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Chlorogenic acid versus amaranth's caffeoylisocitric acid – gut microbial degradation of caffeic acid derivatives

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Abstract

The almost forgotten crop amaranth has gained renewed interest in recent years due to its immense nutritive potential. Health beneficial effects of certain plants are often attributed to secondary plant metabolites such as phenolic compounds. As these compounds undergo significant metabolism after consumption and are in most cases not absorbed very well, it is important to gain knowledge about absorption, biotransformation, and further metabolism in the human body.

Whilst being hardly found in other edible plants, caffeoylisocitric acid represents the most abundant low molecular phenolic compound in many leafy amaranth species. Given that this may be a potentially bioactive compound, gastrointestinal microbial degradation of this substance was investigated in the present study by performing *in vitro* fermentation tests using three different fecal samples as inocula. The (phenolic) metabolites were analyzed using high-performance liquid chromatography/electrospray ionization tandem mass spectrometry (HPLC-ESI-MS/MS). Furthermore, quantitative polymerase chain reaction (qPCR) analyses were carried out to study the influence on the microbiome and its composition. The *in vitro* fermentations led to different metabolite profiles depending on the specific donor. For example, the metabolite 3-(4-hydroxyphenyl)propionic acid was observed in one fermentation as the main metabolite, whereas 3-(3-hydroxyphenyl)propionic acid was identified in the other fermentations as important. A significant change in selected microorganisms of the gut microbiota however was not detected.

In conclusion, caffeoylisocitric acid from amaranth, which is a source of several esterified phenolic acids in addition to chlorogenic acid, can be metabolized by the human gut microbiota, but the metabolites produced vary between individuals.

Keywords:

amaranth, caffeoylisocitric acid, chlorogenic acid, *in vitro* fermentation, gastrointestinal microbiome

1. Introduction¹

Amaranth (*Amaranthus sp.*) can be used either as a grain, a leafy vegetable, or as an ornamental plant (Zehring et al., 2015). It was an important staple food of the New World in pre-Columbian times but it fell into disuse during the Spanish conquest and was afterwards largely neglected (Council, 1984). However, as an inexpensive source of a diverse variety of compounds with several health benefits and a corresponding high nutritive potential, interest in amaranth as a food source has notably increased again, especially in developing countries in Eastern Africa (Prakash & Pal, 1991; Shukla, Bhargava, Chatterjee, Pandey, & Mishra, 2010). As a result, amaranth is currently regarded as a third millennium crop plant (Rastogi & Shukla, 2013).

Amaranth is a member of the *Amaranthaceae* family with more than 60 species worldwide (Council, 1984). Whilst in Europe and America the grains or seeds of amaranth are considered as pseudo-cereals, in parts of Africa and Asia the leaves are mainly consumed as vegetables (Das, 2016). In some studies, antioxidant,

¹ CA, caffeic acid; C-IA caffeoylisocitric acid; C-QA chlorogenic acid; 3,4-DHPPA, 3-(3,4-dihydroxyphenyl)propionic acid; high-performance liquid chromatography/electrospray ionization tandem mass spectrometry, (HPLC-ESI-MS/MS); 3-HPPA; 3-(3-hydroxyphenyl)propionic acid; 4-HPPA, 3-(4-hydroxyphenyl)propionic acid; 3-PPA, 3-phenylpropionic acid; quantitative polymerase chain reaction, qPCR.

antimalarial, and even antiviral properties of amaranth are described, but the identification of the compounds responsible for such properties is still lacking (Das, 2016; Neugart, Baldermann, Ngwene, Wesonga, & Schreiner, 2017; Shukla et al., 2010; Zehring et al., 2015). Similar to many other promising food and pharmaceutical plants, positive nutritional characteristics are often attributed to bioactive compounds such as secondary plant metabolites (Das, 2016).

One of the major groups of these secondary plant metabolites are phenolic compounds. They are characterized by an aromatic ring with one or more hydroxyl groups (Robbins, 2003). From the very large group of phenolic compounds, phenolic acids are in general phenols which contain a carboxylic acid function. Plant phenolic acids are divided into two groups: hydroxycinnamic acids and hydroxybenzoic acids (Robbins, 2003). In most plants, hydroxycinnamic acids are additionally esterified to sugars, organic acids, or lipids to alter their solubility in cell organelles such as in the vacuole (Clifford, 1999; Marin, Miguelez, Villar, & Lombo, 2015). The most prominent conjugated phenolic acid described is chlorogenic acid (3-O-caffeoylquinic acid, C-QA) which is essentially ubiquitous in the plant kingdom (**Figure 1A**) (Clifford, 1999; Khanam, Oba, Yanase, & Murakami, 2012). A multitude of biological effects has been assigned to C-QA as reviewed by Clifford (1999) turning it into a popular compound often claimed as a health promoting agent. C-QA derivatives have been postulated to be protective against, for example, cardiovascular diseases, type 2 diabetes, and to act as modulator of chemopreventive phase II-enzymes via the Nrf2/ARE pathway (Boettler et al., 2011; Mills et al., 2015). However, C-QA may also have adverse effects, as it may be responsible for stimulating gastric acid secretion causing gastric irritations (Weiss et al., 2010).

In comparison with coffee which is already well characterized, only a few further non-esterified phenolic acids such as vanilic acid, gallic acid, caffeic acid, *p*-coumaric acid, ferulic acid, and *m*-coumaric acid have been described in amaranth thus far (Khanam et al., 2012). The identification of hydroxycinnamic acid esters from the cotyledons of *Amaranthus cruentus* was described in the late 1980s, but detailed knowledge about the phenolic compound composition is still limited (Alvarez-Jubete, Wijngaard, Arendt, & Gallagher, 2010; Strack, Leicht, Bokern, Wray, & Grotjahn, 1987). A new study on the quantification of esterified phenolic acids in amaranth, depending on genotype, has shown that two isomers of caffeoylisocitric acids (C-IA, **Figure 1 B**), and various caffeoylaldaric acids are the main esterified phenolic acids present, as determined in the leaves of 14 different amaranth genotypes tested. Furthermore, feruloylisocitric acids, feruloylaldaric acids, and coumaroylaldaric acids were also present in minor concentrations (Schröter et al., 2017). In the study by Schröter et al. (2017) it has also been shown that the concentration of C-IA is different depending on the amaranth species. For example, the highest concentration were found in *A. hybridus* cv. IP-7 had with $19.39 \pm 1.00 \text{ mg}\cdot\text{g}^{-1}$ dry weight, whereas the concentration in other species such as *A. tricolor* cv. Arkasugna are with $0.66 \pm 0.16 \text{ mg}\cdot\text{g}^{-1}$ dry weight much lower. However, such phenolic acid derivatives are considered quite 'exotic' and their potential for being bioactive with regard to nutritional physiology is not yet defined. Nevertheless, when claiming bioactivity for a certain secondary plant metabolite, it is necessary to obtain information about the absorption, distribution, and biotransformation of these compounds in humans. For phenolic compounds there are several factors that influence the uptake of a substance which was already shown for the prominent and structurally similar C-QA (e.g. Genothier, Verny, Besson, Remesy, and Scalbert, 2003).

The degradation by the colonic microbiota plays an important role, as re-absorption rates of phenolic compounds might differ very drastically depending on their chemical structure (Scalbert & Williamson, 2000). While free phenolic acids such as caffeic acid (CA, chemical structure see **Figure 6**) are nearly completely absorbed in the small intestine, measurements of the recovery of C-QA in ileostomy effluents showed that only 30% of C-QA is taken up by passive diffusion (Manach, Williamson, Morand, Scalbert, & Remesy, 2005; Tomas-Barberan et al., 2014). This is related to the fact that C-QA is more hydrophilic than CA and there are no specific esterases that are able to hydrolyze the ester bonds (Marin et al., 2015). Instead, C-QA reaches the colon and undergoes degradation by the gut microbiota (Ludwig, de Pena, Cid, & Crozier, 2013; Tomas-Barberan et al., 2014). So far, researchers focused on the investigation of C-QA derivatives, as they are consumed worldwide in terms of products, such as coffee, apples, and potatoes (Manach, Scalbert, Morand, Remesy, & Jimenez, 2004). *In vitro* studies on the microbial conversion of C-QA presented a rapid degradation of the substrate within the first few hours and metabolites formed, such as 3-(3,4-dihydroxyphenyl)propionic acid, 3-(3-hydroxyphenyl)propionic acid and benzoic acid (Gonthier et al., 2006; Rechner et al., 2004). Due to the inter-individual microbiota composition of each person, the transformation of plant phenolics may be extremely different. As a result of the polyphenol – bacteria interaction, phenolic compounds may affect the host health positively by promoting beneficial and inhibiting potentially pathogenic bacteria (Braune & Blaut, 2016; Duenas et al., 2015).

With regard to the different phenolic acid derivatives and their specific physicochemical properties such as polarity, solubility, molecular size, and conformation, caffeoylisocitric acid might differ tremendously from C-QA suggesting a

different behavior in the human gastrointestinal tract. With regard to amaranth it is of interest to determine whether or not the nutritive potential of amaranth arises from those exotic phenolic acid esters such as C-IA and/or their biotransformation.

The aim of the present study was to determine the *in vitro* degradation of C-IA and C-QA with fecal microbiota from three different donors to investigate the biotransformation of C-IA by the human gut microbiota. Furthermore, qPCR analyses were performed to determine the initial composition of the microbiota and a possible change during the fermentations.

2. Material and Methods

2.1 Extraction and purification of caffeoylisocitric acid

The isolation of caffeoylisocitric acid (C-IA) was carried out following the protocol described by Schröter et al. (2017). Briefly, 8 mL of 60% methanol were added to 500 mg amaranth (dry matter) and the compounds were extracted under continuous shaking for 30 min at room temperature. Afterwards, the mixture was centrifuged for 5 min at $990 \times g$ at 21 °C. The supernatants were collected and the solvent was removed on a rotary evaporator. The solutions were cleaned-up by solid phase extraction (SPE). The SPE fractions were collected, concentrated with a rotary evaporator and then used for semi preparative liquid chromatography analysis. The fractions of the different semi preparative analysis which contained the C-IA were combined and the organic solvent was removed on a rotary evaporator. Finally, the aqueous solution was lyophilized. From the extract of 1 g freeze dried amaranth powder an amount of approx. 10 mg C-IA was obtained. The purity of the isolated C-IA was assessed by HPLC-DAD-ESI-MS. The amount of C-IA was estimated from

the LC-UV signal at 320 nm based on the assumption that C-IA has a similar molecular extinction due to the fact that CA is the only UV-active part of the molecule C-IA. The purity of the C-IA was 91% (HPLC-DAD, 320 nm). The contamination results from another isomer of C-IA. Moreover, no other contaminants were detected neither by HPLC-DAD nor -ESI-MS detection. The identity of C-IA was assessed following the chromatographic and mass spectrometric parameters as described by previous work (Schröter et al., 2017).

2.2 Preparation of the fecal suspension

Aliquots of 0.5 g of fresh fecal sample from three different, healthy donors (donor A, B, and C) were mixed with 12 mL of pre-reduced phosphate buffered saline and homogenized in a Dispomix Drive (Medic Tools AG, Lussiwag, Switzerland). Each of the fecal suspensions was then used as an inoculum for one of the three *in vitro* fermentations. All three donors did not suffer from gastrointestinal disorders and did not receive any antibiotics within three months prior to donation.

2.3 Fermentation medium

The medium used here contains a range of minerals, vitamins, supplements and carbon sources to enable a wide range of gut bacteria to grow. It has been used in fermentor experiments of the human microbiota (e.g. Walker, Duncan, Leitch, Child, and Flint, 2005) and seems to sustain a diverse microbiota composition. The composition of the fermentation medium per liter distilled water was as follows: 4.5 g NaCl, 3.2 g NaHCO₃, 2 g K₂HPO₄, 1 g casein hydrolysate, 1 g yeast extract, 1 g potato starch, 0.5 g MgSO₄ × 7 H₂O, 0.45 g CaCl₂ × 2 H₂O, 0.25 g xylan from oat spelts, 0.25 g pectin from apple, 0.25 g amylopectin from potato, 0.25 g arabinogalactan from larch wood, 0.005 g FeSO₄ × 7 H₂O, 0.01 g haemin, 0.05 g bile

salts, 0.6 ml resazurin (0.1%), 2 mL mineral solution (500 mg EDTA, 200 mg FeSO₄ × 7 H₂O, 30 mg H₃BO₃, 20 mg CoCl₂ × 6 H₂O, 10 mg ZnSO₄ × 7 H₂O, 7.5 mg NaSeO₃, 3 mg MnCl₂ × 7 H₂O, 3 mg NaMoO₄ × 2 H₂O, 2 mg NiCl₂ 6 H₂O, 1 mg CuCl₂ × 2 H₂O per liter), 3.6 mL volatile fatty acid (VFA) solution (17 mL acetic acid, 1 mL *n*-valeric acid, 1 mL *iso*-valeric acid, 1 mL *iso*-butyric acid neutralized with 20 mL 10 M NaOH), and 0.5 g cysteine HCl. The fermentation medium was boiled for 1 min while flushing with CO₂ and aliquots were dispensed into Hungate tubes with butyl rubber stoppers and screw caps under a CO₂ atmosphere (Bellco Glass, Shrewsbury, UK). Finally the Hungate tubes were autoclaved.

2.4 *In vitro* fermentations with fecal sample microbiota

Three independent *in vitro* fermentations (fermentation A, B and C) with the substrates C-IA and C-QA as a reference were carried out at an initial pH value of 6.5 with a final volume of 10 mL. As the pH could not be controlled, it is estimated that the pH drops by 0.5-1 units during fermentation based on previous experiments with similar levels of carbon source present. Immediately before the incubation of the fermentation media with the fecal suspension was performed, 100 µL of the substrate C-QA or C-IA (final concentration 200 µM, pre-dissolved in DMSO) and 14 µL vitamin solution (10 g nicotinamide, 5 g *p*-aminobenzoic acid, 4 g thiamine, 2 g biotin, 2 g pantothenate, 1 g menadione, 0.5 g cobalamine per liter; filter-sterilised) were added to 9.4 mL of the fermentation medium while flushing with CO₂. Subsequently, 0.5 mL freshly prepared fecal suspension were added (0.2 % final fecal concentration) and were incubated for 48 h on a rotator (Stuart SB3, Bibby Scientific, Stone, UK) at 37 °C and 25 rpm. Initially (0 h) and after 3 h, 7 h, 24 h, and 48 h, two aliquots of 0.75 mL each were taken from the fermentation mixtures under a CO₂ atmosphere. The first aliquot was frozen with liquid nitrogen and stored at -80 °C until HPLC-ESI-

MS/MS analysis. The second aliquot was centrifuged for 10 min at $10,000 \times g$ at $4\text{ }^{\circ}\text{C}$ (Jouan MR 18.22, Sartorius AG, Göttingen, Germany). For DNA extractions and qPCR analysis, only the pellets from the 48 h samples and $400\text{ }\mu\text{L}$ of the fecal suspension from every donor (see 2.2) were used. The latter's were taken to determine the initial microbiota of each donor. Samples were immediately re-suspended in $122\text{ }\mu\text{L}$ MT buffer followed by adding $800\text{ }\mu\text{L}$ sodium phosphate buffer and transferred to Lysing Matrix E tubes (solutions and tubes are used from the FastDNA® spin kit for soil, MP Biomedicals, Illkirch, France). Storage was at $-80\text{ }^{\circ}\text{C}$. Residual pellets were stored as a backup at $-80\text{ }^{\circ}\text{C}$ without buffer treatment.

Every fermentation was conducted in triplicate except the first fermentation of C-QA which was done in duplicate as one incubation failed. Negative control incubations without the addition of C-QA or C-IA were done for every fermentation in triplicate. For that, $100\text{ }\mu\text{L}$ DMSO were added instead of the substrate ('control samples I'). The no-substrate controls were performed in order to estimate the metabolite formation out of the phenolic substances being naturally present in the fecal samples. This is related to the fact that the donors might have consumed food the day before which also contained phenolic acids.

Furthermore, to estimate the interactions between the medium and the substrates, aliquots of 9.9 mL medium were filled into Hungate tubes and mixed with $100\text{ }\mu\text{L}$ of C-QA or C-IA and $14\text{ }\mu\text{L}$ vitamin solution ('control samples II').

2.5 Extraction of chlorogenic acid, caffeoylisocitric acid and its metabolites

$400\text{ }\mu\text{L}$ fermentation mixture were mixed with $15\text{ }\mu\text{L}$ of ascorbic acid solution (20 % in water) for adjusting the pH and as a protector against autoxidation. Afterwards, the

samples were centrifuged for 10 min at $18,000 \times g$ and $4\text{ }^{\circ}\text{C}$ and the supernatants were transferred into a tube. To extract all compounds (remaining substrates and metabolites), $350\text{ }\mu\text{L}$ ethyl acetate were added to the supernatants and pellets, respectively and vortexed. As all substances are of different polarity, the ethyl acetate extraction used in this study was a compromise. However, previous studies have shown its appropriateness (own results, data not shown).

The ethyl acetate phases were combined. The pellets were discarded and the extractions were carried out twice again with the supernatants and $350\text{ }\mu\text{L}$ ethyl acetate as described above. The ethyl acetate phases were collected. All combined ethyl acetate phases were dried under a gaseous stream of nitrogen, residues dissolved in $400\text{ }\mu\text{L}$ methanol/water (1:1, v/v), and vortexed for 20 seconds. Finally, the samples were centrifuged at $18,000 \times g$ and $4\text{ }^{\circ}\text{C}$ for 10 min and $50\text{ }\mu\text{L}$ were transferred into a vial for HPLC-ESI-MS/MS analysis.

2.6 HPLC-ESI-MS/MS analysis

HPLC analyses were done using a 1260 Agilent Series system consisting of a binary pump, an online-degasser, an autosampler, and a column oven. The separation of all substances was carried out on an Ascentis[®] Express F5 column, $15\text{ cm} \times 3\text{ mm}$, $5\text{ }\mu\text{m}$ equipped with an Ascentis[®] Express F5, $5\text{ cm} \times 3\text{ mm}$, $2.7\text{ }\mu\text{m}$ pre-column (both from Sigma Aldrich Chemie GmbH, Taufkirchen, Germany) at $40\text{ }^{\circ}\text{C}$. For the chromatographic analysis, a binary gradient system (eluent A: 0.1% formic acid in water, eluent B: acetonitrile) was used as follows with a flow rate of 0.65 mL/min : 2% B at 0 - 1.5 min, 50% B at 15 min, 90% B at 15.1 - 20 min, 2% B at 20.1 - 25 min. The injection volume was $5\text{ }\mu\text{L}$.

MS analyses were performed in negative ion mode using a QTrap 6500 triple quadrupole mass spectrometer (AB Sciex Germany GmbH, Darmstadt, Germany) equipped with an ESI interface. The electrospray voltage was set at -4500 V and the temperature at 450 °C. Nitrogen was used for the curtain gas (3.5 bar), ion source gas 1 (3.5 bar), ion source gas 2 (4.1 bar), and collision gas. For each substance, the optimal declustering potential, entrance potential, collisions energy, collision exit potential, and characteristic fragments were determined. The fragment with the highest intensity was used for quantification. The fragments with lower intensity were used as qualifiers. The method included the following substances: C-QA, C-IA, caffeic acid (CA), benzoic acid, 3-(3,4-dihydroxyphenyl)propionic acid (3,4-DHPPA), 3-(3-hydroxyphenyl)propionic acid (3-HPPA), 3-(4-hydroxyphenyl)propionic acid (4-HPPA), 3-phenylpropionic acid (3-PPA), *p*-coumaric acid, 3,4-dihydroxybenzoic acid, 3-(3,4-dihydroxyphenyl)acetic acid, hippuric acid, methylhippuric acid, and phenylacetic acid. For quantification, an external calibration was used. The data were generated and analyzed with the software Analyst[®] 1.6.2 from AB Sciex Germany GmbH (Darmstadt, Germany).

2.7 DNA extractions

DNA extractions from the fecal slurries and from the aliquots of the fermentations mixtures were carried out for further qPCR analyses. The DNA was extracted with the FastDNA[®] spin kit for soil (MP Biomedicals, Illkirch, France), using 400 µL of the fecal inoculum and the cell pellets from the 48 h samples, which were already re-suspended in buffer. The DNA extracts were stored at -80 °C until qPCR analysis.

2.8 Fluorimetric DNA quantification and qPCR analysis

DNA was quantified using a Qubit dsDNA BR Assay kit on a Qubit™ 3.0 Fluorimeter (Thermo Fisher Scientific, Renfrew, UK) as per manufacturer's instructions.

Quantitative real-time PCR (qPCR) was performed in duplicate with iTaq™ Universal SYBR® Green Supermix (Bio-Rad) in a total volume of 10 µl in the presence of 1 µg/ml herring sperm DNA with primers at 500 nM and 2 ng of DNA in optical-grade 384-well plates sealed with optical sealing tape. Amplification was performed with a CFX384™ Real-time System (Bio-Rad) with the following protocol: one cycle of 95 °C for 3 min, 40 cycles of 95 °C for 5 s and annealing temperature as per **Table 1** for 30 s, 1 cycle of 95 °C for 10 s and a stepwise increase of the temperature from 65 °C to 95 °C (at 5 s per 0.5 °C) to obtain melt curve data. As described previously (Ramirez-Farias et al., 2009) standard curves consisted of dilution series of amplified bacterial 16S rRNA genes from reference strains. Samples were amplified with universal primers against total bacteria and specific primers against *Bifidobacterium* spp., Bacteroidetes, Ruminococcaceae, Lachnospiraceae, *F. prausnitzii*, *Blautia* spp., and the *Roseburia/Eubacterium rectale* group (**Table 1**).

2.9 Statistical analysis of the qPCR results

To determine a possible change in the microbiota caused by either C-QA or C-IA, statistical analysis was performed. Data were analyzed with IBM SPSS Statistics 22 (Ehningen, Germany). For every donor, the data for the 'control samples I', C-QA, and C-IA out of the triplicate fermentations were examined for normality. The normally distributed data were then investigated for significant differences between the 'control samples I' and either C-QA or C-IA with an independent t-test. A confidence level of 99% ($p < 0.01$) was defined as significant.

3. Results

3.1 Analysis of caffeoylisocitric acid

Caffeoylisocitric acid (C-IA) was extracted from amaranth and enriched with a solid phase extraction approach. Afterwards, further isolation was carried out by semi-preparative HPLC with diode array detection. To confirm the successful isolation and purity of C-IA, mass spectrometric analyses were done by determining the exact mass of the molecular ion as well as the characteristic fragments. C-IA has a nominal mass of 354 atomic mass unit (amu). For the isolated substance, an m/z of 353.05008 (error = 3.8 ppm) was determined, which represents the monoisotopic pseudomolecular ion $[M-H]^-$. Furthermore, characteristic fragments with m/z values 191.01891 (error = 4.3 ppm), 173.00835 (error = 4.7 ppm), 154.99740 (error = 7.7 ppm) and 111.00813 (error = 5.7 ppm) were recorded (**Figure 2**). The first fragments occur from the fragmentation at the ester bond. The m/z 191.01891 results from the ios-citric acid after the loss of the CA followed by the loss of H_2O resulting in a m/z value of 173.00835. The last two dominant signals can be assigned to fragments resulting from subsequent neutral losses of H_2O and CO_2 . In general, the qTOF measurements confirmed the successful isolation of C-IA.

3.2 *In vitro* fermentation of chlorogenic acid

In all *in vitro* fermentations, C-QA was metabolized significantly by the fecal microbiota. The degradation and metabolite formation within the *in vitro* fermentations of C-QA are presented in **Figure 3**. The results represent the mean and the standard deviation of three independent incubations per donor.

Within the first 7 h after the inoculation of the fermentation media with the fecal slurry from donor A ('fermentation A'), an increase of caffeic acid (CA) was detected (**Figure 3A**). While CA was subsequently totally degraded by the microbiota between

7 h and 24 h, a simultaneous increase in the concentration of 3-(3,4-dihydroxyphenyl)propionic acid (3,4-DHPPA, chemical structure see **Figure 6**) was recorded. 3,4-DHPPA reached its concentration maximum after 24 h. Transformation to 3-(4-hydroxyphenyl)propionic acid (4-HPPA, chemical structure see **Figure 6**) was detected between 24 h and 48 h.

In contrast to the *in vitro* fermentation of C-QA with the fecal sample of donor A, a different metabolite profile was identified for the *in vitro* fermentation with the fecal samples from donor B ('fermentation B'). Intermediary CA, was not detected. Instead, the concentration of the metabolite 3,4-DHPPA, as well as the concentration of the metabolite 3-(3-hydroxyphenyl)propionic acid (3-HPPA, chemical structure see **Figure 6**) increased within the first seven hours (**Figure 3B**). 3,4-DHPPA was then further degraded to 3-HPPA and was not detectable after 24 h. The concentration of 3-HPPA increased to 50 μ M within 24 h and remained nearly constant for the subsequent 24 hours.

The metabolite profile from the degradation of C-QA during the early stage of fermentation with the fecal samples C ('fermentation C') was quite similar to fermentation with the fecal sample B (**Figure 3C**). An increase of the concentration of the metabolites 3,4-DHPPA and 3-HPPA was identified within the first 7 h. At this point, the concentration of 3-HPPA was lower than the concentration of 3,4-DHPPA whilst the concentration of 3-HPPA remained nearly constant up to 48 h. For 3,4-DHPPA, a small decrease at 24 h, and a nearly constant concentration during the time period to 48 h was identified. However, the concentration of the latter was always higher than the concentration of 3-HPPA.

In all three fermentations, a nearly constant level of the metabolite 3-phenylpropionic acid (3-PPA, chemical structure see **Figure 6**) was detected over the whole

fermentation period. When taking the results of the 'control samples II' (substrate and medium) additionally into consideration, the presence of 3-PPA probably arises from the interaction of the substrate with the medium, as the same amounts were identified in the 'control samples II' (data not shown).

3.3 *In vitro* fermentation of caffeoylisocitric acid

As well as in the fermentations with C-QA, the fecal microbiota from of all three donors was able to metabolize C-IA. **Figure 4** displays the degradation and metabolite formation within the *in vitro* fermentations of C-IA.

In the fermentation with the fecal sample from donor A, metabolite formation within the first 7 h was not detected (**Figure 4A**). Only small amounts of CA were formed. Between 7 h and 48 h, microbial transformation was apparent, resulting in the formation of the metabolite 3,4-DHPPA to at least 30 μM at 48 h. At the same time, the metabolite 4-HPPA was detected in small amounts.

In fermentation with the fecal sample from donor B, the maximum concentration of 3,4-DHPPA was reached after 7 h. At 24h the metabolite 3,4-DHPPA was totally metabolized and was not detected anymore(**Figure 4B**). Continuous formation of 3-HPPA was registered from 3 h to 48 h.

In fermentation C, the concentrations of 3,4-DHPPA and 3-HPPA increased within the first 7 h (**Figure 4C**). Between 7 h and 48 h, a further slight increase in the formation of 3-HPPA was detected, while the concentration of 3,4-DHPPA fell to a certain extent and remained essentially constant.

As already presented for the fermentation of C-QA, the concentration of 3-PPA in the fermentations with C-IA was nearly constant (data not shown). In contrast to the fermentation with C-QA, the initial level was much higher (100 μM). Furthermore, the

concentration of 3-PPA was similar to the concentration in the 'control samples II' (substrate + medium) in all fermentations. Therefore, the metabolite 3-PPA is not shown in **Figure 4**.

3.4 Fluorimetric DNA quantification and qPCR analysis

To investigate whether the presence of the substrates influenced microbiota composition, qPCR was carried out against major bacterial groups. This included total bacteria, Bacteroidetes, Ruminococcaceae (Firmicutes), Lachnospiraceae (Firmicutes) and *Bifidobacterium* spp. (Actinobacteria), as well as some dominant genera within the Ruminococcaceae (*Faecalibacterium prausnitzii*) and Lachnospiraceae (*Roseburia* spp. & *Eubacterium rectale* as well as *Blautia* spp.). All data showed normal distribution. No significant changes ($p < 0.01$) between the 'control samples I' and C-QA or C-IA after 48 h of incubation were found for any of the bacterial groups analyzed here (**Figure 5**).

4. Discussion

In the present work, *in vitro* fermentations with human fecal microbiota were performed in order to estimate the microbial metabolism of C-IA in comparison to the well-known phenolic acid derivative C-QA. While C-QA is present in a wide range of plants e.g., coffee, C-IA is one of the major phenolic acids in amaranth. Because of the different chemical nature of the esterification, it is of interest to determine if the gut microbiota can also convert C-IA to more lipophilic metabolites which can then be absorbed via the colon.

The major end products of all fermentations were the metabolites 3,4-DHPPA and either 3-HPPA or, 4-HPPA, which is in accordance with *in vitro* fermentations of C-

QA described in the literature (Gonthier et al., 2006; Rechner et al., 2004; Tomas-Barberan et al., 2014). In general, the microbial degradation of C-QA showed the same metabolites as the degradation of C-IA within the same donor. As a first step, the substrate is degraded to CA, which is attributed to the cleavage of the ester bond. Furthermore CA is converted to 3,4-DHPPA, corresponding to the reduction of the double bond. Either 4-HPPA or 3-HPPA were then formed out of 3,4-DHPPA resulting from the dehydroxylation at the C3- or C4-position of the phenolic ring (**Figure 6**). The citrate liberated during biotransformation of C-IA cannot be detected with the used method but may be utilized by some other members of the microbiota, as has been demonstrated for lactic acid bacteria and *Clostridium indolis* (Biddle et al., 2014; Martino, Quintana, Espariz, Blancato, & Magni, 2016).

Interestingly, fermentation with the microbiota from donor A lead to the formation of the metabolite 4-HPPA, whilst fermentation by the microbiota from donors B and C resulted in the formation of the metabolite 3-HPPA which likely reflects differences in the prevailing metabolic pathways in different donors. The two metabolites are generated by dehydroxylation of the aromatic ring in different positions. Chesson et al. (1999) reported that dehydroxylation at C4-position, resulting in 3-HPPA, is the major metabolite deriving from ferulic acid and CA, whereas dehydroxylation at the C3-position, leading to 4-HPPA, is rarely found. When taking the results of the present study into account, it is also recognized that the concentration of 4-HPPA in the fermentations of C-QA and C-IA (present in donor A) is notably lower than the concentration of 3-HPPA found in the fermentations with donors B and C. Furthermore, the early metabolite CA was only detected in fermentation with the fecal sample from donor A. This is in agreement with differences in the time courses of metabolite formation during the fermentations.

For C-QA as well as for C-IA, the rate of the metabolite formation increased in the order of fermentations $A < C < B$. For example, while in fermentation A 3,4-DHPPA was first detected after 24 h for both substrates, the metabolite was already present at its highest concentration after 7 h in fermentation B and C, and 3-HPPA was also present. For fermentation B the metabolite was then completely metabolized until 24 h alongside further accumulation of 3-HPPA, whereas in fermentation C, both 3,4-DHPPA and, at a lower concentration, 3-HPPA were still detected after 48 h.

In general, the substrate degradation, metabolite formation and the time courses of C-QA and C-IA within a donor are very similar. Looking at the fermentations with the fecal slurry from donor A (**Figure 3A and Figure 4A**), the same metabolites (CA, 3,4-DHPPA, 4-HPPA) were detected at the same time points. In contrast to the fermentation of C-QA, where the concentration of 3,4-DHPPA acid had its maximum at 24 h, there is still a further increase of its concentration between 24 h and 48 h when fermenting C-IA. For fermentation B, the same metabolites at the same time points were also present in the fermentation of C-QA and C-IA, but the concentration of 3-HPPA was already higher at 7 h in the C-QA fermentation than the concentration of 3,4-DHPPA. The *in vitro* fermentation of C-IA with the fecal slurry from donor C showed very similar behavior in metabolite formation as in the one of C-QA.

The determination of 3-PPA in every fermentation and control samples at the same concentration level leads to the conclusion that 3-PPA is not a microbial product resulting from the degradation of C-QA or C-IA. It is rather formed by the interaction between the substrate and the medium (see 3.2 and 3.3).

In all fermentations, the concentrations of the metabolites were generally low, and the initial substrates C-QA and C-IA were nearly not detectable even at the beginning of the fermentations. Taking the 'control samples II' into account, where the substrate

was co-incubated with the media, the low concentrations are related to the fact that the substrate already interacts with the medium or is degraded due to the fermentation conditions. Substrate concentration in the control samples and fermentation samples were very similar. As a result, the metabolites can only occur in lower concentration as well. Apart from that, the extraction efficiency/recovery of the metabolites with ethyl acetate depends on its solubility. As C-QA and C-IA are quite hydrophilic, it is assumed that the substrates still remain in the aqueous medium (the fermentation mixture) to a certain extent and were not completely extracted with ethyl acetate. As already mentioned under 2.5, this extraction method was a compromise, as the different compounds represent a broad polarity and solubility range. For every metabolite, the recovery rates were determined in previous studies (data not shown), so it can be guaranteed that the formation of further metabolites in significant concentrations would have been detected as well.

Microbiota analysis did not reveal major differences in composition after 48 hours incubation in the presence of C-QA or I-CA in comparison to the no-substrate control. The analysis performed here targeted the major bacterial groups within the microbiota. However, it cannot be excluded that changes may have occurred for less abundant groups or at a lower phylogenetic scale. All media contained carbohydrates, casein hydrolysate and yeast extract to ensure that the bacteria had an appropriate energy source available, thus microbiota changes were mainly driven by the growth substrates present. The absence of major differences between incubations indicates that the phenolic acids studied here had no major inhibitory effects on the microbiota. In contrast, Mills et al. (2015) have noticed changes in some bacterial groups such as *Bifidobacterium* spp and the *C. coccoides* – *E. rectale* group when fermenting C-QA.

Figure 6 shows schematically the microbial degradation of C-IA based on the *in vitro* data obtained in the present study. Studies on the bioavailability of C-QA compared to CA showed that the absorption of the metabolites of C-QA mainly takes place in the colon after C-QA is degraded by the microbiota, whereas CA is to some extent already absorbed in the upper intestinal tract (Olthof, Hollman, & Katan, 2001; Tomas-Barberan et al., 2014). Due to the similar chemical structures of C-QA to C-IA, it was assumed that C-IA has also a very low absorption rate in the small intestine and fermentation in the colon is much more relevant than absorption of the initial, intact compound. The first step of degradation may involve esterases of microbial origin that cleave the ester bond of C-IA. In a study described by Couteau, McCartney, Gibson, Williamson, and Faulds (2001), some bacterial species being responsible for the esterolysis of conjugated hydroxycinnamic acids have been identified. The results showed that selected strains of *Escherichia coli*, *Bifidobacterium lactis*, and *Lactobacillus gasseri* have esterase activity with the potential to cleave C-QA and ethyl ferulate. Furthermore, Couteau et al. (2001) also described that CA was not further degraded by *E. coli*, *B. lactis*, and *L. gasseri* after the hydrolysis of C-QA. Those results underlined the hypothesis of Peppercorn and Goldman (1971), who demonstrated that twelve strains of human gut bacteria were not able to carry out more than one step in the degradation pathway of CA. Therefore, the following degradation steps observed in the present study – reduction of the double bond and further dehydroxylation – are probably conducted by further microorganisms (present in the fecal sample). It is also possible that cross feeding occurs whereby one strain gives good growth on a primary substrate, which is partially degraded to a product that is a secondary substrate for another strain; therefore, the composition of the microbiota might be influenced to a certain extent (Pfeiffer & Bonhoeffer, 2004).

Even though the metabolite CA was not identified in every fermentation, it can be hypothesized that the degradation of both substrates goes via the formation of CA. It seems that CA is an intermediary metabolite, which is further degraded rapidly to 3,4-DHPPA by a reduction of the double bond. In the studies described by Booth and Williams (1963a, 1963b), the reduction of the double bond of CA by intestinal microbiota of different animal species was described for the first time and 3,4-DHPPA, 3-HPPA, and *m*-coumaric acid were detected as metabolites. This reduction was confirmed by Scheline (1968) and described as being a general reaction for cinnamic acid derivatives when incubating with rat cecal contents (Soleim & Scheline, 1972). In contrast to the study described by Booth and Williams (1963a), the metabolite *m*-coumaric acid was not identified in the work of Scheline (1968). The results of the latter study correspond to the results of the present study. Scheline (1968) explained that the capacity of the cecal microorganisms to reduce the double bond greatly exceeds the capacity to dehydroxylate the aromatic ring primarily.

In 1966, a strain of *Pseudomonas* sp. was isolated from rat feces being able to dehydroxylate CA to 3-HPPA and *m*-coumaric acid (Perez-Silva, Rodriguez, & Perez-Silva, 1966). With every degradation step, the metabolites become more lipophilic allowing absorption via passive diffusion into the enterocytes of the colon. Once the metabolites are absorbed, they enter the blood stream, reach the portal vein and the liver where they become conjugated. Here, the metabolites undergo several transformations until they are secreted with the urine or the bile (Marin et al., 2015). Metabolites which are not absorbed or re-absorbed will be excreted with the feces (Marin et al., 2015).

In vivo studies on the metabolism of C-QA or CA showed the formation of benzoic acid or its conjugates such as hippuric acid (Gonthier et al., 2003; Olthof, Hollman,

Buijsman, van Amelsvoort, & Katan, 2003). Benzoic acid is partly formed *in vivo* in the liver from 3-PPA by shortening the side chain via β -oxidation (Gonthier et al., 2003). Therefore, 3-PPA is assigned to be a precursor metabolite for benzoic acid (Gonthier et al., 2006). It is not clear whether 3-PPA is formed from the microorganisms within a longer fermentation period or if the conversion from 3-HPPA to 3-PPA results from the mammalian organism. For that, it might be possible that even though 3-PPA was not identified as a main metabolite in the *in vitro* fermentations and it was rather formed by the interaction of the substrate with the medium, it would have been formed by a dehydroxylation of 3-HPPA or 4-HPPA during a longer fermentation time.

In conclusion, the substrates C-QA and C-IA were degraded in all *in vitro* fermentations performed in the present study. The main reactions were the cleavage of the ester bond, followed by the reduction of the double bond and dehydroxylation in C3- or C4- position of the aromatic ring. By comparing the degradation of C-QA with C-IA, very similar metabolite formations and time courses were determined.

With regard to the consumption of amaranth, C-IA can be metabolized by the human gut microbiota. Furthermore, it is assumed that these metabolites can be also absorbed via the colon and undergo further hepatic metabolism. Despite the fact that C-QA occurs more ubiquitous, amaranth is a relevant source of other esterified phenolic compounds.

Conflict of interest

The authors declare no conflict of interest.

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References

- Alvarez-Jubete, L., Wijngaard, H., Arendt, E. K., & Gallagher, E. (2010). Polyphenol composition and in vitro antioxidant activity of amaranth, quinoa buckwheat and wheat as affected by sprouting and baking. *Food Chemistry*, *119* (2), 770-778.
- Biddle, A. S., Leschine, S., Huntemann, M., Han, J., Chen, A., Kyripides, N., Markowitz, V., Palaniappan, K., Ivanova, N., Mikhailova, N., Ovchinnikova, G., Schaumberg, A., Pati, A., Stamatis, D., Reddy, T., Lobos, E., Goodwin, L., Nordberg, H. P., Cantor, M. N., Hua, S. X., Woyke, T., & Blanchard, J. L. (2014). The complete genome sequence of *Clostridium indolis* DSM 755(T). *Standards in Genomic Sciences*, *9* (3).
- Boettler, U., Volz, N., Pahlke, G., Teller, N., Kotyczka, C., Somoza, V., Stiebitz, H., Bytof, G., Lantz, I., Lang, R., Hofmann, T., & Marko, D. (2011). Coffees rich in chlorogenic acid or N-methylpyridinium induce chemopreventive phase II-enzymes via the Nrf2/ARE pathway in vitro and in vivo. *Mol Nutr Food Res*, *55* (5), 798-802.
- Booth, A. N., & Williams, R. T. (1963a). Dehydroxylation of caffeic acid by rat and rabbit caecal contents and sheep rumen liquor. *Nature*, *198*, 684-685.
- Booth, A. N., & Williams, R. T. (1963b). Dehydroxylation of Catechol Acids by Intestinal Contents. *Biochemical Journal*, *88* (3), P66-&.
- Braune, A., & Blaut, M. (2016). Bacterial species involved in the conversion of dietary flavonoids in the human gut. *Gut Microbes*, 1-19.
- Chesson, A., Provan, G. J., Russell, W. R., Scobbie, L., Richardson, A. J., & Stewart, C. (1999). Hydroxycinnamic acids in the digestive tract of livestock and humans. *Journal of the Science of Food and Agriculture*, *79* (3), 373-378.
- Clifford, M. N. (1999). Chlorogenic acids and other cinnamates - nature, occurrence and dietary burden. *Journal of the Science of Food and Agriculture*, *79* (3), 362-372.
- Cole, J. R., Wang, Q., Cardenas, E., Fish, J., Chai, B., Farris, R. J., Kulam-Syed-Mohideen, A. S., McGarrell, D. M., Marsh, T., Garrity, G. M., & Tiedje, J. M. (2009). The Ribosomal Database

- Project: improved alignments and new tools for rRNA analysis. *Nucleic Acids Research*, 37 (Database issue), D141-145.
- Council, N. R. (1984). *Amaranth: Modern Prospects for an Ancient Crop*. Washington, DC: The National Academies Press.
- Couteau, D., McCartney, A. L., Gibson, G. R., Williamson, G., & Faulds, C. B. (2001). Isolation and characterization of human colonic bacteria able to hydrolyse chlorogenic acid. *Journal of Applied Microbiology*, 90 (6), 873-881.
- Das, S. (2016). *Amaranthus: A Promising Crop of Future*. Singapore: Springer Science+Business Media.
- Duenas, M., Munoz-Gonzalez, I., Cueva, C., Jimenez-Giron, A., Sanchez-Patan, F., Santos-Buelga, C., Moreno-Arribas, M. V., & Bartolome, B. (2015). A survey of modulation of gut microbiota by dietary polyphenols. *BioMed Research International*, 2015, 850902.
- Fuller, Z., Louis, P., Mihajlovski, A., Rungaparnestry, V., Ratcliffe, B., & Duncan, A. J. (2007). Influence of cabbage processing methods and prebiotic manipulation of colonic microflora on glucosinolate breakdown in man. *British Journal of Nutrition*, 98 (2), 364-372.
- Gonthier, M. P., Remesy, C., Scalbert, A., Cheynier, V., Souquet, J. M., Poutanen, K., & Aura, A. M. (2006). Microbial metabolism of caffeic acid and its esters chlorogenic and caftaric acids by human faecal microbiota in vitro. *Biomedicine & Pharmacotherapy*, 60 (9), 536-540.
- Gonthier, M. P., Verny, M. A., Besson, C., Remesy, C., & Scalbert, A. (2003). Chlorogenic acid bioavailability largely depends on its metabolism by the gut microflora in rats. *Journal of Nutrition*, 133 (6), 1853-1859.
- Khanam, U. K. S., Oba, S., Yanase, E., & Murakami, Y. (2012). Phenolic acids, flavonoids and total antioxidant capacity of selected leafy vegetables. *Journal of Functional Foods*, 4 (4), 979-987.
- Ludwig, I. A., de Pena, M. P., Cid, C., & Crozier, A. (2013). Catabolism of coffee chlorogenic acids by human colonic microbiota. *Biofactors*, 39 (6), 623-632.
- Manach, C., Scalbert, A., Morand, C., Remesy, C., & Jimenez, L. (2004). Polyphenols: food sources and bioavailability. *The American Journal of Clinical Nutrition*, 79 (5), 727-747.

- Manach, C., Williamson, G., Morand, C., Scalbert, A., & Remesy, C. (2005). Bioavailability and bioefficacy of polyphenols in humans. I. Review of 97 bioavailability studies. *The American Journal of Clinical Nutrition*, *81* (1 Suppl), 230S-242S.
- Marin, L., Miguelez, E. M., Villar, C. J., & Lombo, F. (2015). Bioavailability of Dietary Polyphenols and Gut Microbiota Metabolism: Antimicrobial Properties. *BioMed Research International*, *2015*, 18.
- Martino, G. P., Quintana, I. M., Espariz, M., Blancato, V. S., & Magni, C. (2016). Aroma compounds generation in citrate metabolism of *Enterococcus faecium*: Genetic characterization of type I citrate gene cluster. *International Journal of Food Microbiology*, *218*, 27-37.
- Matsuki, T., Watanabe, K., Fujimoto, J., Takada, T., & Tanaka, R. (2004). Use of 16S rRNA gene-targeted group-specific primers for real-time PCR analysis of predominant bacteria in human feces. *Applied and Environmental Microbiology*, *70* (12), 7220-7228.
- Mills, C. E., Tzounis, X., Oruna-Concha, M. J., Mottram, D. S., Gibson, G. R., & Spencer, J. P. E. (2015). In vitro colonic metabolism of coffee and chlorogenic acid results in selective changes in human faecal microbiota growth. *British Journal of Nutrition*, *113* (8), 1220-1227.
- Neugart, S., Baldermann, S., Ngwene, B., Wesonga, J., & Schreiner, M. (2017). Indigenous leafy vegetables of Eastern Africa - A source of extraordinary secondary plant metabolites. *Food Research International*, <https://doi.org/10.1016/j.foodres.2017.02.014>.
- Olthof, M. R., Hollman, P. C., Buijsman, M. N., van Amelsvoort, J. M., & Katan, M. B. (2003). Chlorogenic acid, quercetin-3-rutinoside and black tea phenols are extensively metabolized in humans. *Journal of Nutrition*, *133* (6), 1806-1814.
- Olthof, M. R., Hollman, P. C. H., & Katan, M. B. (2001). Chlorogenic acid and caffeic acid are absorbed in humans. *Journal of Nutrition*, *131* (1), 66-71.
- Peppercorn, M. A., & Goldman, P. (1971). Caffeic acid metabolism by bacteria of the human gastrointestinal tract. *Journal of Bacteriology*, *108* (3), 996-1000.

- Perez-Silva, G., Rodriguez, D., & Perez-Silva, J. (1966). Dehydroxylation of caffeic acid by a bacterium isolated from rat faeces. *Nature*, *212* (5059), 303-304.
- Pfeiffer, T., & Bonhoeffer, S. (2004). Evolution of cross-feeding in microbial populations. *American Naturalist*, *163* (6), E126-E135.
- Prakash, D., & Pal, M. (1991). Nutritional and Antinutritional Composition of Vegetable and Grain Amaranth Leaves. *Journal of the Science of Food and Agriculture*, *57* (4), 573-583.
- Ramirez-Farias, C., Slezak, K., Fuller, Z., Duncan, A., Holtrop, G., & Louis, P. (2009). Effect of inulin on the human gut microbiota: stimulation of *Bifidobacterium adolescentis* and *Faecalibacterium prausnitzii*. *British Journal of Nutrition*, *101* (4), 541-550.
- Rastogi, A., & Shukla, S. (2013). Amaranth: A New Millennium Crop of Nutraceutical Values. *Critical Reviews in Food Science and Nutrition*, *53* (2), 109-125.
- Rechner, A. R., Smith, M. A., Kuhnle, G., Gibson, G. R., Debnam, E. S., Srai, S. K. S., Moore, K. P., & Rice-Evans, C. A. (2004). Colonic metabolism of dietary polyphenols: Influence of structure on microbial fermentation products. *Free Radical Biology and Medicine*, *36* (2), 212-225.
- Robbins, R. J. (2003). Phenolic acids in foods: an overview of analytical methodology. *Journal of Agricultural and Food Chemistry*, *51* (10), 2866-2887.
- Scalbert, A., & Williamson, G. (2000). Dietary intake and bioavailability of polyphenols. *Journal of Nutrition*, *130* (8), 2073s-2085s.
- Scheline, R. R. (1968). Metabolism of phenolic acids by the rat intestinal microflora. *Acta Pharmacologica et Toxicologica (Copenh)*, *26* (2), 189-205.
- Schröter, D., Baldermann, S., Schreiner, M., Witzel, K., Maul, R., Rohn, S., & Neugart, S. (2017). Natural diversity of hydroxycinnamic acid derivatives, flavonoid glycosides, carotenoids and chlorophylls in leaves of six different amaranth species. *submitted*.
- Shukla, S., Bhargava, A., Chatterjee, A., Pandey, A. C., & Mishra, B. K. (2010). Diversity in phenotypic and nutritional traits in vegetable amaranth (*Amaranthus tricolor*), a nutritionally underutilised crop. *Journal of the Science of Food and Agriculture*, *90* (1), 139-144.

- Soleim, H. A., & Scheline, R. R. (1972). Metabolism of Xenobiotics by Strains of Intestinal Bacteria. *Acta Pharmacologica Et Toxicologica*, 31 (5-7), 471-&.
- Strack, D., Leicht, P., Bokern, M., Wray, V., & Grotjahn, L. (1987). Hydroxycinnamic Acid-Esters of Isocitric Acid - Accumulation and Enzymatic-Synthesis in *Amaranthus-Cruentus*. *Phytochemistry*, 26 (11), 2919-2922.
- Tomas-Barberan, F., Garcia-Villalba, R., Quartieri, A., Raimondi, S., Amaretti, A., Leonardi, A., & Rossi, M. (2014). In vitro transformation of chlorogenic acid by human gut microbiota. *Mol Nutr Food Res*, 58 (5), 1122-1131.
- Walker, A. W., Duncan, S. H., Leitch, E. C. M., Child, M. W., & Flint, H. J. (2005). pH and peptide supply can radically alter bacterial populations and short-chain fatty acid ratios within microbial communities from the human colon. *Applied and Environmental Microbiology*, 71 (7), 3692-3700.
- Weiss, C., Rubach, M., Lang, R., Seebach, E., Blumberg, S., Frank, O., Hofmann, T., & Somoza, V. (2010). Measurement of the intracellular pH in human stomach cells: a novel approach to evaluate the gastric acid secretory potential of coffee beverages. *Journal of Agricultural and Food Chemistry*, 58 (3), 1976-1985.
- Zehring, J., Reim, V., Schröter, D., Neugart, S., Schreiner, M., Rohn, S., & Maul, R. (2015). Identification of novel saponins in vegetable amaranth and characterization of their hemolytic activity. *Food Research International*, 78, 361-368.

Figure captions

Figure 1. Chemical structure of chlorogenic acid (A) and caffeoylisocitric acid (B).

Figure 2. Mass fragmentation pattern of caffeoylisocitric (m/z 353.05008) acid obtained by qTOF analysis.

Figure 3. Metabolite formation of chlorogenic acid (C-QA) for three independent *in vitro* fermentations with different donors (A, B, and C). Each data point represents the mean and standard deviation of triplicates.

Figure 4. Metabolite formation of caffeoylisocitric acid (C-IA) for three independent *in vitro* fermentations with different donors (A, B, and C). Each data point represents the mean and standard deviation of triplicates.

Figure 5. Bacterial composition of the initial fecal samples and fecal incubations (48 h) from donor A, B, and C, in 'control samples I' and in the presence of chlorogenic acid (C-QA) or caffeoylisocitric acid (C-IA). Data represents the mean and standard deviation of triplicates.

Figure 6. Schematic microbial degradation pathway of caffeoylisocitric acid (C-IA) in the colon. (CA, caffeic acid; 3,4-DHPPA, 3-(3,4-dihydroxyphenyl)propionic acid; 3-HPPA; 3-(3-hydroxyphenyl)propionic acid; 4-HPPA, 3-(4-hydroxyphenyl)propionic acid; 3-PPA, 3-phenylpropionic acid).

* Metabolites that are not absorbed via the colon will be excreted with the feces.

Table 1: Quantitative PCR primers used in this study.

Target	Name	Sequence	Primer annealing temp (°C)	Reference
Universal	UniF	GTGSTGCAYGGYYGTCGTCA	60	Fuller et al. (2007)
	UniR	ACGTCRTCCMCNCCTTCCTC		
<i>Bifidobacterium</i> spp.	BifF	TCGCGTCYGGTGTGAAAG	60	Fuller et al. (2007)
	g-Bifid-R	GGTGTTCCTCCCGATATCTACA		
Bacteroidetes	Bac303F	GAAGGTCCCCCACATTG	60	Ramirez-Farias et al. (2009)
	Bfr-Fmrev	CGCKACTTGGCTGGTTCAG		
Ruminococcaceae	sg-Clept-F	GCACAAGCAGTGGAGT	60	Matsuki et al. (2004), modified ¹
	sg-Clept-Fm2	GCACAAGCGGTGGATT		
	sg-Clept-R3	CTTCCTCCGTTTTGTCAA		
Lachnospiraceae	Erec482F	CGGTACCTGACTAAGAAGC	60	Ramirez-Farias et al. (2009)
	Erec870R	AGTTYATTCTTGCGAACG		
<i>Faecalibacterium prausnitzii</i>	FPR-2F	GGAGGAAGAAGGTCTTCGG	60	Ramirez-Farias et al. (2009)
	Fprau645R	AATCCGCCTACCTCTGCACT		
<i>Blautia</i> spp.	BlautiaF2	CGCGTGAAGGAAGAAGTATC	63	this study ²
	BlautiaR2	GAGCCTCAACGTCAGTTACC		
<i>Roseburia</i> spp. & <i>E. rectale</i>	RrecF	GCGGTRCGGCAAGTCTGA	63	Ramirez-Farias et al. (2009)
	Rrec630mR	CCTCCGACACTCTAGTMCGAC		

¹Equal amounts of both forward primers were used to improve coverage of the Ruminococcaceae family with a total concentration of 500 nM. The assay was validated against 16S rRNA gene amplicons of 53 gut bacteria, including 12 Ruminococcaceae, which were the only positive species.

²New primers were designed as described previously (Ramirez-Farias et al., 2009) by inspection of alignments downloaded from the Ribosomal Database Project (RDP; Cole et al. (2009)) and by RDP Probe match analysis. Primers were validated against 16S rRNA gene amplicons of 44 gut bacteria, including 25 Lachnospiraceae species. Only the target group (*Blautia obeum* A2-162, *Blautia hydrogenotrophica* DSM 10507, *Blautia producta* DSM 14466, *Blautia hansenii* DSM 20583) was found to be positive.

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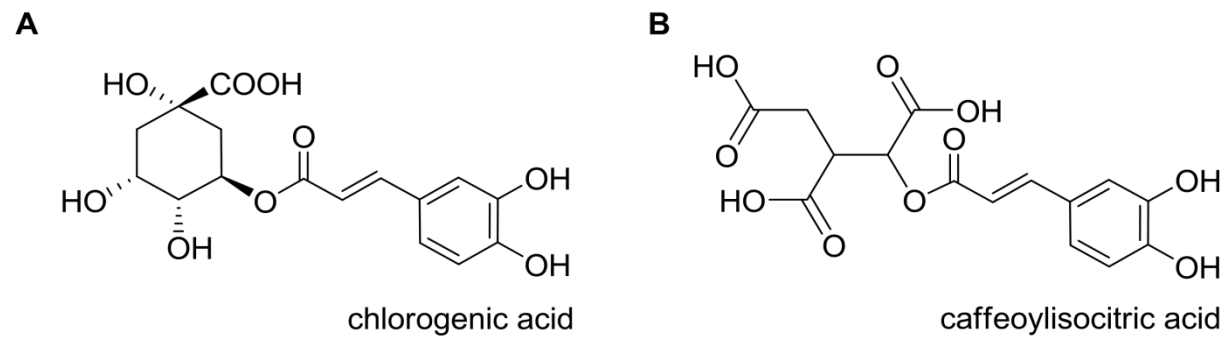


Fig. 1

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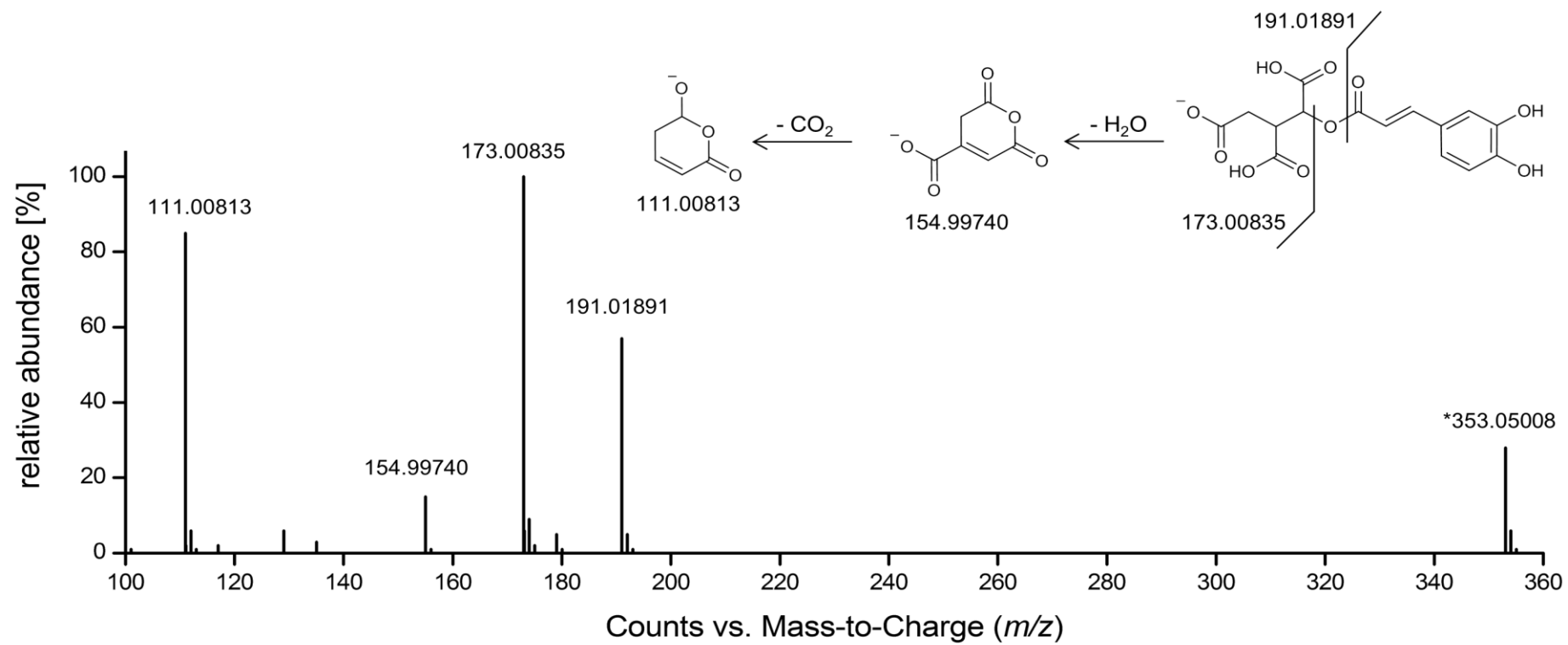


Fig. 2

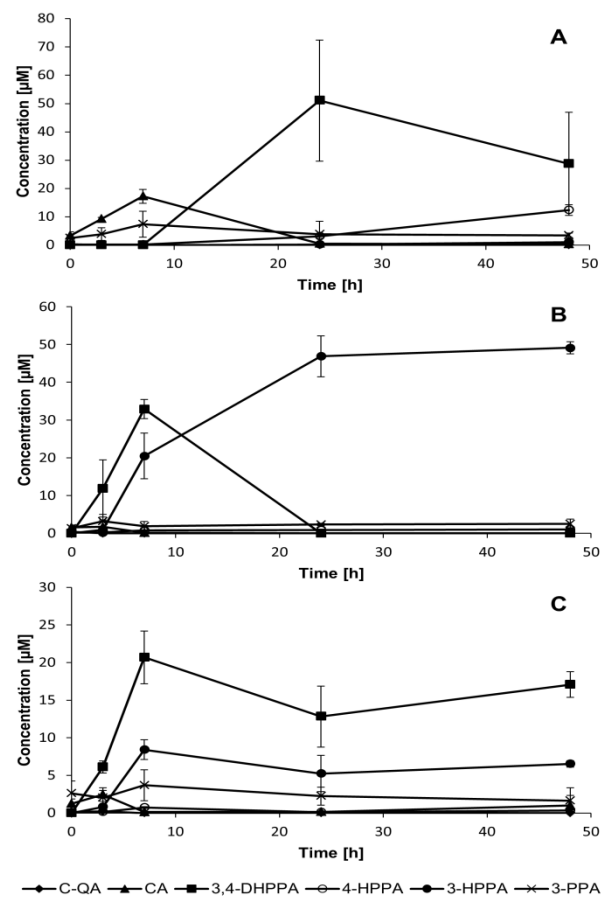


Fig. 3

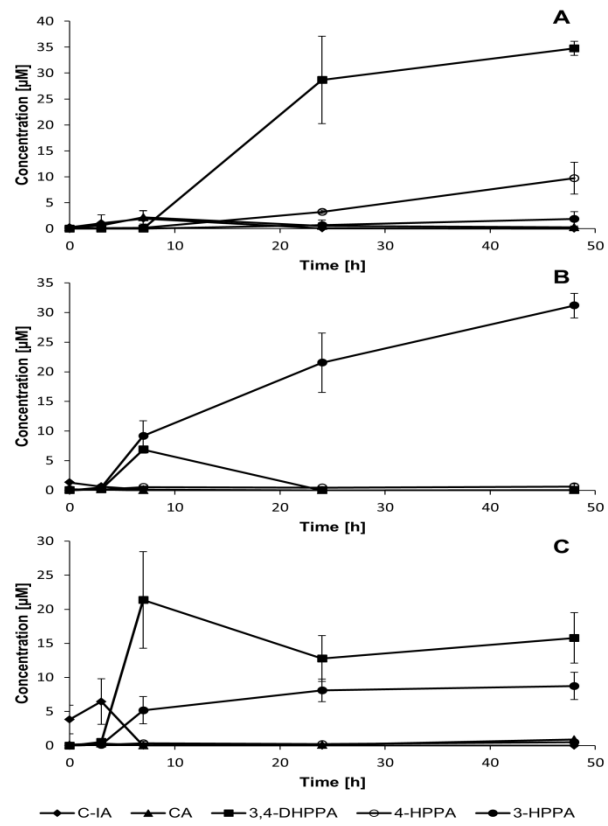


Fig. 4

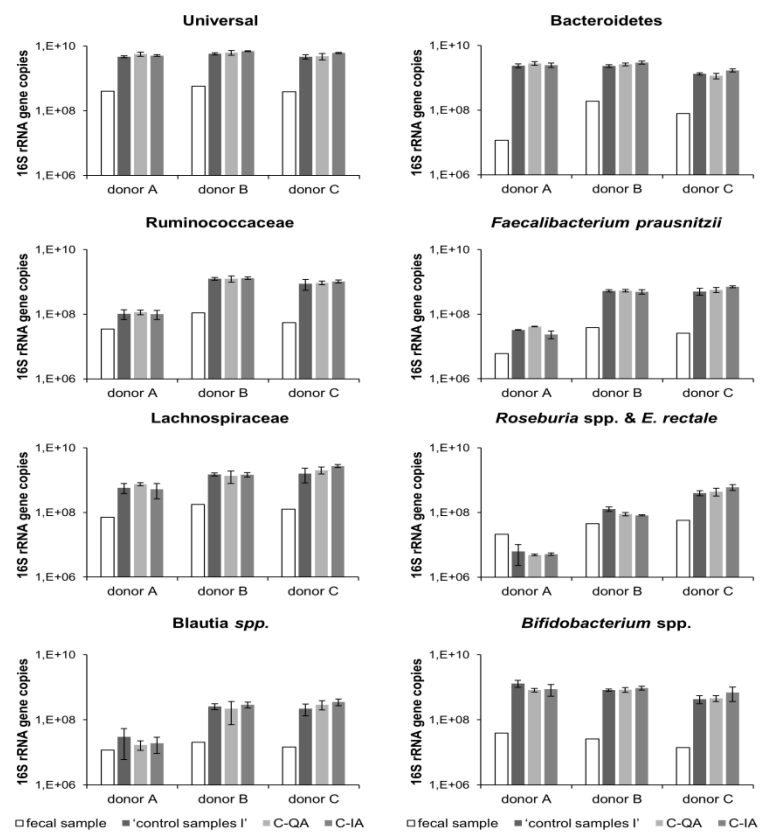


Fig. 5

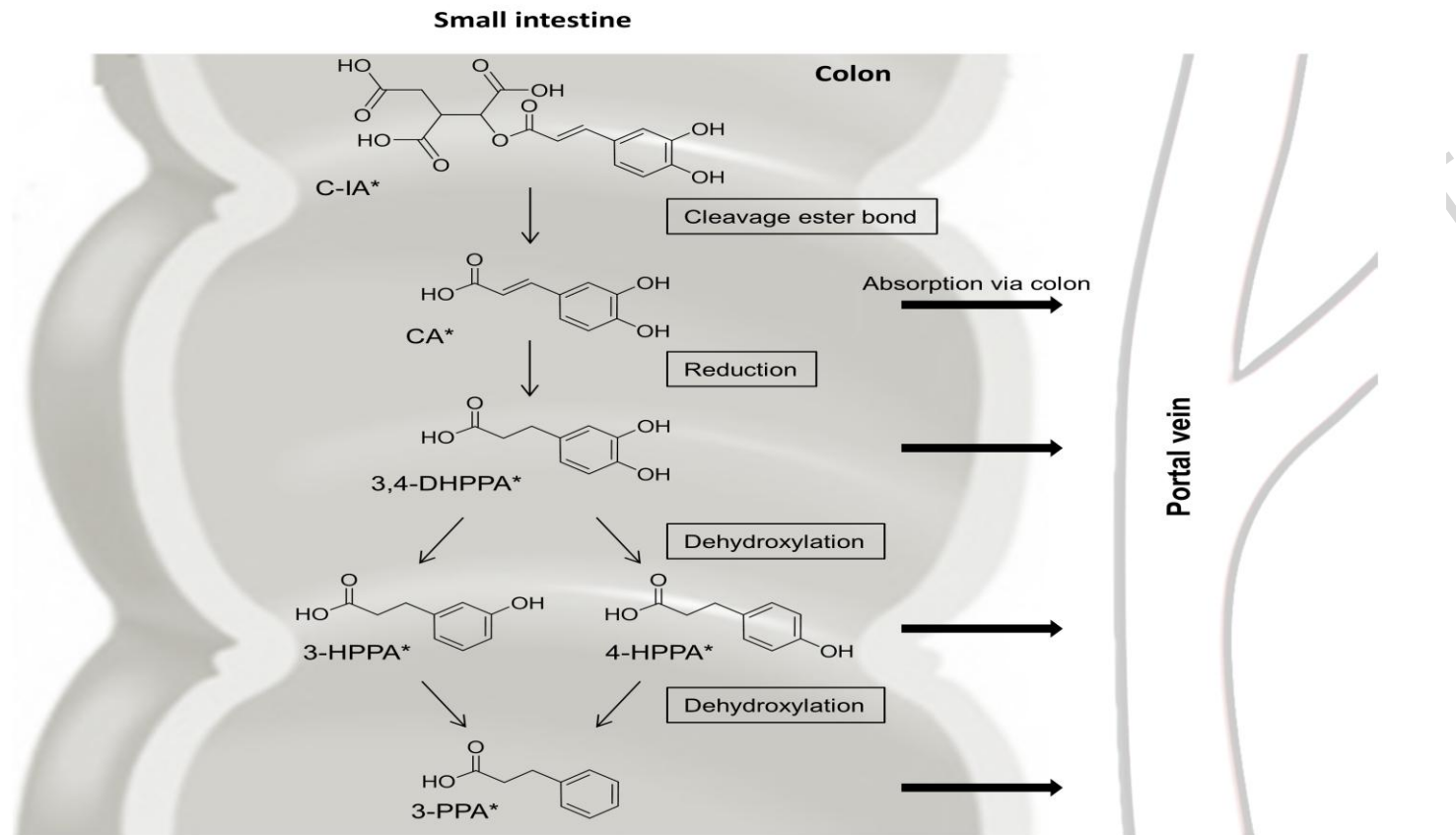


Fig. 6

HIGHLIGHTS

- Microbial degradation of caffeoylisocitric acid from amaranth was characterized
- Metabolites of caffeoylisocitric acid and chlorogenic acid fermentations are very similar
- Composition of the microbiota was not affected

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