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Optimization of an *in vitro* gut microbiome biotransformation platform with chlorogenic acid as model compound: from fecal sample to biotransformation product identification

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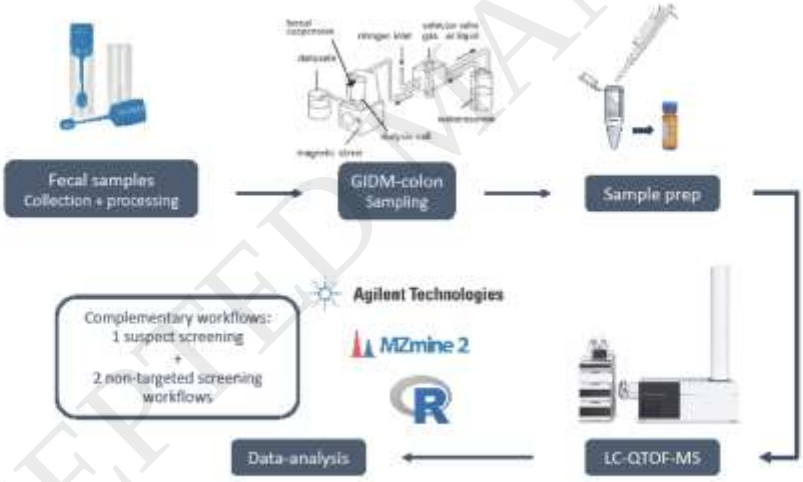
Graphical abstract



Highlights:

- This study optimized a ready-to-use *in vitro* platform to investigate the colonic biotransformation of xenobiotics by the gut microbiome
- Processing of fecal samples, before use in the *in vitro* gastrointestinal dialysis model with colon stage, was optimized to ensure resemblance with the *in vivo* situation using 16s rDNA gene sequencing methods.
- The effect of four different sample preparation procedures on the biotransformation product identification was evaluated.

- By using liquid chromatography coupled to high-resolution mass spectrometry and complementary suspect and non-targeted screening data-analysis workflows, the number of identified biotransformation products was increased.

Abstract

Recent data clearly show that the gut microbiota plays a significant role in the biotransformation of many endogenous molecules and xenobiotics, leading to a potential influence of this microbiotic metabolism on activation, inactivation and possible toxicity of these compounds. To study the colonic biotransformation of xenobiotics by the gut microbiome, *in vitro* models are often used as they allow dynamic and multiple sampling overtime. However, the pre-analytical phase should be carefully optimized to enable biotransformation product identification representative for the *in vivo* situation. During this study, chlorogenic acid was used as a model compound to optimize a ready-to-use gut microbiome biotransformation platform using an *in vitro* gastrointestinal dialysis-model with colon phase together with an instrumental platform using liquid chromatography coupled to high resolution mass spectrometry (LC-QTOF-MS). Identification of the biotransformation products of chlorogenic acid was performed using complementary suspect and non-targeted data analysis approaches (MZmine + R and MPP workflow).

Concerning the pre-analytical phase, (i) the influence of different incubation media (Wilkins-Chalgren Anaerobic Broth (WCB) and (versus) phosphate buffer) and different incubation times (prior to implementation in the colonic stage of the dialysis model) on fecal bacterial composition and concentration were investigated and (ii) four different sample preparation methods (centrifugation, extraction, sonication and freeze-drying) were evaluated targeting colonic biotransformation of chlorogenic acid. WCB as incubation medium showed to introduce substantial variation in the bacterial composition of the fecal samples, while the sterile phosphate buffer guaranteed a closer resemblance to the *in vivo* composition. Furthermore, incubation during 24 h in sterile phosphate buffer as medium showed no significant increase or decrease in anaerobic bacterial concentration, concluding that incubation prior to the colonic stage is not needed. Concerning sample preparation, centrifugation, sonication and extraction gave similar results, while freeze-drying appeared to be inferior. The extraction method was selected as an optimal sample preparation method given the quick execution together with a good instrumental sensitivity.

This study optimized a ready-to-use platform to investigate colonic biotransformation of xenobiotics by using chlorogenic acid as a model compound. This platform can be used in the future to study differences in colonic biotransformation of xenobiotics using fecal samples of different patient groups.

Keywords: Chlorogenic acid, gut microbiome, *in vitro* gastrointestinal dialysis model, 16s rDNA gene sequencing, liquid chromatography-high resolution mass spectrometry

1. Introduction

Every human being can be seen as an ecosystem on its own. Our body houses 10 times more bacterial cells, than human cells, and to lesser extent viruses, fungi, archaea, and protozoans [1]. The gastrointestinal tract (GIT) accommodates between 10^{12}-10^{13} microorganisms with species diversity increasing longitudinally from mouth to colon, the latter harboring the most bacteria of the whole GIT [2]. The human GIT houses eight phyla, *Firmicutes, Bacteroidetes, Proteobacteria, Fusobacteria, Verrucomicrobia, Cyanobacteria, Spirochaeates* and *Actinobacteria*, with the first two accounting for 90% of the gut diversity. The composition of the gut flora largely depends on the genetic background of the host, age, health status, immune system, drug use, life style, hygiene and type of diet leading to high interindividual variation [3]. The gut microbiota executes various important functions, including some that the host himself is unable to perform: synthesis of vitamins K2 and B12, development and regulation of the immune and nervous system, protection against pathogenic species, maintaining barrier function, regulation of host fat storage and energy homeostasis and stimulation of intestinal angiogenesis [2, 3].

As compared to the human genome, the cumulative genome content of the gut microbiota, also known as the gut microbiome, is estimated to contain 100 to 300-fold more genes than the human genome and contains 3.3-10 million nonredundant genes [1, 2, 4]. It is therefore not surprising that the gut microbiome is able to influence the physiology of the host and that it is considered an additional metabolizing organ [4]. Important differences between hepatic and bacterial metabolism have been observed. Liver metabolic pathways consist mostly out of oxidative phase I reactions, by the cytochrome P450 enzymes (CYP450), and conjugation phase II reactions, by uridine 5'-diphospho(UDP)-glucuronosyltransferases (UGTs), sulfotransferases etc., typically leading to the conversion of lipophilic compounds into more hydrophilic compounds to enhance excretion, while the major biotransformation reactions by the gut microbiome are reductive, due to the high reduction potential of anaerobic bacteria, and hydrolytic reactions. In addition, the gut flora is responsible for processing glutathione conjugates of xenobiotics excreted in the bile, deacylation, demethylation, decarboxylation, dehydroxylations, dealkylation, dehalogenation, deamination, acetylation, and oxidation/dehydrogention [1, 5, 6]. These insights have led to the understanding that the gut microbiome can directly and indirectly influence the activity and toxicity of pharmaceuticals and other xenobiotics [6]. Examples of direct interferences are the conversion of a prodrug to the active compound (e.g. azo reduction by azoreductase, of azo-antibacterial pro-drugs based on sulphanilamide such as sulfasalazine, leads to activation of this prodrug), inactivation, detoxification, change of efficacy and direct binding to xenobiotics or dietary components [7, 8]. Examples of indirect interferences are the alteration of host genes expression (e.g. lower expression of CYP450 enzymes), competition and/or inhibition of host enzymes by microbial biotransformation products and reactivation of drugs by deconjugation of phase II biotransformation products after enterohepatic circulation [7, 8]. Besides oral and rectal administrated drugs, other administration routes (e.g. intravenous) can result in the presence of the drug in the colon by secretion or diffusion from the systemic circulation or by enteric and hepatic secretion into the intestinal lumen [1]. Most new pharmaceutical drug candidates present biopharmaceutical problems like low solubility and/or low permeability leading to longer contact with the gut microbiome. This shows the need for more biotransformation studies including the role of the gut microbiome [9].

In vivo animal or clinical studies to investigate the colonic biotransformation of xenobiotics experience some disadvantages. First of all these studies are time-consuming, labor-intensive and expensive [10]. During *in vivo*

studies, access to the gut is difficult to accomplish as it would require a long catheter trough the throat or nose, or a tube stuck up the rectum. Furthermore, animal studies mostly lead to the sacrifice of the animals to be able to sample the different compartments of the gut [5, 11]. *In vitro* GIT models offer an alternative approach to study the colonic metabolism. In general, *in vitro* models of the GIT contain multiple compartments to mimic the different parts of the digestive system (mouth, stomach, small and large intestine) by adjusting temperature, pH, enzymes and peristalsis [10]. If a colon or fermentation compartment is included, simulation of the gut flora is needed, usually derived from a fecal suspension.

Easy access to the different compartments allows dynamic and multiple sampling over time which can help in the understanding of the different steps of the gut fermentation [10]. The disadvantage of *in vitro* models is that they are not fully representative for the *in vivo* conditions. Therefore, it is important to create conditions that closely mimic the *in vivo* situation to obtain a high level of physiological significance [5, 10]. In *in vitro* models, a distinction can be made between static short-term batch incubations (i.e. test-tube containing fecal suspension and substrate), single stage reactors or semi-continuous systems (i.e. comprising a static bioreactor to which needed enzymes are added manually and stirred) and multi-compartmental continuous models (i.e. emphasize dynamic changes in the digestive tract: changes in temperature, pH and digestive secretions, direct connection between compartments, peristalsis) among the wide range of available *in vitro* GIT and fermentation models [5, 10]. Static batch incubations are frequently used but are not able to provide accurate results since they tend to over-simplify the *in vivo* situation. Better representation of the *in vivo* situation can be acquired with semi-continuous (e.g. gastrointestinal dialysis model with colon phase (GIDM-Colon)) or multi-compartmental continuous models (e.g. SHIME®, TIM-2.) [11].

Chlorogenic acid is a hydroxycinnamic acid and phenolic acid which can be found in our daily diet. It is the major phenolic acid in coffee, but can also be found in tea, fruits and vegetables . The gastrointestinal metabolism of chlorogenic acid in humans and rats has been previously investigated by multiple studies (supplementary information Table 1) which made it a suitable model compound. The aim of this study was to optimize a complete and ready-to-use setup to investigate the colonic metabolism of xenobiotics [12-20]. First, the incubation medium and time of incubation of the fecal slurry samples in the medium were optimized in order to preserve the *in vivo* bacterial composition. Secondly four different sample preparation methods were evaluated to obtain extracts compatible with the instrumental detection based on liquid chromatography coupled to high-resolution accurate-mass mass spectrometry. Thirdly, data analysis strategies for the elucidation of biotransformation pathways were optimized using complementary suspect and non-targeted screening workflows [21].

2. Materials and methods

2.1. Chemicals and reagents

Sodium phosphate dibasic (Na_2HPO_4, ≥ 99%), sodium phosphate monobasic dihydrate ($NaH_2PO_4.2H_2O$, ≥ 99%), thioglycollate broth, pepsin from porcine gastric mucosa, bile extract porcine, pancreatin from porcine pancreas, theophylline (≥ 99%, anhydrous), quinic acid (98%), chlorogenic acid (≥ 95%), caffeic acid (≥ 98%), 3-(4-hydroxyphenyl)propionic acid (98%) were acquired from Sigma-Aldrich (St Louis, MO, USA). Deionized water (milliQ) and sodium bicarbonate ($NaHCO_3$, > 99.7%, ACS grade) were obtained from respectively Millipore (Burlington, Massachusetts, USA) and Acros Organics (Pittsburgh, Pennsylvania, USA). Hydrochloric acid (HCl, 32 wt.% for analysis), formic acid (98-100%, Suprapur) and sodium hydroxide pellets (NaOH) were acquired from

Merck (Darmstadt, Germany). Acetonitrile (ACN) and methanol (MeOH, ≥ 99.9%, LC-MS grade) were obtained from Fisher Scientific (Hampton, New Hampshire, USA). Ultrapure water (Purelab flex apparat) was acquired from ELGA Veolia (UK). Nitrogen gas (N_2, AZOTE N28) and a gas mixture of hydrogen, carbon dioxide and nitrogen (5% H_2, 5% CO_2 and 90% N_2, Alphagaz Mix) were obtained from Air Liquide Belge (Liege, Belgium).

2.2. Materials

Stirred ultrafiltration cells (model 8200, 200 mL, 63.5 mm diameter), the related controller (controller MF2 and reservoir RC800) and ultrafiltration discs (Ultracel, MWCO 1000 Da, 63.5 mm diameter) were acquired from Amicon Ltd (USA). A shaking warm water bath from VWR (Radnor, Pennsylvania, USA) was used during the gastric stage of the GIDM-Colon. Dialysis tubing (MWCO 12-14 kDa, Visking size 6 Inf Dia 27/32 – 21.5 mm: 30 M) and an immersion circulator (model 1122S) were acquired respectively from Medicell Ltd. (London, UK) and VWR, for use during the small intestinal stage. A Jacomex glove box T3 from TCPS (Belgium) was used to create an anaerobic environment during the colonic stage.

A Branson 5510DHT ultrasonic cleaner (40 kHz), a vortex mixer (100-2500 rpm) and a FreeZone 1 Liter Benchtop Freeze Dry System (model 7740030) were acquired from respectively Branson Ultrasonics (Danburry, USA), VWR and Labconco (Missouri, USA). A Sigma 1-15PK centrifuge and centrifugal filters (modified nylon membrane, 0.2 µm, 500 µL sample capacity) were obtained respectively from Sigma Laborzentrifugen GmbH (Germany) and VWR.

2.3. Fecal samples

2.3.1. Collection, processing and characterization of the fecal samples

Nine fecal samples were collected from human donors that met the following inclusion criteria: women, 25-45 years old, not pregnant, non-smoking, body mass index (BMI) < 25, no risk factors for metabolic diseases, non-vegetarian, normal defecation, no history of gastrointestinal disease, no intake of antibiotics six months, or pre- or probiotics supplements three months prior to fecal donation and no history of immunosuppressive or chemotherapeutic treatment. A complete list of all inclusion criteria can be found in supplementary information. Ethical approval for the project was acquired from the Ethical Committee of the Antwerp University Hospital (reference number: 16/43/442).

Donors collected the feces using Protocult collection containers (Ability Building Center, Rochester, USA). An overview of the fecal sample treatment before use in the GIDM-Colon is shown in Fig. 1. After collection, fecal samples were held at room temperature together with an anaerocult bag from Merck and treated within 3 h before storage at -80 °C. The fecal slurry of 10% (w/v) feces in sterile phosphate buffer (0.1 M, pH 7.0, 0.58% w/v Na_2HPO_4, 1.03% w/v $NaH_2PO_4.2H_2O$, 3.45% thioglycolate broth) was prepared in an anaerobic glove box. Homogenization and elimination of solid particles was performed using a Stomacher® lab blender (VWR, Leuven, Belgium) for three minutes. Aliquots of 2 mL fecal slurry were stored at -80 °C.

2.3.2. DNA quantification and 16S rDNA sequencing

DNA was isolated from fecal samples using QIAamp DNA Stool Mini Kit (Qiagen). The concentration of all isolated DNA was determined using a Qubit 2.0 Fluorometer (ThermoFisher Scientific) with the dsDNA HS Assay

Kit. Presence of bacterial DNA was confirmed by 16S rDNA-targeting PCR which was visualized on a 2.0% agarose gel to confirm amplification of fragments with an appropriate length.

2.3.3. 16S rDNA amplification of V3 and V4 regions

Amplification of the V3-V4 region of the 16S rDNA was performed in triplicate with 2x KAPA HiFi HotStart Ready Mix (Kapa Biosystems) with the following cycling profile: 95 °C for 3 min, [95 °C for 30 sec, 62 °C for 30 sec, and 72 °C for 50 sec]x25, 72 °C for 10 min.

2.3.4. Library preparation

An index PCR was performed with the Nextera XT Index Kit with dual indices. The concentration of each individual library was measured, mixed with a denatured PhiX control library, and diluted to a final concentration of 4 pM. This library was loaded onto a MiSeq V2 500 cycle cartridge and sequenced (2 x 250 bp) with the MiSeq (Illumina.Inc., USA)

Raw sequence reads were quality assessed using fastqc and data analysis was done using microbial genomics module inbuilt in CLC Genomics workbench v9.5.3 (clcbio, qiagen). Briefly, contigs were created by heuristically merging paired-end reads based on the Phred quality score of both reads. Contigs were aligned to the SILVA 16S database v.132 [22] and binned based on the sequence similarity. Taxonomic classification was performed binned contigs with the SILVA v.132 database.

2.3.5. Incubation of the fecal slurry suspension

Before use in the GIDM-Colon, 2 mL fecal slurry aliquots of all donors were pooled and a suitable medium was added before incubation leading to a 10% (v/v) pooled fecal slurry suspension (Fig. 1). Two different incubation mediums for the pooled fecal slurry suspension were tested. The use of the first medium, Wilkins-Chalgren Anaerobe Broth (WCB) was based on the protocol described by Breynaert et al. [12]. A sterile phosphate buffer (0.1 M, pH 7) was tested as a second incubation medium. The pooled fecal suspensions were incubated (continuously stirred) for 17 h in the anaerobic glove box and samples of both pools were taken before and after 17 h incubation and characterized by 16S rDNA gene sequencing as described above.

To optimize the incubation time, a pooled fecal slurry suspension was incubated for 24 h in phosphate buffer, and aliquoted every 2 h, to determine the anaerobic colony forming units per gram (CFU/g). The same experiment was carried out with WCB, with a sample after 17 h of incubation (*cf.* the original protocol).

Serial dilutions (from 10^{-1} to 10^{-8}) of the samples were prepared with brain heart infusion (BHI) broth (BD Biosciences, Franklin Lakes, NJ, USA), plated on an anaerobic agar OXOID0972 (Oxoid, Basingstoke, UK) and incubated for 72 h at 37 °C in a bug box anaerobic workstation (Ruskinn Technology Ltd, Bridgend, UK). The colonies were counted after 72 h with spiral plater (EddyJet).

2.4. Protocol GIDM-Colon

To study the colonic biotransformation of the model compound chlorogenic acid, a validated *in vitro* continuous flow dialysis-model with colon phase (GIDM-Colon, Fig. 2) was used as described by Breynaert et al. [12]. Ultrafiltration cells of the intestinal stage were equipped with a semi-permeable dialysis membrane (detailed figure in supplementary information Fig. SI-1) to simulate one-way absorption through passive diffusion from intestinal

lumen to mucosa. One blank sample (containing no chlorogenic acid), one negative control sample (containing no fecal slurry suspension) and two replicate samples were used.

2.4.1. Gastric stage

During the gastric stage, 78 mg of chlorogenic acid was dissolved in 1 mL 16% (w/v) pepsin (19.6% protein, 622 U/mg protein) together with 49 mL 0.1 M HCl solution and set at a pH of 2 using 6 M HCl. Samples were incubated for 1 h in a shaking water bath at 37 °C (120 strokes/min). After the gastric stage, 1 mL of sample was collected and stored at –80 °C for further analysis.

2.4.2. Small intestinal stage

The content of the gastric stage was immediately manually transferred to ultrafiltration cells to simulate the small intestinal stage and 50 mL of ultrapure water was added. Dialysis bags containing 1 M $NaHCO_3$ were used to alter the pH from 2 till 7.5 in 30 min. The volume of 1 M $NaHCO_3$ needed to alter the pH was determined on the blank sample. Ultrafiltration cells were placed in a water bath (35-37 °C), continuously stirred and connected with a water tank and a N_2 gas input using push bottom control switches (supplementary information Fig.SI-1). N_2 gas puts pressure on the cells (2 bar) to enable dialysis. After 30 min of dialysis, 15 mL of a pancreatin-bile solution was added to each cell. This solution was prepared dissolving 0.4% (w/v) of pancreatin (32 000 FIP-U lipase, 143 600 FIP-U amylase, 16 400 FIP-U protease) and 0.766% (w/v) of bile in 0.1 M $NaHCO_3$. Dialysis was performed for an extra 2 h. After the small intestinal stage, 1 mL samples were collected from the retentate and dialysate fraction and stored at –80 °C for further analysis.

2.4.3. Colonic stage

In order to simulate the colonic stage, the pH of the retentate samples was adjusted to 5.8-6.0 using 1 M HCl and ultrafiltration cells were transferred to an anaerobic glove box (0.5% oxygen, 35-37 °C). 50 mL of 10% (v/v) fecal slurry suspension was added to each ultrafiltration cell with exception of the negative control sample. Instead, 50 mL of sterile phosphate buffer solution was added to the negative control sample. Ultrafiltration cells were continuously stirred and pressure was introduced on top of the ultrafiltration cells (0.8 bar N_2) to obtain dialysis. Samples (1 mL) were taken after 0, 2, 4, 6 and 24 h from the retentate fraction and after 2, 4 and 6 h from the dialysate fraction and stored at –80 °C.

2.5. Sample preparation procedure

Four different sample preparation methods (centrifugation, extraction, sonication and freeze-drying) were evaluated for the colonic retentate samples to obtain extracts that are compatible with the instrumental detection. With exception of the samples prepared using only the centrifugation step, all colonic retentate samples were immediately frozen at –80 °C after sampling as mentioned above. Centrifugation samples were first centrifuged (4 °C, 14 000 rpm, 8 min) after sampling and 1 mL of supernatant was transferred to an eppendorf tube before storage at –80 °C. Following the sample preparation method, three different dilution factors (1/10, 1/20 and 1/40) were evaluated together with the chosen optimal sample preparation technique. The extraction efficiency of the different procedures was visualized as the sum of the peak areas of all biotransformation products identified at a given dialysis-time within one sample preparation procedure. The number of identified biotransformation products was used as a second evaluation-criteria to evaluate the different sample preparation techniques.

2.5.1. Centrifugation

Before analysis samples were thawed at room temperature (15-25 °C). Samples were spiked with an internal standard (10 µL of 0.4% (w/v) theophylline solution) and vortexed during 30 s. 25 µL of the samples was transferred to a new eppendorf and 975 µL of ultrapure water/ACN (1/1, v/v) was added. Samples were transferred to a 0.2 µm nylon centrifugal filter and centrifuged (5 min, 8000 rpm) before analysis with liquid chromatography coupled to quadrupole time-of-flight mass spectrometry (LC-QTOF-MS).

2.5.2. Extraction and sonication

Samples were thawed at room temperature (15–25 °C) and spiked with 10 µL of 0.4% (w/v) theophylline solution (as internal standard). One mL of ice-cold MeOH (-80 °C) was added to quench remaining ongoing biotransformation reactions. Extraction samples were vortexed for 60 s, and sonication samples were sonicated for 60 min. Samples were further diluted by transferring 25 µL of sample to 475 µL of ultrapure water/ACN (1/1, v/v) and filtered as described above.

2.5.3. Freeze-drying

Samples were lyophilised using the automatic start-up software of the FreeZone 1 Liter Benchtop Freeze Dry System (Model 7740030, Labconco). This method maintained a temperature of –40 °C with a high vacuum of 0.133 mbar over time to remove the ice via sublimation. After lyophilisation, 1 mL of ice-cold MeOH (-80 °C) and 10 µL of 0.4% (w/v) theophylline solution (as internal standard) were added to the freeze-dried samples. Samples were sonicated for 45 min and additionally vortexed. Finally, the samples were diluted and filtered as described for the centrifugation samples.

2.6. Analysis of extracts resulting from the GIDM-Colon experiment

2.6.1. Instrumental parameters

Extracts resulting from the GIDM-Colon experiment were analyzed using an Agilent 1290 Infinity ultra-high-performance liquid chromatography instrument coupled to an Agilent 6530 Accurate-Mass Q-TOF (Agilent, Santa Clara, CA, USA). Chromatographic separation was performed on a Luna Omega PS C18 column (100 x 2.1 mm; 3 µm particle size) from Phenomenex (Utrecht, the Netherlands) using a mobile phase composed of ultrapure water with 0.04% (v/v) formic acid (A) and 80/20 (v/v) ACN/ultrapure water with 0.04% (v/v) formic acid (B) with a constant flow of 0.3 mL/min. The gradient elution method was constructed as follows: for 3 min A was used at 100%, after which B increased till 8% at 18 min. B was further increased till 95% at 28 min. Consecutively, the column was rinsed with 95% B for 4 min, and re-equilibrated at 100% A for 4 min before the next injection. The injection volume was 5 µL. The eluent was directed to the waste during the first minute of each run to protect the ion source from extensive contamination as well as during the 4 min of re-equilibration. The column temperature was kept constant at 40 °C.

All samples were analyzed in negative ionization mode using Agilent Jet Stream electrospray ionization (ESI) with following parameters: 300 °C drying gas temperature, 8 L/min drying gas flow, 25 psig nebulizer pressure, 350 °C sheath gas temperature, 11 L/min sheath gas flow, 3500 V sampling capillary voltage and 500 V nozzle voltage.

The QTOF mass spectrometer was operated in the 2 GHz (extended dynamic range) mode in data-dependent MS/MS acquisition. In case it was needed, reanalysis of extracts in targeted MS/MS mode was applied for interesting features where no MS/MS spectrum was obtained in the data-dependent MS/MS mode. Calibration of the mass axis was performed in negative ion mode with respect to two ions with an *m/z*-value of 119.0363 and 966.0007. Instrumental variation was evaluated by monitoring the relative standard deviation of the internal standard peak area in the samples throughout the analysis. The acquisition parameters were set for an *m/z* range from 50 to 1000 at a scan rate of 2.5 scans/s and 6.67 scans/s for MS and MS/MS spectra, respectively. Collision energies were applied at 5, 10 and 20 eV. All data were stored in centroid mode.

2.6.2. Data analysis workflows

This study applied three complementary data processing workflows for the detection and identification of biotransformation products. The workflows were developed in-house and were based on earlier optimised workflows used in *in vitro* human liver biotransformation investigations for xenobiotics [23, 24]. Next to a suspect screening approach based on literature and *in silico* biotransformation prediction, two non-targeted screening approaches were applied to the data.

Using the suspect screening, a list of possible biotransformation products was received from the *in silico* prediction software BioTransformer (Biotransformer.ca, v1.0.0). Using the webtool, the SMILES string of chlorogenic acid was uploaded and 'Human Gut Microbial Transformation' was selected as metabolic transformation. The generated csv file contained InChIKey, synonyms, major isotope mass, molecular formula, type of biotransformation reaction and precursor ID. The molecular formulas and corresponding exact masses were stored in a csv database. Microbial biotransformation products of chlorogenic acid described in literature and not predicted by BioTransformer were added to the database. Table SI-1 in supplementary information gives an overview of the different consulted papers together with the number of identified biotransformation products.

Identification was based on the accurate mass, isotopic pattern, fragmentation pattern of the product ions and injection of an analytical standard if available. Similar criteria were set as in previous studies [23, 24]: (a) maximal mass variation of ± 10 ppm between theoretical and measured parent ions; (b) product ions may not exceed the maximal mass variation of ± 25 ppm; (c) the identified biotransformation products were not present in the blank or negative control sample, unless mentioned otherwise in the manuscript and (d) the biotransformation product was present in both replicates at a certain sampling time.

One of the untargeted screening workflows used the software packages MzMine and R. Raw data files were uploaded in MZmine 2.33 [25]. Molecular features were detected using the centroid algorithm followed by a chromatogram building step. A noise amplitude algorithm was used to deconvolute the chromatograms. Deisotoping was performed by retaining the lowest *m/z* as the representative isotope. Peaks with a peak width lower than 0.05 min or more than 1 min were discarded. Next, the random sample consensus (RANSAC) alignment algorithm aligned the peaks across samples. Finally, any missing peaks were extracted with the same RT and *m/z* gap filler algorithm. The obtained *m/z* features were exported as a csv file, imported into R and processed using a previously published in-house developed script [24, 26]. Fold changes of each feature were calculated between samples and blank samples, as well as the p-value from a student t-test. Features with a log-10 fold change greater or equal to 1 and a p-value <0.05 were retained. For each *m/z* value, the log-10 fold change was plotted to the log-

10 p-value leading to a volcano plot (supplementary information Figure SI-2). Corresponding molecular formulas to the *m/z* values were calculated using the Generate Formulas algorithm in the MassHunter Qualitative Analysis software (version B.07.00, Agilent Technologies, Santa Clara, CA, USA). Identification was based on the same criteria as mentioned for the suspect screening.

Finally, the third data analysis workflow combined the MassHunter Qualitative (version B.07.00, Agilent Technologies, Santa Clara, CA, USA) and MassProfiler Professional (v12.6, Agilent Technologies, Santa Clara, CA, USA) software-packages. The Find by Molecular Feature algorithm in MassHunter Qualitative software extracted the features from the data without restrictions concerning retention time or *m/z* value. The data were imported in Mass Profiler Professional. Samples were classified per type (blank, negative control or replicate) and incubation time (0 h, 2 h, 4 h, 6 h or 24 h). Features present in one or more blanks were discarded by filtering the data on frequency. Additional filtering was performed on the features by retainment of the features present in both replicates at a given incubation time. The Generate Formulas algorithm in MassHunter Qualitative software was used to predict the molecular formulas based on the found *m/z* values. Identification was based on the same criteria as mentioned for the suspect screening.

3. Results and discussion

3.1. Processing and incubation of fecal samples

3.1.1. Optimization of incubation medium

Detailed results of the 16S rDNA gene sequencing are shown in supplementary information Fig. SI-3. The principle component analysis (PCA) plot in Fig. 3 represents the comparison of the microbial composition between the samples of the individual volunteers, the pooled sample and the 17 h incubated pooled fecal suspension in WCB or sterile phosphate buffer based on the 16S rDNA gene sequencing results. Incubation in WCB led to a clear switch in bacterial composition of the pool, while this effect was less pronounced for the sterile phosphate buffer as medium. The 17 h incubated fecal suspension pool in phosphate buffer showed a closer resemblance to the composition of the initial pool (i.e. before incubation) and to the composition of the individual volunteers. These results suggest that the choice of a sterile phosphate buffer as incubation medium gave a closer resemblance to the *in vivo* situation leading to a more representative *in vitro* model.

3.1.2. Optimization of incubation time

As phosphate buffer showed to be the most appropriate incubation medium for maintaining the resemblance with the *in vivo* bacterial composition, an additional experiment regarding the effect of the incubation time in phosphate buffer on the number of bacteria was necessary. No clear increase or decrease of the CFU/g bacteria in the fecal slurry suspension could be observed over 24 h of incubation. A detailed overview of the results can be found in supplementary information (Table SI-2, Fig. SI-4). A pooled fecal suspension sample incubated for 17 h in WCB was added as comparison and contained 1.48 x 10^9 CFU/g. In comparison to phosphate buffer, a clear effect of incubation in WCB was observed, however the disadvantage of this method, with a shift in bacterial composition, was already described above. The minor influence of phosphate buffer on the bacterial concentration in comparison to WCB was expected since phosphate buffer did not contain any growth factors or energy sources. Furthermore, experiments with fecal suspensions in phosphate buffer incubated with chlorogenic acid as model compound showed colonic biotransformation activity. With regard to these results and the increased risk of introducing

variation during incubation, it was concluded that the 17 h incubation time in WCB, as described by Breynaert et al., showed no advantage over phosphate buffer [12]. Elimination of this 17 h incubation phase will reduce the total time of the GIDM-Colon experiments thus increasing the throughput of the total platform.

3.2. Optimization of sample preparation procedure

3.2.1. Extract preparation

An overview of the extraction efficiency of the different sample preparation procedures can be found in Fig. 4. The sum of the peak areas of all biotransformation products identified at a given dialysis-time was plotted against the different procedures. The results showed that freeze-drying as a sample preparation method overall resulted in lower peak areas of colonic biotransformation products in comparison to the three other methods, which showed similar results. Furthermore, biotransformation product M11 (Table 1) was not detected by freeze-drying in comparison to the other sample preparation methods.

The inferiority of freeze-drying as a sample preparation method could be explained by irreversible binding of hydrophilic biotransformation products to matrix components (microbial membranes and/or cell wall macromolecules) and loss of these molecules due to precipitation during extraction steps such as centrifugation [27, 28]. Using the centrifugation, extraction and sonication method, no big differences in relative peak area were detected, suggesting that sonication and extraction did not cause more leakage of intracellular biotransformation products compared to the centrifugation method or all biotransformation products of chlorogenic acid were excreted in the extracellular environment by the fecal bacteria. Although the method using sonication appeared to be slightly better in comparison to extraction (based upon availability of MS/MS data), the extraction method was selected as an optimal method. A big advantage of the latter method is the limited time needed to prepare the samples, leading to the ability to perform more experiments in a shorter time period.

3.2.2. Dilution

One biotransformation product (M16) was not detected using the 1/40 retentate dilution, since the 1/40 dilution influenced the sensitivity of the detection of biotransformation products more negatively compared to the 1/20 and 1/10 dilution. Using a 1/10 dilution would introduce a bigger risk concerning apparatus contamination compared to a 1/20 dilution, while the same number of identified biotransformation products was reached using both dilutions. Usage of a 1/20 dilution was selected as the most optimal method, since it was the highest dilution (i.e. less risk of contamination of the LC-MS system and ion suppression) that resulted in highly sensitive detection of biotransformation products of chlorogenic acid.

3.3. Analysis of extracts

3.3.1. Injection solvent

When analyzing extracts immediately after sample preparation by LC-MS, poor chromatographic peak shapes were observed (supplementary information Fig. SI-5A). However, reinjection of the samples after overnight storage at –20 °C led to an improvement of peak shape (supplementary information Fig. SI-5B). These results could be explained by the findings of Gu et al. [29]. They showed that phase separation of an ACN-water mixture could occur at –17 °C. Injection of the samples after storage at –20 °C, without homogenization (e.g. vortexing), would lead to the injection of the aqueous phase, as this is the most dense solvent. The composition of the injected

sample would show a higher resemblance to the starting mobile phase, leading to better chromatographic peaks. This hypothesis was confirmed with an additional experiment (supplementary information Fig. SI-6 and SI-7). Homogenization of the samples after storage led to poor quality peak shapes. Samples evaporated to dryness and reconstituted in mobile phase A showed high quality peak shapes, and no influence of storage at -20 °C or homogenization on peak shape was detected. This highlights the importance to check the compatibility of the extracts to be analyzed with the mobile phase used in the LC set-up.

3.3.2. Evaluation data-analysis workflows

In total, 23 colon biotransformation products of chlorogenic acid were identified (Table 1) when combining the three workflows. An overview of the results can be found in Fig. 5. If the suspect screening or the 'MZmine + R' workflow would have been used, only 16 biotransformation products would have been identified, while the MPP workflow would have led to the identification of 17 colonic biotransformation products. One, four and five biotransformation products were unique for the suspect screening, MPP and MZmine + R workflow respectively. These data confirm the complementarity of the three different data-analysis workflows as previously described by Vervliet et al. [24].

4. Conclusions

This paper presents the optimization of a complete and ready-to-use *in vitro* bioanalytical platform for the identification of colonic biotransformation products of xenobiotics by the gut microbiome, starting from the GIDM-Colon developed by Breynaert et al. [12]. First, WCB as incubation medium showed to introduce variation in the bacterial composition of the pooled fecal slurry suspension. A sterile phosphate buffer was chosen as incubation medium as a closer resemblance to the *in vivo* composition of fecal bacteria was guaranteed. Furthermore, incubation of a 10% (v/v) fecal slurry suspension using a sterile phosphate buffer (0.1 M, pH 7) as medium showed no big increase or decrease in anaerobic bacterial concentration over 24 h of incubation. Future experiments to simulate the human colon microbiome *in vitro* as a tool to investigate colonic biotransformation, can be performed without fecal incubation, reducing the risk of introducing changes in the initial fecal bacterial composition. Reduction of this risk enables a better resemblance to *in vivo* human colonic biotransformation during *in vitro* gastrointestinal dialysis experiments.

Centrifugation, sonication, extraction and freeze-drying were used to evaluate the influence of the sample preparation procedure on detection of colonic biotransformation products. Centrifugation, sonication and extraction methods gave similar results, while freeze-drying clearly showed to be inferior. Extraction was selected as the optimal method considering the shorter time period needed to obtain samples suitable for LC injection. Furthermore, a 1/20 dilution of samples prior to LC-QTOF-MS analysis was chosen as most optimal dilution in comparison to a 1/10 and 1/40 dilution.

The optimization of the GIDM-Colon system and the pre-analytical sample preparation phase, as well as the combination of complementary data-analysis workflows (Suspect screening, MZmine + R and MPP workflow) for biotransformation product identification have resulted in the establishment of a unique ready-to-use platform to investigate colonic biotransformation of different compounds. Moreover, this platform can be used to investigate interindividual differences in colonic biotransformation, by using different fecal donors. When using this platform to investigate colonic biotransformation of different compounds, chlorogenic acid will be implemented as a positive control.

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Conflicts of interest

The authors declare they have no conflict of interest.

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Figures and tables

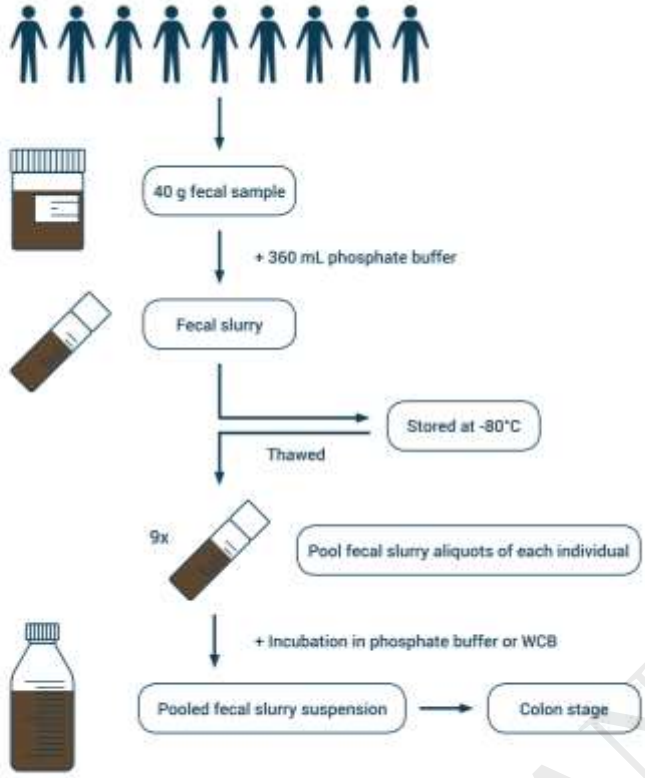

Fig. 1 Overview of the fecal sample treatment before use in the gastrointestinal dialysis model with colon-stage.

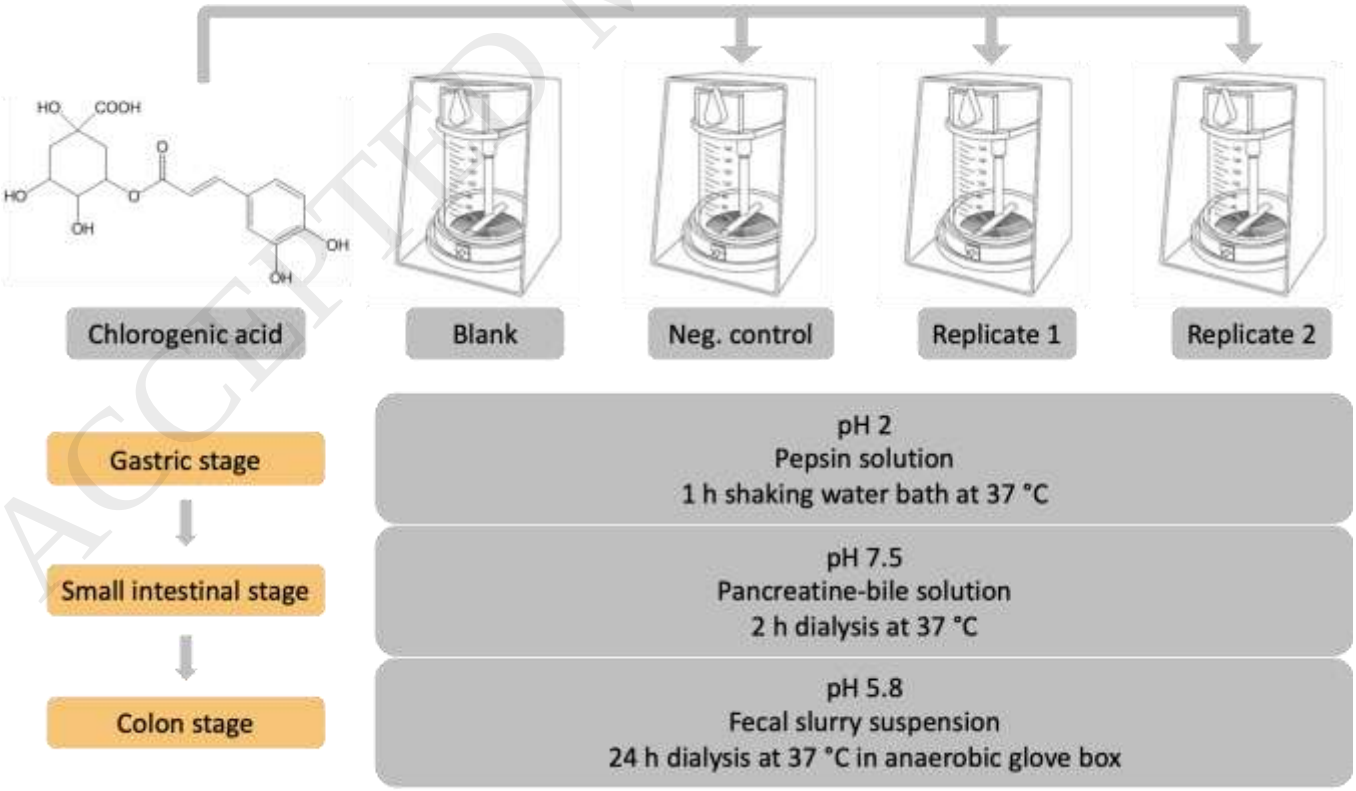

Fig. 2 Experimental setup during the gastrointestinal dialysis model with colon stage (GIDM-Colon).

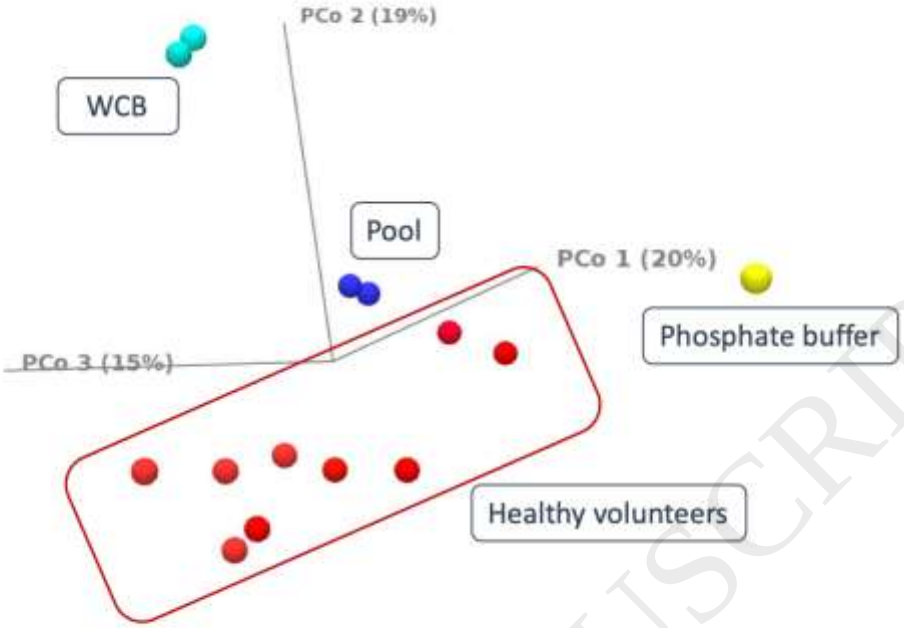

Fig. 3 PCA plot showing the comparison between the 16S rDNA gene sequencing results of the samples of the healthy volunteers (Control samples, red), the pooled fecal slurry (blue), the pooled fecal slurry suspension after 17 h of incubation in phosphate buffer (yellow) and the pooled fecal slurry suspension after 17 h of incubation in WCB (turquoise).

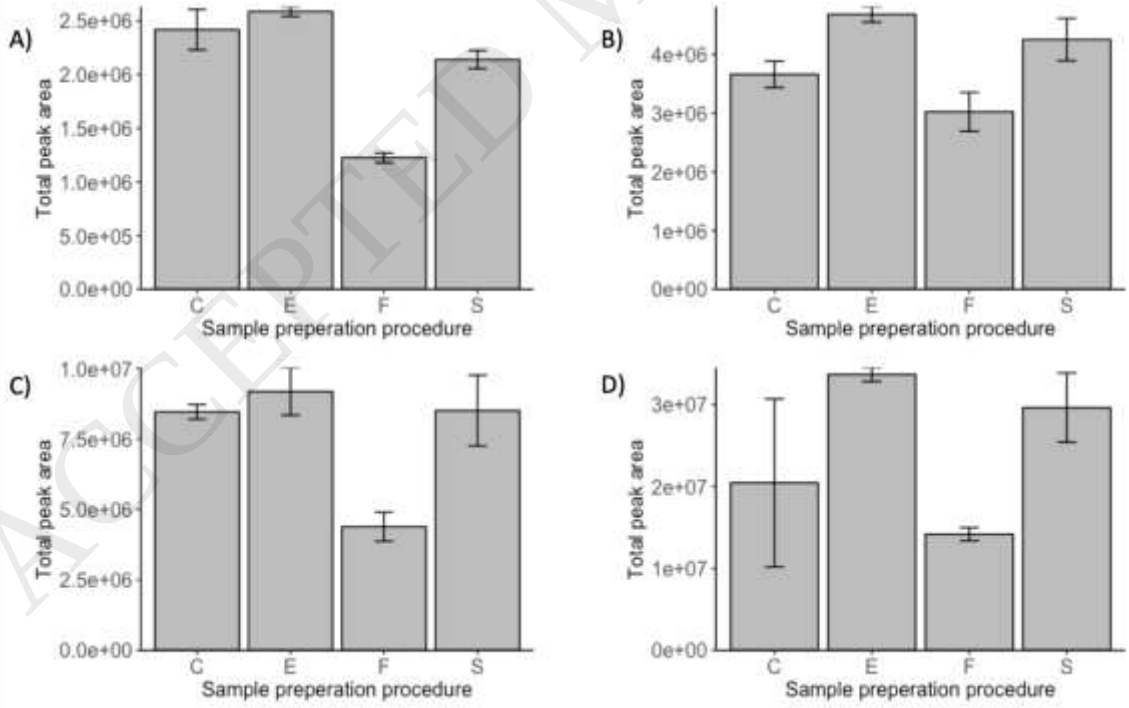

Fig. 4 Sum of the peak areas of the identified biotransformation products for each sample preparation procedure at different dialysis-times in the GIDM-colon: 2 h (A), 4 h (B), 6 h (C) and 24 h (D).

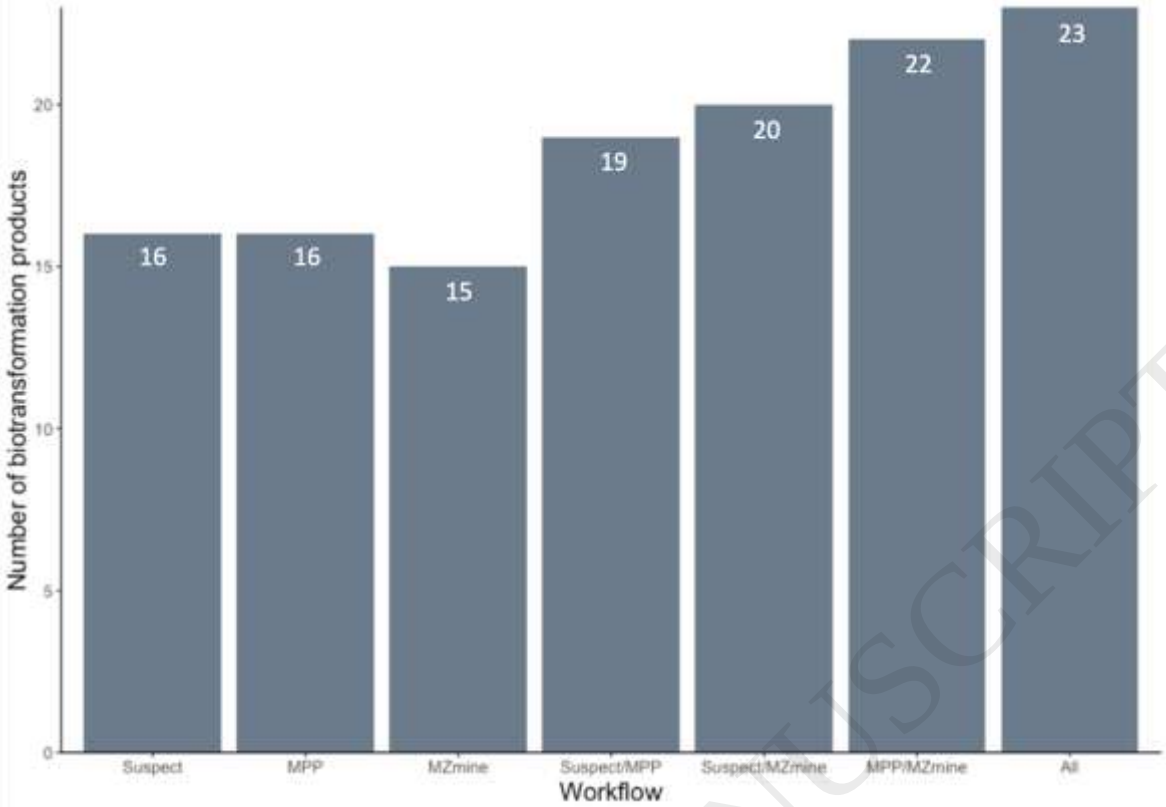

Fig. 5 Comparison of the different data-analysis workflows based on the number of identified biotransformation products.

Table 1 Metabolite ID, compound name, molecular formula, level of confirmation as proposed by Schymanski et al.[30], detected by which workflow, retention time, exact mass, detected parent ion, mass difference. Level 1 (L1): Structure confirmed by analytical reference standard; level 2a (L2a): Probable structure by library spectrum match; level 3 (L3): tentative candidates based on experimental data; level 4 (L4): Unequivocal molecular formula based on MS1 and isotope ratios. MPP: MassProfiler Professional. *A: Confirmed using mzCloud.org

ID	Compound name	Molecular formula	Level of confirmation*	Workflow	t_r (min)	Exact mass (g/mol)	Parent ion (*m/z*)	Δ Mass (ppm)	MS/MS product ions
M1	Trihydroxycyclohexane carboxylic acid	C7H12O5	L3	Suspect, MZmine	1.1	176.0685	175.0613	-0.03	157.0493 [C7H9O4]-; 113.0609 [C6H9O2]-; 95.0493 [C6H7O]-; 73.0297 [C3H5O2]-; 44.9992 [CHO2]-
M2	NA	C10H12O6	L4	MPP	1.1	228.0617	227.056	-7.22	NA
M3	NA	C10H12O7	L4	MZmine	1.1	244.0599	243.0523	6.64	NA
M4	Quinic acid	C7H12O6	L1	All	1.12	192.0635	191.0564	0.47	85.0297 [C4H5O2]-; 73.0271 [C3H5O2]-; 59.0157 [C2H3O2]-
M5	Dihydroxycyclohexane carboxylic acid	C7H12O4	L3	Suspect, MPP	1.12	160.0733	159.0662	-1.41	113.0594 [C6H9O2]-
M6	NA	C10H12O8	L4	MZmine	1.12	260.0516	259.0446	-6.08	191.0583 [C7H11O6]-; 172.9757 [C5HO7]-; 127.0425 [C6H7O3]-; 85.0305 [C4H5O2]-
M7	NA	C12H16O9	MS, MS/MS	MZmine	1.13	304.078	303.0713	-4.69	191.0556 [C7H11O6]-; 143.0108 [C9H3O2]-; 135.0848 [C9H11O]-; 111.0449 [C6H7O2]-; 109.0260 [C6H5O2]-; 89.0141 [C3H5O3]-; 85.0311 [C4H5O2]-; 60.9936 [CHO3]-
M8	NA	C18H20O8	L4	MZmine	10.9	364.115	363.1079	-2.14	181.0496 [C9H9O4]-; 137.0596 [C8H9O2]-

M9	6-(3-(3,4-dihydroxyphenyl)-1-hydroxypropoxy)cyclohexane-1,2,4-triol	C15H22O7	L3	MPP	11	314.1352	313.1282	-4.21	203.0314 [C11H7O4]-; 181.0496 [C9H9O4]-; 135.0442 [C8H7O2]-; 131.0704 [C6H11O3]-; 107.0490 [C7H7O]-
M10	Dihydrocaffeic acid	C9H10O4	L1	All	11.42	182.0579	181.0507	-0.01	137.0593 [C8H9O2]-; 121.0286 [C7H5O2]-; 119.0465 [C8H7O]-; 109.0287 [C6H5O2]-; 59.0138 [C2H3O2]-
M11	Hydroxybenzaldehyde	C7H6O2	L2a[A]	Suspect, MZmine	11.52	122.0367	121.0294	-0.84	93.0310 [C6H5O]-
M12	Caffeic acid	C9H8O4	L1	All	15.21	180.0414	179.0342	-4.63	135.0431 [C8H7O2]-; 117.0353 [C8H5O]-; 91.0000 [C6H3O]-; 44.9992 [CHO2]-
M13	Caffeic acid quinone	C9H6O4	L3	All	15.4	178.0265	177.0191	-0.83	105.0331 [C7H5O]-; 45.0001 [CHO2]-
M14	Dihydrochlorogenic acid	C16H20O9	L3	Suspect, MPP	16.28	356.1094	355.1022	-3.74	191.0540 [C7H11O6]-; 173.0446 [C7H9O5]-; 137.0630 [C8H9O2]-
M15	3-((3-(3,4-dihydroxyphenyl)-3-hydroxypropanoyl)oxy)-1,4,5-trihydroxycyclohexane-1-carboxylic acid	C16H20O10	L3	Suspect, MPP	16.51	372.1045	371.0983	-0.84	353.0867 [C16H17O9]-; 191.0556 [C7H11O6]-
M16	NA	C10H12O5	L4	All	19.14	212.0674	211.0603	-5.14	165.0551 [C9H9O3]-; 121.0656 [C8H9O]-; 106.0426 [C7H6O]-
M17	3-Phenyllactic acid	C9H10O3	L2a[A]	All	16.79	166.0628	165.0556	-1.26	147.0415 [C9H7O2]-; 119.0472 [C8H7O]-; 103.0533 [C8H7]-; 91.0547 [C7H7]-; 72.9901 [C2HO3]-; 44.9972 [CHO2]-
M18	1-Caffeoylglycerol	C12H14O6	L3	All	19.92	254.0791	253.0719	0.11	179.0328 [C9H7O4]-; 161.0236 [C9H5O3]-; 135.0443 [C8H7O2]-; 133.0298 [C8H5O2]-
M19	NA	C10H12O4	L4	Suspect, MPP	21.26	196.0729	195.0658	-3.32	NA
M20	NA	C8H10O2	L4	All	21.63	138.0679	137.0607	-1.34	NA

M21	NA	C12H14O5	L4	MPP	21.96	238.0838	237.0763	-1.43	161.0231 [C9H5O3]-; 135.0441 [C8H7O2]-; 119.0358 [C4H7O4]-
M22	Methyl caffeate	C10H10O4	L2a[A]	All	22.52	194.058	193.0507	0.47	161.0224 [C9H5O3]-; 133.0288 [C8H5O2]-; 104.0237 [C7H4O]-
Parent	Chlorogenic acid	C16H18O9	L1	Suspect	18.51	354.095	353.0878	-0.27	191.0546 [C7H11O6]-; 173.0451 [C7H9O5]-; 161.0214 [C9H5O3]-; 135.0413 [C8H7O2]-