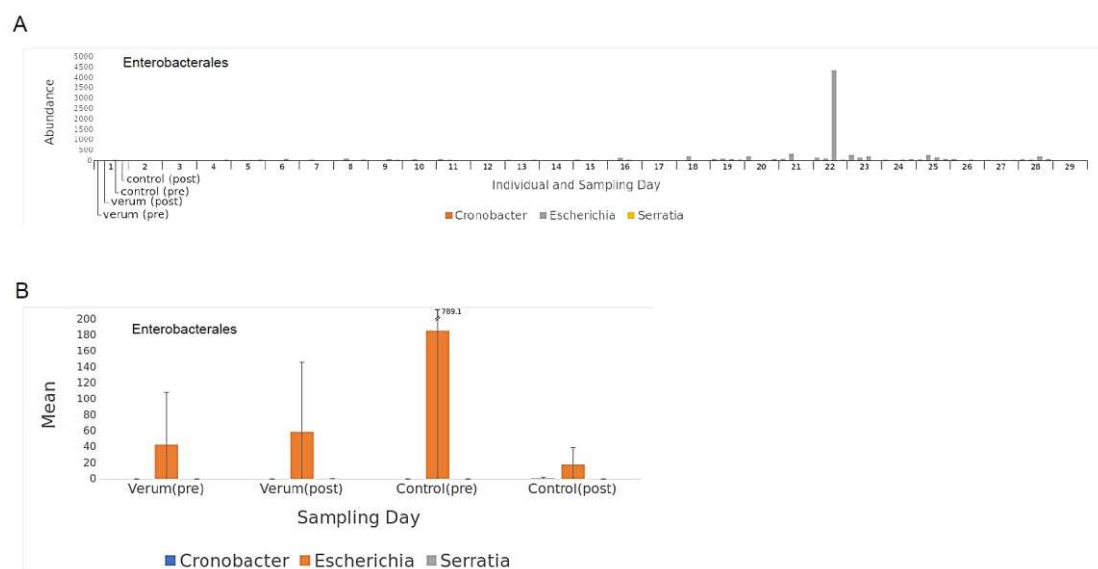

Figure 4. Abundance of potentially uropathogenic bacteria. Absolute abundance per individual and sampling day of bacterial genera from the order Enterobacterales (A), and for each genus the mean and standard deviation per sampling day (B).

3.3. Bacterial Diversity is not affected by Nasturtium Intervention

In addition to the quantitative composition, the diversity of the samples was investigated. The results suggest that the bacterial microbiome of each subject remained relatively stable, although there were large differences in diversity between individuals again. Different alpha diversity metrics

— Observed OTUs, Shannon, Berger-Parker, Bootstrap, Chao and Inverse-Simpson indices — were used as measures of richness (biodiversity) and evenness (equitability) [35]. Nasturtium intervention did not significantly affect bacterial alpha diversity measures, as seen in Figure 2**B** representatively given for the Shannon index. For beta diversity, NMDS of thetaYC (Yue and Clayton theta similarity coefficient) distances between samples based on their 97% OTU composition and abundances showed that bacterial communities were also not significantly affected by nasturtium intervention (Supplementary Figure S1). This is in agreement with non-significant AMOVA (p-value = 1).

3.4. High-Level Phenotypes

After metagenomic prediction with PICRUSt [27], high-level phenotypes were determined, and some KEGG level three modules were predicted using BugBase [29]. As seen in Supplementary Figure S2, treatment did not have a significant effect on these phenotypes.

3.5. Correlation of gut Microbiome and serum Metabolome

It has been shown that the composition of the diet and the gut microbiome can strongly influence the level of metabolites in the human serum [36]. We analyzed SCFA and amino acid content in serum samples before and after both intervention phases.   As given in Supplementary Figure S3, neither amino acid level (Supplementary Figure S3**A-G**) nor mean SCFA level (Supplementary Figure S3**H-J**) in serum were affected by the nasturtium intervention.

In our previous analysis, we found in 18 of 34 subjects a >25% increase in the primary study parameter, serum prostaglandin E2 (PGE$_2$), upon nasturtium intervention (verum), while it was >25% decreased in nine subjects [19]. PGE$_2$ is a multifunctional molecule that orchestrates a wide range of biological responses like tissue homeostasis and inflammation and it is one of the most abundant prostanoids in the human body [37,38]. We thus used the same subgroups of participants here for further investigating whether these large variations in PGE$_2$ response upon nasturtium intervention are associated with a particular change in the composition of the gut microbiome, as previously proposed [39], or serum metabolome. To assess a possible relationship between changes in gut microbiota and circulating metabolites in these subgroups, we calculated the Spearman's rank correlation coefficient between changes in OTUs and metabolite abundances caused by the verum treatment (Figure 5**A,B**). As an approximation for this difference in abundance (displayed in the left side bar in Figure 5**A,B**) the median of the differences between the log2 transformed OTU abundances (pre vs. post treatment of verum) was used. After Benjamini-Hochberg correction of the computed p-values, Spearman's test showed no significance (q-values > 0.05). Unadjusted p-values are shown below to indicate trends.
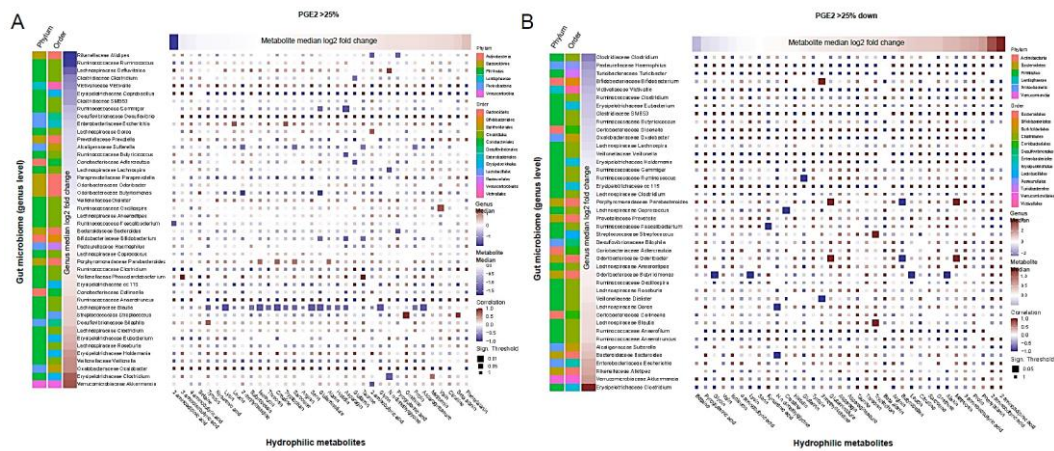

Figure 5. Spearman correlation analysis of the gut microbiome and metabolome. Spearman correlation analysis is conducted on the difference in the log2-fold changes of bacteria genera between pre vs. post treatment with *verum* and the log2-fold changes of metabolites between pre vs. post treatment with *verum*. Spearman correlation of the difference in bacteria genera and the difference in

hydrophilic metabolites (**A**) for subjects with a >25% upregulated serum PGE$_2$, and for the difference in bacteria genera and the difference in hydrophilic metabolites for subjects with a >25% downregulated serum PGE$_2$ (**B**). Correlation with an unadjusted p-value < 0.05 or 0.01 are emphasized by bigger squares in the correlation matrix. Positive correlation coefficients are displayed in red, negative ones in blue. To the left of the matrix, color-coded bars of the phylum and order of the respective genera are displayed. Also, to the left and above the correlation matrix the median of the absolute difference in the log2-transformed abundance of bacteria genera (left) and metabolites (top) between *verum* and control is displayed as a color-coded bar, where blue corresponds to negative and red to positive values. No correlations were significant after FDR correction.

Among individuals with a >25% upregulated serum PGE$_2$, a lower abundance of five genera, namely Ruminococcus, Clostridium (Clostridiaceae), SMB53, Defluviitalea, and Gemmiger, all belonging to the order Enterobacteriales, was detected after verum intervention (Figure 5**A**). Additionally, the genera Alistipes, Victivallis, and Coprobacillus were found with lower abundances in these subjects. Two genera of the family Lachnospiraceae, namely Clostridium (Lachnospiraceae) and Roseburia, and three genera of Erysipelotrichaceae, Eubacterium, Holdemania, and Clostridium (Erysipelorichaceae) were increased in this subgroup after nasturtium intervention. The genera Veillonella, Oxalobacter, and Akkermansia were also increased.

Analysis for the subgroup with a >25% decrease in serum PGE$_2$ revealed a reduced abundance of the genera Clostridium (Clostridiaceae), Haemophilus, Turicibacter, Bifidobacterium, and Victivallis upon verum intervention in these subjects. Intervention-dependent increased abundance was observed in the genera Sutterella, Bacteroides, Escherichia, Alistipes, Akkermansia and Clostridium (Erysipelotrichaceae) (Figure 5**B**). Changes of the genus Bifidobacterium positively correlated here with changes of the hydrophilic serum metabolite 3-methylhistidine (p = 0.017) (Figure 5**B**).

We also compared the bacteria abundance between both subgroups (see Supplementary Figure S4**A**). Here, some bacterial populations changed in opposite directions after verum treatment. Rikenellaceae Alistipes, Ruminococcaceae Ruminococcus, Enterobacteriaceae Escherichia, and Veillonellaceae Dialister were reduced in the subgroup with >25% increase in PGE$_2$, but the same species were increased or showed no change upon intervention in the subgroup with >25% PGE$_2$ reduction. However, after Benjamini-Hochberg correction, the Welch's t-test showed no significance of this effect (q-values > 0.05). In contrast, Alcaligenaceae Sutterella was decreased in the subgroup with a >25% increase of PGE$_2$ while it was increased in the subgroup with a >25% decrease of PGE$_2$ (based on the unadjusted p-values, p = 0.0127). Erysipelotrichaceae Eubacterium was increased in the subgroup with a >25% increase of PGE$_2$, while it was decreased in the subgroup with a >25% decrease of PGE$_2$ (based on the unadjusted p-values, p = 0.0344).

In the subgroup with >25% increase in PGE$_2$ after the intervention the changes in Alistipes for each subject were negatively correlated with the changes in 4-aminobutyric acid (p = 0.037) and pyroglutamic acid (p = 0.037), and positively correlated with the changes in 2-methylbutyric acid (p = 0.014) in serum. The changes in Defluviitalea positively correlated with the changes in N,N-dimethylglycine (p = 0.036), the changes in Clostridium (Clostridiaceae) was negatively correlated with the changes in glutamine (p = 0.043). The changes in Gemmiger negatively correlated with the changes in asparagine levels, (p = 0.006) and SMB53 negatively correlated with the changes in 2-methylbutyric acid (p = 0.006). The changes in Clostridium (Erysipelotrichaceae) were negatively correlated with the changes in serum content of N,N–dimethylglycine (p = 0.010) and positively correlated with changes in methionine (p = 0.050). Changes in Akkermansia positively correlated with changes in 2-methylbutyric acid (p = 0.022), and negatively correlated with changes in 2-aminobutyric acid (p = 0.048) and acetic acid (p = 0.022) (Figure 5**A,B**). In the subgroup with >25% decrease in PGE$_2$ after the intervention the changes of the genus Bifidobacterium positively correlated with changes of the hydrophilic serum metabolite 3-methylhistidine (p = 0.017) (Figure 5**B**).

Correlations between bacterial genera and SCFAs in the subgroup with >25% increase serum PGE$_2$ are shown in Supplementary Figure S4**B**. However, after FDR correction, no q-values were below 0.05. The lack of significance may be partly explained by the low sample number in both

subgroups. No significant correlations were observed between changes in microbiota and changes in SCFAs for the subgroup with >25% decrease in serum PGE_2 (not shown).

3.6. Nasturtium Intervention Increases hBD-1 in Urine and EBC

Beta defensins are small cationic antimicrobial peptides, which are expressed in epithelial cells of the respiratory [40] and urinary tract [41]. They kill or inhibit the growth of bacteria through a multiplicity of antimicrobial mechanisms, which include direct membrane disruption, inhibition of bacterial cell wall synthesis, or neutralizing secreted toxins [42]. While hBD-1 and hBD-2 are active preferably against Gram-negative bacteria [43], intervention of BITC-containing nasturtium significantly increased hBD-1 level in urine (Figure 6**A**), and EBC (Figure 6**B**), but not in serum samples (Figure 6**C**).
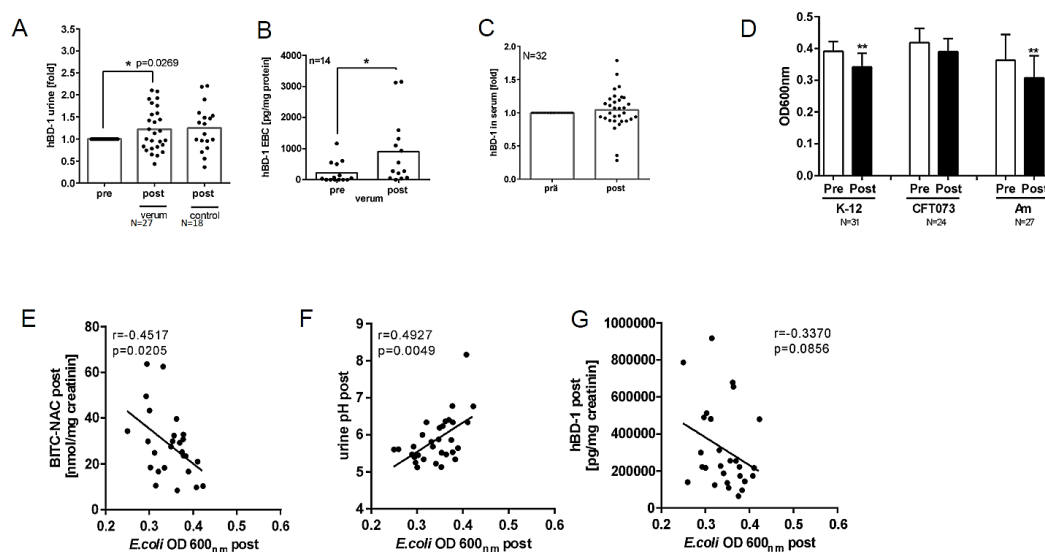

Figure 6. Antimicrobial effects of nasturtium. Quantification of hBD-1 in the samples before (pre) and after (post) nasturtium intervention was done by ELISA in urine (**A**), exhaled breath condensate (**B**), and serum (**C**). Each dot represents results from one subject, tested in duplicates. The line represents the mean. *$p < 0.05$ and **$p < 0.01$. Urine samples were incubated for 12 h at 37 °C with 10^5 CFU/mL of one of the three *E. coli* cultures. Antibacterial activity was measured at OD 600 nm as an indicator for bacterial growth (**D**). Correlations for *E. coli* bacterial growth and post intervention BITC-NAC concentration (n = 26) (**E**), pH (n = 31) (**F**), and hBD-1 concentration (n = 27) (**G**) were calculated using Pearson (**F**) and Spearman (**E, G**) analysis. Statistical significance was described as $p < 0.05$ and $p < 0.01$.

3.7. Nasturtium Intervention Increases the Antibacterial Activity of Urine Samples

In order to investigate whether BITC-containing nasturtium could improve the antibacterial potential of urine, we used the collected spot urine samples from subjects before and after *verum*, and tested them against *E. coli* bacteria. Pre-to-post analysis showed that *verum* intervention significantly inhibited the growth of all three *E. coli* cultures, albeit this effect was weak (Figure 6**D**). Interestingly, the urinary content of BITC-NAC metabolite positively correlated with *E. coli* inhibition (r = 0.4517, p = 0.0205; Figure 6**E**). The correlation between hBD-1 level in urine and bacterial inhibition was insignificant (Figure 6**G**), but there was also a significant positive correlation between the urinary pH and *E. coli* growth (r = 0.4927, p = 0.0049; Figure 6F).

4. Discussion

The primary advantage of using plant-based antimicrobials is that they can be administered without exhibiting the side effects often associated with the use of synthetic chemicals. In addition,

antimicrobial resistance to their bioactive constituents is negligible, due to the multiple mechanisms of the constituents' action potentially preventing selection of resistant bacteria strains [2,44]. On the other hand, the question of *in vivo* efficacy arises; in most cases this has yet to be proven. We could demonstrate here that no relevant perturbations to the gut microbiome in terms of evident changes in species richness or diversity could be detected upon nasturtium intervention, neither in the BITC-containing group nor the control without preformed BITC. In addition, the level of SCFA as well as different amino acids in serum of subjects demonstrated high stability during the whole course of the intervention. Considering the recommended use of nasturtium against infections of the upper respiratory tract and urinary tract [6,9], this could be advantageous. In contrast to our results, in rats exposed to broccoli, which contains the health promoting ITC sulforaphane, compositional changes in the gut microbiota have been reported [45]. Another human study reported that sulfate-reducing bacteria were present in significantly lower levels in six of nine subjects after consuming *Brassica* vegetables over a two-week period [46]. Critically, however, bacteria of the genera *Bilophila* and *Desulfovibrio* were represented in these samples by an average of 0.049% and 0.033% of the total population, respectively. To our knowledge, in this study no error rate using mock community data was given, which would have been useful for assessing the reliability of these findings. Using a well-defined bacteria mock community in our study, we determined an error rate of 0.73%, which is in the range reported by others [47]. Thus, here we did not evaluate bacterial communities below one percent abundance. As with the present study, the usual eating behavior of the subjects was maintained in their study, and supplemented with the investigated plant. Subjects were only instructed to avoid GSL/ITC-containing foods during wash-out and intervention. More detailed data have been reported by [48], in a completely controlled 18-day human feeding study (N=18). This was conducted with 200 g cooked broccoli, and 20 g of raw daikon radish as source for myrosinase per day. The authors found the relative abundance of Firmicutes significantly decreased, while both Bacteroidetes and the genus *Bacteroides* increased [48]. Subjects consumed all their meals on site, which might have been a critical advantage in avoiding microbiome changes due to individual food preferences. This could also explain their clear findings even on phylum level. The broccoli used in all these mentioned studies mainly contains glucoraphanine, which is degraded to the aliphatic ITC sulforaphane [49]; nasturtium predominantly contains the aromatic structured BITC. For aliphatic ITC, a weaker antimicrobial activity has been reported *in vitro* compared to the aromatic ITCs [50,51]. Thus, the higher total intake in GLS as compared to our study could possibly have contributed to more pronounced effects on the gut microbiome despite a smaller study size.

Some studies support the hypothesis that uropathogenic bacteria responsible for UTIs originate from the intestine [52–54]. Here, *E. coli* are the most common uropathogens found in patients with UTI [53], which are then also often found in patients´ intestines [52]. Nowadays, the interaction between the gut microbiome and UTI is intensively investigated as the "gut-UTI axis" [54]. Magruder et al. [54] found, for example, that antibiotic treatment altering the composition of the microbiome led to increases in the prevalence of the uropathogenic bacteria *Escherichia* or *Enterococcus*. Analysis of the number of species from the family *Enterobacteriaceae* — to which *E. coli* belongs — revealed no relevant changes upon intervention in the present study. This bacterial family was present in 28 of 29 subjects. However, one must keep in mind that the present study was conducted with healthy subjects, and we did not record whether the subjects had a history of UTI. Further, we performed V3–V4 sequencing of the microbiome, which limits species identification, and this could be considered here as a disadvantage, as well. In the future, patients actually suffering from UTI or having a history of UTI could be screened for the presence of certain uropathogenic bacteria in their gut microbiome, and then further considered for another intervention study on nasturtium.

For spot urine samples from BITC-containing nasturtium treated subjects a significant, albeit weak, growth inhibitory effect against *E. coli* bacteria was found. BITC-metabolite concentration in subjects´ urine then highly correlated with the observed antibacterial activity. Additionally, hBD-1 — a quick-acting, anti-infective peptide in human host defense — was significantly upregulated upon nasturtium intervention. To date, there is little literature on the bioactivity of the ITC-NAC conjugates in general and to our knowledge, none on the antimicrobial activity of the metabolite in particular.

Preliminary evidence from inhibition experiments with cancer cells suggests here that the NAC-conjugates are also active at a comparable potency [55]. This might be due to their pH-dependent dissociation capacity back into free ITC [56]. So far, several *in vitro* studies have shown sensitivity of both Gram-negative and -positive bacteria to the antimicrobial properties of BITC [8,57]. BITC was effective against species of *Escherichia* and *Klebsiella*, as well as *Bacillus*, *Listeria*, *Salmonella*, *Serratia*, and *Staphylococcus* [57]. The concentration of applied BITC was, however, relatively high, which might provide a good explanation for the discrepancy between our results and these *in vitro* reports. For our experiments, only spontaneous urine samples were available instead of samples containing maximal expected BITC-metabolite levels. These urine samples then contained 41.3 ± 40.79 nM BITC-NAC. However, it is well known, that the concentration of the ITC metabolite can reach up to several mM in the urine [58,59]. Platz et al. [58] demonstrated for example, that upon a single consumption of 10 g fresh nasturtium juice, the urinary excretion of BITC-NAC peaked between 4 and 6 h at 830–2063 μM. Thus, our results are in principle useful to confirm the antimicrobial potential expected from ingested nasturtium in humans, but cannot provide information on the true antibacterial activity in urine.

In the human body, the main route of reactive, lipophile ITC is their absorption in the small intestine, and subsequent excretion via the urine as mercapturic acid pathway metabolites [60–62]. In animal experiments, only a minor part of <10% BITC/BITC-conjugate has been detected to be excreted via faeces [63,64]. The intact hydrophilic and biologically inactive GLS are expected to transit to the large intestine. There, some bacteria strains with β-thioglucosidase activity were shown to be involved in their further processing into the active ITC [65]. Interestingly, a) other intestinal bacteria, e. g. *Bifidobacteria*, seem to metabolize GLS rather to their corresponding inactive nitriles or amines [66,67]; and b) results of one study on gnotobiotic rats even suggest that the human microflora may result in a lower net absorption of ITC by the distal digestive tract due to breakdown to other final products than ITC. Thus, while ITC can reach the bladder at very high concentrations, much lower concentrations could be expected to reach the large intestine, which might help in protecting the gut microbiome from the antimicrobial properties of the ITC.

5. Conclusion

The effect of nasturtium on diversity and species richness of the bacterial gut microbiome was investigated here in the context of a secondary parameter analysis in healthy participants. Our analyses did not reveal any evidence for changes of the microbiome induced by BITC added to the individual diet. However, statistically significant antimicrobial effects of the intervention were found in breath condensate and urine. Furthermore, associations between serum PGE$_2$ and specific gut bacteria or serum metabolites found in an exploratory subgroup analysis were identified. In future studies, establishment of a baseline gut microbiome, generated from at least three different sampling points before intervention, could help account for interindividual variations and for the dynamic nature of the microbiome. This procedure has been suggested earlier [68] and could help to increase the sensitivity in assessment of the effect of dietary intervention.

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