Metabolomics reveals impact of seven functional foods on metabolic pathways in a gut microbiota model

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HIGHLIGHTS

- Metabolomics was employed to assess 7 functional foods impact on gut microbiota.
- Insights regarding how functional foods alter gut metabolic pathways is presented.
- Increased GABA production was observed in polyphenol rich functional food.
- Purine alkaloids served as direct substrate in microbiota metabolism.

GRAPHICAL ABSTRACT

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Functional foods gut microbiota interaction

ABSTRACT

Functional food defined as dietary supplements that in addition to their nutritional values, can beneficially modulate body functions becomes more and more popular but the reaction of the intestinal microbiota to it is largely unknown. In order to analyse the impact of functional food on the microbiota itself it is necessary to focus on the physiology of the microbiota, which can be assessed in a whole by untargeted metabolomics. Obtaining a detailed description of the gut microbiota reaction to food ingredients can be a key to understand how these organisms regulate and bioprocess many of these food components.

Abbreviations: GC, Green Coffee; BC, Black Coffee; GT, Green Tea; BT, Black Tea; Fl, Opuntia ficus-indica (prickly pear); POM, pomegranate (Punica granatum); SUM, sumac (Rhus coriaria); SCFAs, short chain fatty acids; GI, gastrointestinal; GIT, gastrointestinal tract.

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Introduction

In humans, the gastrointestinal (GI) tract harbors approximately 10^{14} bacterial cells (i.e., 10 times the number of eukaryotic cells in the body), [1]. Bacteria contributing to this intestinal microbiota niche belong to more than 1,000 different species harboring more than three million bacterial genes and amount to a biomass of approximately 2 kg, which can be considered as an internal “organ” and provide many functions that are crucial for the hosts well-being. Moreover, the study of intestinal microbiota cannot be separated from its environmental context. For instance, host genetics, geographical location, nutrition, antibiotics, and other treatments affect the microbiota and its metabolic machinery [2]. The human GI tract hosts a nutrient-rich environment that supports a commensal microbiome providing crucial functions that cannot be carried out alone by the host [3,4]. These functions are both metabolic (colon fermentation and production of short chain fatty acids), protective (improving barrier and increasing the resistance to colonization by opportunistic pathogens, secretion of antimicrobial peptides etc.), and structural (maturity of the intestinal epithelium and the immune system) [1]. Microbes present at mucosal sites can also become part of the tumor microenvironment of aerodigestive tract malignancies [5]. In counterpoise, gut microbiota also functions in detoxification of dietary or drug components, can reduce inflammation, and help to maintain a balance in host cell growth and proliferation [6]. Thus, interrogation of gut microbiota metabolism as such and in response to dietary intervention requires a holistic perspective. Assigning microbial communities, their members, and aggregate biomolecular activities into these categories will require a substantial research commitment. Beyond metagenomics, functional approaches, such as metatranscriptomics, metaproteomics, and metabolomics (together referred to as met-omics), are now also rapidly enhancing our knowledge on the gut microbiome. Metatranscriptomics and metaproteomics are doing so by providing a snapshot of the gene expression and protein production, respectively, while metabolomics is providing information on the concentration of small molecules, such as peptides, amino acids, and lipids [7].

Daily diet has an impact on not only our nutrition but also health and wellness. This has necessitated an increase in the development and characterization of food products with additional effects than just energy, mineral or vitamin supply, the so-called functional foods. Functional foods can be defined as dietary supplements that in addition to their nutritional values, can beneficially modulate body functions towards enhancing physiological responses or reducing a risk of certain disease [8] to the extent that these can be nutraceuticals, i.e. foods with clearly established medicinal properties. Upon food ingestion, several mechanical, chemical and enzymatic processes occur within the GIT to mediate its digestion into nutrients or active ingredients, which are then absorbed to be suitable for use by the body [9].

Gut microbiota mediated metabolic activity can contribute to the digestion of various dietary compounds as well as transformation of xenobiotics as in functional foods and supply of micronutrients, thus affecting their potential health effects. In contrast, functional food components can themselves also affect the growth and the metabolic activity of gut microbiota accordingly their composition and or potential functions [10]. For instance, tea polyphenols exhibited an inhibitory effect on certain gut microbiota species such as; Bacteroides spp., Clostridium spp. (C. perfringens and C. difficile), E. coli and Salmonella typhimurium with caffeic acid showing the highest inhibitory activity [11]. In parallel, gut microbiota can also affect the pharmacological properties of ingested food products via its biotransformation while in the gut pending if not absorbed earlier in the GIT [12]. For example, tannins are solely metabolized by microbial enzymes leading to the formation of conjugated derivatives, which have a different pharmacological profile and being subject to rapid excretion through urine or bile secretions back into the gastro intestinal tract [13]. Whilst most studies have indeed focused on how gut microbes bio transform functional foods, few reports are known to us, on how gut microbiota metabolism is affected by these food supplements [14]. Including; the wide range alterations in the gut microbiota composition imparted by animal-based vs. plant-based diets [15] as well as, the increased abundance of Bifidobacterium species in breast-fed infants over formula-fed ones [16]. Nevertheless, further studies are needed to investigate the impact of such changes on the normal homeostasis of GI tract.

The main interaction between organisms is of chemical nature. Obtaining a detailed description of how functional foods interact with gut microbiota or do affect its biotransformations can be a key to understand how these organisms regulate our daily food.

Metabolomics is the systematic study of the small-molecule metabolite profiles of living organisms at certain status or phenotype. Such dense chemical information can be acquired through utilizing hyphenated mass spectrometry techniques such as; gas or liquid chromatography-mass spectrometry (GC-MS and LC-MS) [17]. Moreover, untargeted GC/MS-based metabolomics is routinely used to detect and monitor low molecular weight and non-polar primary and secondary metabolites, the latter known to be abundant within a plant matrix [18]. Since the microbiota...
in humans but also in domestic animals is highly diverse and individual, and thus incomprehensible and irreproducible, we used a simplified but therefore more reliable gut microbiota model system. Hence we established cultivating a selection of eight bacterial species that are representing the core functions of the large intestine microbiota [19]. These consortium is comprised of 8 bacterial species namely: Anaerostipes cacao, Bacteroides thetataoamazon, Bifidobacterium longum, Blautia producta, Clostridium butyricum, Clostridium ramosum, Escherichia coli and Lactobacillus plantarum. These species belong to the most abundant phyla of the human gut microbiota representing the extended simplified intestinal human microbiota (SIHUMix) with functionally important biochemical pathways and interactions that likely occur in the human gut.

In order to cover at least part of the most commonly consumed food products worldwide either as beverages, condiments, food color and or herbal drugs [20], we selected the following plant products: green (GC) and black (BC) coffee (Coffea arabica), green (GT) and black (BT) tea (Camellia sinensis), Opuntia ficus-indica (Fl), pomegranate (POM) (Punica granatum) and sumac (SU) (Rhus coraria). For example, various coffee constituents are reported to exhibit antioxidant properties and to protect against cardiovascular, inflammatory and neurodegenerative diseases [21,22]. While, many health benefits have been attributed to tea products consumption such as antihypertensive [23], anti-hyperlipidemic [24], antioxidant [25] and CNS stimulant effects. On the other hand, the ability to form a stable community [19]. Co-cultured bacterial species included: Anaerostipes cacao (DSMZ 14662), Bacteroides thetataoamazon (DSMZ 2079), Bifidobacterium longum (NC 2705), Blautia producta (DSMZ 2950), Clostridium butyricum (DSMZ 10702), Clostridium ramosum (DSMZ 1402), Escherichia coli K-12 (MG1655) and Lactobacillus plantarum (DSMZ 20174). All bacteria were cultivated in Brain-Heart-Infusion (BHI) medium under anaerobic conditions at 37 °C and 175 rpm shaking for 72 h prior to inoculation. All strains were shown to be able to grow equally in the media. BHI media was prepared by mixing 37 g brain heart infusion, 0.5 g L-cysteine hydrochloride, 0.001 g resazurin, 10 ml Vitamin K hemin solution and 5 g yeast extract in one L of sterile water. Gut bacteria cultured in Brain-Heart-Infusion medium (optical density of 0.1) was left to grow under anaerobic condition at 37 for 18 h till optical density reached 1.7 prior to functional food extract addition. Details on isolated microbiota consortium strains and its potential metabolic functions is depicted in Suppl. Table S1 and S2, respectively. For the control including only SIHUMix strains and each functional food extract, cultivation was perfomed in triplicates leading to 48 samples in total.

**Gut microbiota culture**

The microorganisms consortium used in this study is described as: the extended simplified intestinal human microbiota – SIHUMix. Microorganisms of the SIHUMix community, a model for the intestinal microbiota, were selected according to their occurrence in humans, the spectrum of fermentation products formed and the ability to form a stable community [19]. Co-cultured bacterial species included: Anaerostipes cacao (DSMZ 14662), Bacteroides thetataoamazon (DSMZ 2079), Bifidobacterium longum (NC 2705), Blautia producta (DSMZ 2950), Clostridium butyricum (DSMZ 10702), Clostridium ramosum (DSMZ 1402), Escherichia coli K-12 (MG1655) and Lactobacillus plantarum (DSMZ 20174). All bacteria were cultivated in Brain-Heart-Infusion (BHI) medium under anaerobic conditions at 37 °C and 175 rpm shaking for 72 h prior to inoculation. All strains were shown to be able to grow equally in the media. BHI media was prepared by mixing 37 g brain heart infusion, 0.5 g L-cysteine hydrochloride, 0.001 g resazurin, 10 ml Vitamin K hemin solution and 5 g yeast extract in one L of sterile water. Gut bacteria cultured in Brain-Heart-Infusion medium (optical density of 0.1) was left to grow under anaerobic condition at 37 for 18 h till optical density reached 1.7 prior to functional food extract addition. Details on isolated microbiota consortium strains and its potential metabolic functions is depicted in Suppl. Table S1 and S2, respectively. For the control including only SIHUMix strains and each functional food extract, cultivation was perfomed in triplicates leading to 48 samples in total.

**Materials & methods**

**Plant material and extraction**

Methanol extracts were prepared from finely powdered green GC and black coffee BC seeds, green GT and black tea BT leaf, peeled Opuntia ficus-indica red ‘Rose’ Fl fruit powder and sumac SU lyophilized fruits powders by cold maceration over 2 days using 100% methanol until exhaustion. Extracts were then filtered and subjected to evaporation under vacuum at 40°C until complete dryness. Extracts were placed in tight glass vials and stored at −20°C until further analysis. In case of pomegranate POM, seeds were extracted and expressed to obtain a juice, which was then lyophilized until complete dryness and stored as above.

**Chemicals and solvents**

All solvents and chemicals were of analytical grades and purchased from Sigma-Aldrich, St. Louis, USA.

**Gut microbiota functional food incubation assay**

A stock solution was prepared of functional food at a concentration of 50 mg/ml in 50:50 methanol: (BHI) growth medium and stored at 4 °C until inoculation. 100 μl and 1 ml of each functional food stock solution was then aliquoted to a final volume of 10 ml BHI media containing the gut microbe culture to achieve a final concentration of 0.5 and 5 mg/ml, respectively. Blank cultures were prepared by adding an equivalent amount of 50 and 500 μl 100% methanol into the culture medium, kept under the same condition, and compared to the culture receiving no solvent treatment.

**Metabolites extraction and GCMS analysis**

200 μl of aliquoted culture harvested at different time points was spiked with xylitol standard solution dissolved in sterile water to reach a final concentration of 10 μg/ml followed by the addition of 800 μl acetonitrile/methanol mixture with incubation at 4 °C for 30 min till complete protein precipitation. Mixture was then centrifuged at 12,000g using Eppendorf centrifuge for 4 min, with 100 μl of the supernatant then aliquoted and subjected to evaporation under nitrogen stream till complete dryness. For metabolites derivatization, 150 μl of N-methyl-N-(trimethylsilyl)-trifluoroacetic amide (MSTFA) was then added to the residue and incubated at 60 °C for 45 min. Samples were then analyzed using GC–MS (Shi-谙du, Japan). Silylated derivatives were separated on Rtx-5MS (30 m length, 0.25 mm inner diameter, and 0.25 μm film) column. Injections were made in a (1:15) split mode, conditions: injector 280°C, column oven 80°C for 2 min, rate 5 °C/min to 315°C, kept at 315°C for 12 min. He carrier gas at 1 mL/min-1. The transfer line and ion-source temperatures were set at 280 and 180 °C, respectively.

**GC-MS multivariate data analyses**

MS peak abundance of primary silylated metabolites were extracted using MET-IDEA software with default parameter settings for GC–MS [29]. The aligned peak abundance data table
Results & discussion

Two different time aliquots were obtained from functional foods amended cultures, the first one was at 0.5 h a time at which no significant biotransformation is expected to occur, and at 24 h at which most of the biochemical and enzymatic changes would have occurred. Results revealed that while addition of functional foods alter microbiota metabolism through either stimulation or inhibition of its metabolic pathways, not surprisingly functional food metabolites themselves can also act as substrate for microbiota metabolism.

GC-MS metabolite profiling of gut microbiota treated functional foods

GC/MS was employed to characterize microbiota culture metabolism and monitor changes occurring 30 m and 24 h post exposure to the different functional food extracts. Metabolites detected (as such or as volatile per-trimethylsilylated derivatives) comprised mostly microbial low molecular weight primary metabolites viz. organic acids, alcohols, amino acids, fatty acids, inorganic compounds, nitrogenous compounds, nucleic acids, phenolics, steroids and sugars in addition to few secondary metabolites representative of certain functional foods, e.g. catechins in case of tea [17]. A total of 131 metabolite peaks (Suppl. Table S3) was detected from all untreated blank and functional food treated cultures. The relative percentile levels of all metabolite classes detected for cultures harvested at 0.5 and 24 h from functional foods and blank cultures is presented in (Fig. 1), and a representative chromatogram showing main metabolite classes with their elution regions can be found in (Suppl. Fig. S1). GC-MS Metabolite profiling revealed that amino acids form the major class in cultures harvested at 0.5 h ranging from 44% to 60% in those amended with food extracts compared to 62% in blank culture at the same time point (Suppl. Table S3), suggesting that amino acids are mostly derived from the microbial culture itself. Following amino acids, nitrogenous compounds represented the second most abundant class, ranging from 14% to 19% in blank gut microbial culture and those fortified with food extracts at 0.5 h. Compared to amino acids and nitrogenous compounds that showed comparable levels at 0.5 h incubation, sugars (4–20%) showed a larger variation among cultures fortified with different food extracts compared to blank culture with 4.0%, supporting the conclusion that such difference is attributed to functional foods individual compositions. Sugars are a major primary metabolite class in most plant foods as assessed using GC-MS [30]. Other minor classes identified in microbial cultures included inorganic metabolites (2–7%), phenolics (0.02–5.6%) and nucleic acids (1.6–2%).

After incubation for a period of 24 h, samples were aliquoted and analyzed using the same protocol to reveal for metabolite changes occurring in culture (Fig. 2). A general decrease in amino acid levels by 16% (0.8 fold) in functional food treated samples, with the largest decrease observed in sumac treated cultures (0.6 fold) indicate that amino acids may serve as nutrient substrate for gut microbial growth. Amino acids are utilized by bacteria as building blocks for microbial protein assembly essential for bacterial growth, or to be fermented as an energy source [31]. This amino acid decrease trend in functional food treated samples 24 h post incubation is contrasting the untreated blank samples, showing even a slight 1.1-fold increase in amino acid content. For nitrogenous compounds a decrease was revealed in all cultures treated and blank. Most pronounced decreases in functional food treated samples were observed with sumac, green tea and green coffee with 0.6, 0.8 and 0.8 fold reductions, respectively. Bacteria can utilize nitrogenous compounds such as amino propanoate as a single carbon source or energy source [32]. Interestingly, a significant decrease in sugar levels is observed in functional food treated samples by 0.7 fold compared to 0.25 fold decrease in blank samples. The most significant decrease of sugars and amino acids in functional food treated samples was observed in GT and BT (0.1 fold), and GC (0.5 fold), i.e. polyphenol rich plant products. Metabolite classes that showed an opposite pattern, i.e. an increase over time (0.5 versus 24 h) included organic acids and low molecular weight phenolics, most evident in case of POM and GT amended cultures compared to blank. Increase is likely attributed for microbiota degradation of high molecular weight plant phenolics to its simpler organic and phenolic acids. Detection of high

![Fig. 1. Relative percentile levels of metabolite classes detected using GC-MS for cultures harvested at 0.5 and 24 h from functional foods amended culture: BC, BT, FI, GC, GT, POM & SU versus blank.](image-url)
molecular weight phenolics cannot be achieved using GC-MS and has yet to be performed using liquid chromatography mass spectrometry (LC-MS) [33]. Several bacterial species are reported to possess hydrolytic enzymes necessary for plant polyphenols degradation, e.g. through dehydroxylation, decarboxylation, ring cleavage or oxidation, ultimately generating simpler phenolic and organic compounds [34]. Likewise, nucleic acids increase upon incubation, especially in case of a GC amended culture at 6-fold increase after 24 h compared to 0.5 h treatment versus a 3-fold increase in case of the blank culture. A pie chart showing relative percentile levels of the different metabolite classes analyzed in microbiota culture fortified with the different treatments at 0.5 and 24 h is given in Suppl. Fig. S2 A–G. Provided below is an overview of the major changes observed for metabolite classes in blank culture compared to those amended with food extracts.

**Canonical amino acids**

Ornithine, alanine and isoleucine were the most abundant amongst the detected amino acids, and showed a decline of 0.8, 0.14 and 0.32 fold, respectively, upon incubation in all cultures. An exception to this pattern was observed in case of phenylalanine, glutamic and pyroglutamic acid in both treated and blank cultures. Phenylalanine showed ca. 1.8-fold increase in all functional foods compared to 2.5-fold increase in blank culture, likewise pyroglutamic acid showed an average of 1.7-fold increase in all functional foods compared to 4-fold increase in blank at 24 h treatment, i.e. the increase in treated cultures also was reduced vs. blank. Despite, its reduction or even complete depletion upon GC, Fl and POM treatment, in case of the other functional foods, glutamic acid showed a ca. 2.3-fold increase which is slightly reduced vs. the untreated blank with a 4.5-fold increase. This might be attributed to specific producers of these amino acids i.e. E. coli induced production of phenylalanine [35] and L. plantarum mediated production of glutamic and pyroglutamic acid [36], both present in the consortium culture. Amino acids play multiple roles in gut microbiota either via protein fermentation or internally to synthesize essential amino acids to be further utilized as building blocks for cellular composition [37], to serve as signaling molecules between microbial cells [38] or within the host [39], or to be used as an energy source [40]. Nevertheless, such increase failed to lead to an increase in the overall amino acid percentile levels as the aforementioned reduction of other amino acids was much more significant with larger negative fold changes.

**Other nitrogenous compounds**

The most pronounced decrease in nitrogenous compounds was detected for 3-amino propanoate at comparable levels in functional foods amended and blank cultures (ca. 0.16-fold). Human gut microbiota produces various short chain fatty acids (SCFAs) with propanoate as major metabolic product of anaerobic fermentation. Along with butanoate, propanoate is a major component in microbiota metabolic pathways for the synthesis of other SCFAs [41]. It does play a significant role during irritable bowel syndrome if reduced by medication [42]. Bacterial genera such as Bacteroides, Blautia, Eubacterium, Escherichia and Clostridium can ferment nondigestible dietary carbohydrates and amino acids to propionate through either 1,2-propanediol or succinate pathways (Suppl. Fig. S3) [43,44].

In contrast, other nitrogenous compounds viz. gamma amino butyric acid (GABA) increased significantly in all food extract amended cultures over time compared to a reduction in case of blank, likely attributed to bacterial fermentation of plant derived amino acids i.e. L-glutamic acid to GABA [45]. The highest increase in GABA levels was detected in Fl and POM with ca. 3 and 2 fold.
increases, respectively. Whether an increase in GABA levels occurs similarly in the gut upon fermentation of functional foods is unclear, but if so it will contribute to a biological effect yet to be determined in its extent. GABA is an inhibitory neurotransmitter that has many reported pharmacological effects. It also is involved in various neurological disorders including epilepsy, seizures, convulsions, Huntington’s disease, and Parkinsonism [46]. The gut microbiota related brain axis effect (Brain-Gut-Microbiome Axis) is a hot topic regarding all CNS disorders, and alterations in brain-gut-microbiome communication is found to be involved in the pathogenesis of several disorders [47].

Other detected nitrogenous compounds include cadaverine derived from lysine via its decarboxylation [48]. Gut microbiota is reported to synthesize cadaverine under high protein diet [49]. This is in general related to biogenic amino compounds including putrescine and spermidine produced via decarboxylation of other amino acids. Polyamines have been reported to stimulate cell division of gut microbiota, e.g., of E. coli [50] and also to impart health benefits to the host such as regulation of growth and aging, and prevention of metabolic and neurodegenerative disorders [51]. Microbiota cell uptake of polyamines may explain the lower cadaverine levels at 24 h. However, the relatively high levels of putrescine may indicate biosynthesis overwhelming microbiota cell uptake and can be explained by the fact that putrescine is synthesized from ornithine [51,52], detected at higher levels at 0.5 h and showing a decrease with incubation time (Suppl. Fig. S4). Polyamine generation reaction is found to favor of SCFA synthesis, driven by certain bacterial strains such as E. coli [53] also present in this microbial consortium (Suppl. Table S3). In this study, a reduction in putrescine was observed to be concurrent with higher lactic acid and succinic acid levels in certain cultures, which support the competition between polyamine and SCFA synthesis pathways on amino acid substrates.

Sugars

Sugars identified included monosaccharides, disaccharides and sugar alcohols. Generally, sugars showed lower abundance 24 h post incubation as expected due to their utilization as energy source and as carbon source for the generation of SCFA [54] as evidenced in strains of Bifidobacterium [55]. For all treatments there was a decrease, with the exception of SUM (Rhus coriaria) that showed higher sugar levels at 24 h compared to 0.5 h (Suppl. Table S3; Suppl. Fig. S5). A neuroprotectant sugar that could exert its effect at the gut level is trehalose which showed one of the most pronounced decreases, being completely depleted upon incubation in most food fortified cultures and blank as evident by a decrease in e.g. fructose (Suppl. Table S3). Elevated succinate levels within the gut lumen have been associated with microbiome disturbances (dysbiosis), as well as in patients with inflammatory bowel disease (IBD) [62]. Other sources of succinic acid in the body involve the tricarboxylic acid (TCA) cycle within host cells, though unlikely to function here under hypoxic conditions. Lactate, another carbohydrate fermentation product [63] mediated via lactic acid bacteria (LAB) i.e. Lactobacillus plantarum, [64] also increased upon incubation at 24 h, especially in POM, SUM, and FI treated samples at 18.7 (0.2 fold), 15.3% (0.15 fold) and 6.3% (0.05 fold), respectively, compared to trace levels in blank. A similar (secondary metabolite) profile of changes was observed for hydrolysable tannins, showing enrichment in POM and SUM. This might account for the similar metabolic response observed in their respective microbiota cultures.

Phenolics

Phenolics comprise a metabolite class that is almost solely derived from functional food extracts and was close to being absent in the blank culture. Our interest in reporting these metabolites herein is that they are rather important plant natural products with many proven or claimed health benefits including influence on microbes [14]. Thus a potential influence of its composition or its levels on gut microbiota metabolism is likely to impact functional food ultimate health effects. Catechin was found exclusively in GT and BT cultures at 0.5 h and to increase at 24 h ca. 2–3 fold. This can be attributed to its polymers or higher mw conjugates cleaved. e.g. (-)-epicatechin gallate (ECG) is expectedly found enriched in tea samples, and this is supported by a ca. 4-fold increase in gallic acid levels after 24 h. Although catechins were detected almost exclusively in GT and BT, gallic acid was present in most cultures though at lower levels especially for FI, SUM and POM, but it was absent from blank, suggestive of being derived from hydrolysis of plant phenolic conjugates or polymers. e.g. by bacteria such as here in L. plantarum known to act on hydrolysable tannins [24,26]. L. plantarum is the only bacterial species reported to have esterase (tannase) and gallate decarboxylase enzymes necessary for such a degradation. Gallic acid is an important dietary supplement that has several health benefits including antioxidant, anti-inflammatory, antibacterial, anti-allergic, anti-mutagenic and anti-carcinogenic effects [65]. Among the reported significant antibacterial activity of gallic acid was its activity against bacterial strains such as; E. coli [66] and Bifidobacterium [67] available within the used consortium.

Nucleic acids

Nucleic acids i.e. purines were detected in most samples especially GT and GC. Increase in its levels is attributed to gut microbiota metabolism effected by or derived from caffeine catabolism, with caffeine found to be enriched in these two functional foods. Nucleic acids showed a general pattern of an increase upon incubation in most food fortified cultures and blank as observed in case of uracil and to a lesser extent in adenine (Suppl. Table S3). Hypoxanthine was also detected in all amended cultures
as well as in blank as a byproduct of microbial oxidation of nucleic acids through a salvage pathway to act as a nitrogen source as well as energy sources and promoting protective functions to the colonic epithelium [60,68]. Positive correlations of hypoxanthine and uracil with Bacteroidetes species Alloprevotella [69] was reported. In contrast, xanthine was detected exclusively in GC and GT amended cultures as a metabolic microbiota product of methylxanthines i.e. caffeine, theobromine and theophylline enriched in green coffee and tea (Suppl. Fig. S7) [22,23].

Unsupervised and supervised multivariate data analyses of GC/MS dataset

Considering the complexity of acquired data in terms of the large number of specimens and monitored metabolites (96 × 131), Fig. 1, multivariate data analyses were employed to further determine the impact of treatment on microbiota metabolism in an untargeted manner. Untargeted multivariate analysis tools such as principle component analysis (PCA) and orthogonal partial least squares discriminant analysis (OPLS-DA) can help reveal for differences between samples and postulate hypothesis related to the effect of applied functional food on microbiota metabolism. Principle component analysis (PCA) is an untargeted multivariate analysis tool that is used to bring multidimensional datasets into a two-dimensional plane that can be graphically represented as scatter plots. In the two-dimensional plane, data point coordinates on principle component 1 (PC1) and PC2 vectors are assigned to account for the maximum variability of multidimensional data [70,71].

**PCA analysis of the whole dataset**

PCA was applied to the whole sample dataset to determine metabolome heterogeneity among all samples examined with no sort of samples classification. The principle component PC1/PC2 score plot derived from the whole GC–MS data accounted for 42% of the total variance (R2). This is not a high value and shows that variance between samples is high and needs more dimensions to cover all aspects. The whole pool of samples in the score plot, however, could be segregated into two big clusters distributed along PC1 (Fig. 3A). These two major clusters appeared to be based upon incubation time that is (0.5 h versus 24 h incubation time as revealed in (Fig. 3D), suggesting this to be the most-variable parameter overcoming others. Labeling of samples based on the administered functional food extract dose levels (5 mg/mL, 0.5 mg/mL) and blank samples showed no clear segregation of sample in the score plot (Fig. 3B). The same result was also obtained when samples were colored based upon the amended functional food extract type (Fig. 3C), and in all cases they show overlap among specimens. All findings pinpointed that monitored metabolites are mostly derived from microbiota culture and not much represented by metabolites from amended functional foods since either of the identified big clusters (Fig. 3D) contained functional food extract treated samples along with their corresponding blank samples. Hierarchical cluster analysis (HCA) is another multivariate data analysis tool that provides a mean of intuitive graphical abstract of samples clustering pattern [72]. HCA analysis confirmed PCA results (Suppl. Fig. S8) by showing clear samples segregation aliquoted at 0.5 h (highlighted in a green box) compared to samples harvested at 24 h (blue box) disregarding treatment type (either blank or functional food treated samples) or dose level (0.5 mg/mL & 5 mg/mL). Results of whole sample data set modelling either from PCA or HCA fall in agreement with another report showing the impact of transient time on gut microbiota metabolites composition [40]. To pinpoint for metabolites mediating for such segregation and contributing to samples segregation with time course, the corresponding loading plot was inspected. Among detected metabolites, amino acids i.e. phenylalanine, ornithine and to a lesser extent organic acids, especially lactic acid, accounted for most of the variability observed along PC1 (Fig. 4).

**PCA classification based upon functional food type versus blank**

Due to the low model variance coverage (R2) and predictability (Q2) as revealed from whole sample datasets (Fig. 3), another PCA attempt was adopted to model each functional food extract amended culture vs. the blank at the two time points at 0.5 and 24 h (Suppl. Fig. S9). Both functional food and blank samples aliquoted at 0.5 h were positioned on the right side of PC1 with positive p values. Whereas its counterpart aliquots harvested at 24 h were aligned on the left side of PC1, with an overall covered variance from PC1 and PC2 from each dataset (Suppl. Fig. S9) showing in general higher variance coverage than of the whole sample dataset (Fig. 3). The score plots of BC (A), GC (B), BT (C) and GT (D) treated cultures showed consistent patterns of samples segregation being mostly based upon harvest time. Both blank and treated samples aliquoted at 0.5 h showed overlapping masses clustering at the positive side of PC1 versus samples aliquoted at 24 h showing two well-separated clusters distributed along PC2, which indicated no metabolome difference detected at the 0.5 h harvest point between blank samples and functional food. The separation of functional food treated samples at the positive side of PC2 versus its blank samples being located at the opposite side suggested for contributions of functional food metabolites or its biotransformed products in such a discrimination. It should be noted, that other the three additives Fl (E), POM (F) and SLIM (G) showed atypical behavior, in which both functional food and blank sample aliquoted at 24 h showed non-separable masses contrary to the previous models (Suppl. Fig. S9). In contrast, in both POM model score plot (Suppl. Fig. S9F) high scattering along PC2 was observed at either 0.5 h or 24 h incubation time indicating a higher influence of functional food composition on the microbiota metabolism. The same holds for the case of SUM (Suppl. Fig. S9G).

**PCA classification based upon functional food type versus blank at 0.5 h**

To better reveal for contributions from untransformed functional food metabolites in sample segregations as observed in Suppl. Fig. S9, we attempted to model each functional culture amended along with its corresponding blank sample aliquoted at 0.5 h, a time at which no significant biotransformation by microbiota is expected to occur (Suppl. Fig. S10). For BC (A), GC (B), BT (C), and GT (D) lower variance coverage was revealed compared to that in (Suppl. Fig. S9), and overlap between treatment and blank samples at both dose levels indicate that the functional food metabolite interference at 0.5 h is negligible. Such pattern was nevertheless not observed in case of POM (F) and SLIM (G) models showing no overlap between functional food treated samples and blank, suggestive for significant functional food native metabolite interference within 0.5 h, and accounting for specimens segregation. The loading plot of pomegranate model revealed that sugars, especially fructose, short-chain fatty acid (SCFA), here lactic and succinic acid, and the nitrogenous compound and neuroactive amino acid GABA were already upregulated in the treated cultures compared to blank.

**OPLS-DA classification based upon incubation time at 0.5 h versus 24 h**

Considering that unsupervised data analysis failed to provide clear segregation in response to treatment for each respective time
point, supervised orthogonal partial least squares discriminant analysis (OPLS-DA) was further employed to model each treatment separately viz. BC, GC, BT etc. Samples were classified as such by pooling samples for each treatment for the 2 dose levels 0.5 and 5 mg/ml as one class group at 0.5 h (a) versus 24 h (b) as another class group (Suppl. Fig. S11). Compared to PCA (Suppl. Fig. S9), each OPLS model showed clearer sample segregations (Suppl. Fig. S11). Model validation was based on estimating the total variance (R2), prediction goodness parameter (Q2) and p-value as detailed in (Suppl. Table S4). All of the models showed high repeatability, prediction and significantly low regression p-values suggestive of no overfitting of these models. Investigation of the S-loading plot allows to visualize both the covariance and the correlation structure between the X-variables and the predictive score t[1] [73] for each respective OPLS-DA model (Fig. 5) revealed for multiple findings. Metabolites with a positive p[1] value indicate an increase upon incubation, whereas metabolites with a negative p [1] value indicate higher abundance at 0.5 h and a decrease upon incubation (Fig. 5A–H).

Metabolites belonging to amino acids/nitrogenous compounds i.e., alanine, amino propanoate, isoleucine, valine and ornithine exhibited significant model influence at 0.5 h (negative p[1] on S-plot). In contrast, other metabolites related to the same classes such as leucine, methionine, glutamic acid and phenyl alanine showed significant model influence at 24 h (positive p[1] on S-plot). These findings propose mixed microbial metabolic activities that are either to utilize some of these amino acids as a substrate to form other compounds (catabolism) [38] or to be synthesized from other metabolites (anabolism). To provide stronger evidence for metabolites reprogramming and to understand metabolic pathways, a Spearman rank correlation was employed to compute all pairwise correlations between metabolites across the entire dataset, depicted as a metabolite-metabolite correlation heat map (Suppl. Fig. S12). This metabolite correlation analysis shows a negative correlation of ornithine with succinic acid (r² = 0.4) and tartaric acid (r² = 0.35). Similar to ornithine, alanine shows a negative correlation with succinic acid and tartaric acid, in addition to fumaric acid (r² = 0.43) and malic acid (r² = 0.65). Alanine reduced levels are attributed to either serve as building unit in cell wall formation (peptidoglycan) or in its catabolism via aminotransferase into pyruvate [74]. In contrast to this, amino acids such as phenylalanine, threonine and pyroglutamic acid show positive p[1]-values in the S-plot with an increase upon incubation time (Fig. 5F–H). Though threonine is reported to be catabolized into acetate, microbiota can also synthesize it internally [38] and this can account for its increase with elapsed time. Such hypothesis is supported by the correlation analysis showing positive correlation of threonine with acids i.e. maleate (r² = 0.64), succinate (r² = 0.5)
and tartarate ($r^2 = 0.5$) which all showed upregulation with incubation time. Studies revealed that microbiota consumption and synthesis of amino acids is dependent on strain [75] and incubation time [40]. Sampling times for more than 24 h or utilization of several strains for each microorganism in the future can provide better evidence for such a hypothesis.

Fig 5. S-Plot of OPLS model of black coffee (A), Green coffee (B), Black tea (C), Green tea (D), Ficus (E), Pomegranate (F), Sumac (G) and Blank (H) classified based on incubation time. Samples were classified by pooling samples for each treatment at the 2 dose levels 0.5 and 5 mg/ml as one class group at 0.5 h (a) versus 24 h (b) as another class group. Metabolites increasing with time have positive p value while metabolites decreasing with time have negative p value.

SCFA major metabolite markers of gut microbiota, i.e. lactic acid (Fig. 5E–G) and succinic acid, show higher p[1] values in the S-plot of (Fig. 5F), indicating an increase over time at 24 h by up to 17 fold in the pomegranate case. This most probably is due to its role as by-products of microbial metabolism. Other than primary metabolites converting into SCFA, succinic acid showed an increase with
time (+p value in S-plot) acting as fermentation product of secondary metabolites. e.g., chlorogenic acid (CA) was reported to be fermented into succinic acid by microbiota [76], and could account for its 2.4-fold increase in BC at 24 h (Suppl. Table S3). CA is the main active constituent in coffee recognized as its slimming factor.

**OPLS-DA classification based upon functional food type versus blank at 24 h**

Considering that all of the above metabolic changes (Fig. 5) were related to the time course metabolism of microbiota and to help identify other metabolic changes specifically influenced by certain functional food treatment, OPLS-DA was performed by modelling each treatment at 24 h versus its blank sample at the same point for the 2 different dose levels. Derived S-plots for each case are presented in (Suppl. Fig. S4) and with metabolites that showed the strongest variation influence as reflect by their p-values listed in Table 1.

| Metabolite                  | Category          | BC   | BT   | FI   | GC   | GT   | POM  | SU  |
|-----------------------------|-------------------|------|------|------|------|------|------|-----|
| Lactic acid                 | Acid              | (+)  | (+)  | (+)  | (+)  | (+)  | (+)  | (+) |
| Succinic acid               | (+)               | (+)  | (+)  | (+)  | (+)  | (+)  | (+)  | (+) |
| 3-Deoxytetronic acid        | Amino acid        | (+)  | (+)  | (+)  | (+)  | (+)  | (+)  | (+) |
| Fumaric acid                | (+)               | (+)  | (+)  | (+)  | (+)  | (+)  | (+)  | (+) |
| Glycine                     | (+)               | (+)  | (+)  | (+)  | (+)  | (+)  | (+)  | (+) |
| Threonine                   | (+)               | (+)  | (+)  | (+)  | (+)  | (+)  | (+)  | (+) |
| Ornithine                   | (+)               | (+)  | (+)  | (+)  | (+)  | (+)  | (+)  | (+) |
| Valine                      | (+)               | (+)  | (+)  | (+)  | (+)  | (+)  | (+)  | (+) |
| GABA                        | Nitrogenous compound | (+) | (+)  | (+)  | (+)  | (+)  | (+)  | (+) |
| Norvaline ester derivative  | (+)               | (+)  | (+)  | (+)  | (+)  | (+)  | (+)  | (+) |
| Purine                      | Nucleic acid      | (+)  | (+)  | (+)  | (+)  | (+)  | (+)  | (+) |
| Uracil                      | (+)               | (+)  | (+)  | (+)  | (+)  | (+)  | (+)  | (+) |
| 2-Hydroxy-3-methylvaleric acid | Phenolic        | (+)  | (+)  | (+)  | (+)  | (+)  | (+)  | (+) |
| Catechin                    | (+)               | (+)  | (+)  | (+)  | (+)  | (+)  | (+)  | (+) |
| Gallic acid                 | (+)               | (+)  | (+)  | (+)  | (+)  | (+)  | (+)  | (+) |
| D-arabino-3-deoxy Hexonic acid | Sugar          | (+)  | (+)  | (+)  | (+)  | (+)  | (+)  | (+) |
| Ribose-O-methylxolime       | (+)               | (+)  | (+)  | (+)  | (+)  | (+)  | (+)  | (+) |
| Fructose                    | (+)               | (+)  | (+)  | (+)  | (+)  | (+)  | (+)  | (+) |
| Sorbose                     | (+)               | (+)  | (+)  | (+)  | (+)  | (+)  | (+)  | (+) |
| Unknown sugar               | (+)               | (+)  | (+)  | (+)  | (+)  | (+)  | (+)  | (+) |

Symbols (++++) indicate very high influence of S-plot, (+++) high influence, (++) intermediate, and (+) low influence.

**Table 1** Major metabolites differentiating functional food treated samples against blank at 24 h post incubation as revealed from OPLS analysis.

Our results provide insights into gut microbiota altered metabolisms in response to different functional food extracts at the metabolite level and define general and specific biomarkers for each functional food type. In general, functional food amendment to gut microbiota appeared to alter its metabolism in two different scenarios. First, functional food components can serve as a substrate to microbiota metabolism as in case of purine alkaloids such as caffeine acting as precursors of purine by microbiota demethylation. An alternative mechanism is that functional food components modify the extent, existing metabolic pathways are activated within microbiota, showing e.g. GABA production in

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In contrast to these treatment specific markers revealed exclusively for GT and BT, metabolites such as sugars, organic acids, i.e. lactic and succinic acid, and GABA were detected as significant contributors to variation in almost all functional food treated cultures compared with blank samples at 24 h (Suppl. Fig. S4A-G). Gut microbiota such as *Lactobacillus* species and *E. coli* have been reported to secrete GABA as an acid resistance mechanism at low pH which may explain co-appearance of GABA with organic acid increase and other low acidic metabolites such as polyphenols in OPLS –derived S plot of several functional food treatments (Suppl. Fig. S4A, C, E & F). GABA can also be utilized by bacteria as carbon source through conversion to succinate which enters the tricarboxylic acid cycle [78] and this may explain the coexistence of GABA and succinic acid (r2 = 0.40) as strong influencers in S-Plots of functional food treated samples (Suppl. Fig. S4C & F). Such metabolite correlations could not be readily revealed from visual inspection of results and highlight the value of modelling data in results interpretation.

Accumulation of a norvaline derivative (Table 1), a branched chain non-proteinogenic amino acid has been observed in BT and FI treatments (Suppl. Fig. S4C & E). Previous research showed that this compound could only accumulate in high glucose based mineral salt media at limited oxygen supply as modified metabolic route [79]. However, norvaline ester showed higher abundance with elapsed time in functional food treated samples except in case of POM and SUM (*Rhus coriaria*). Pyruvate, a sugar metabolism intermediate, may convert to norvaline via α-isopropyl malate synthase instead of being converted to SCFA, i.e. lactate, which may explain its absence in POM and SUM both showing upregulation of lactate and succinate at 24 h (Suppl. Fig. S4F & G).

**Conclusion**

Our results provide insights into gut microbiota altered metabolisms in response to different functional food extracts at the metabolite level and define general and specific biomarkers for each functional food type. In general, functional food amendment to gut microbiota appeared to alter its metabolism in two different scenarios. First, functional food components can serve as a substrate to microbiota metabolism as in case of purine alkaloids such as caffeine acting as precursors of purine by microbiota demethylation. An alternative mechanism is that functional food components modify the extent, existing metabolic pathways are activated within microbiota, showing e.g. GABA production in
presence of higher acidity induced by metabolites such as polyphenols and organic acids. Nevertheless, it should be noted that current work does not look at microbial strains growth in response to different functional food treatments, further analysis using 16S ribosomal RNA sequencing and as well viability of each individual strain in the presence of each tested functional food should be pursued.

This study demonstrated the metabolic pathways adopted by microbiota in the generation of SCFA through either nitrogen metabolism of amino acids or carbon metabolism of sugars lead to an ultimate increase of SCFA under consumption of (most members) of these metabolic precursors (Fig. 6A and B). However, most of these changes are related to the change the microbiota environment encounters with time and is not specific for certain functional foods with a few exceptions.

Fig. 6A outlines the major changes in microbiota primary metabolite classes viz. sugars, nitrogenous compounds, acids and amino acids in response to functional food treatments and the underlying mechanism (Fig. 6B). Here three (partially overlapping) groups can be separated: caffeine containing additives, changing expectedly nitrogenous compound metabolism, polyphenolics rich additives like tea, changing a number of properties outlined in detail above, and pomegranate and sumac, which inflict the largest differentiations through their sugar (fructose) and phenolics content.

Further studies that apply high resolution visualization of metabolite pools, such as nanoscale secondary ion mass spectrometry, coupled with stable isotope tracers will serve to provide further insight into the potential roles of free metabolites and their exact metabolic origin considering the complexity of microbial metabolisms. It also remains to clarify, if additional information on non-volatile metabolites, which might be captured by LC-MS, and a correlation to the strain composition changes with time, will be useful or not. These data, coupled with the ongoing outputs from rapidly developing additional “omics” studies (i.e., genomics, transcriptomics, and proteomics) will aid in further elucidating the metabolic cross-talk both within and between partners, which is essential for maintaining a functional healthy gut environment.

Obviously neither our functional food selection nor that of the microorganisms does cover all types, and no setup can do that. But we see that the platform presented here can be employed to assess other systems, containing e.g. other metabolite classes like saponins, sulfur organics like in garlic or onion, or even of complex mixture as typical in traditional Chinese medicine TCM.

The above mentioned limitation of this study to metabolites amenable to GC–MS detection, i.e. those of primary origin except for a few simple phenolics, may underpin the impact of secondary metabolites not detected by this analytical platform such as plant polyphenols or not represented in this study at all as with plant terpenoids in leading to a variation in microbiota composition and metabolism. Still, clear evidence for functional food comparable metabolites composition impact on microbiota culture metabolism was observed, exemplified in xanthine rich extracts i.e. GC, GT, BC, BT versus hydrolysable tannins in POM and SUM. Further, clinical studies ought to be considered in the future by analyzing stool metabolism in humans exposed to selected functional foods for defined periods, which would provide a true translation of this in vitro yet fundamental assay results to future research in the field of functional food – gut microbiota interaction.

**Fig. 6.** Diagram sketch outlining the major alterations in microbiota metabolic pathways. (A) Radar Chart summarizing the relative change in abundance of primary metabolites amino acids, nitrogenous compounds, sugars, and organic acids after incubation for 24 h compared to their relative abundance at 0.5 h (identified in dashed gray line). Measurement points indicate abundance in functional food treated samples. All points outside the gray dashed frame indicate increased abundance with time whereas points within the gray frame indicate lower abundance with time. While organic acid increase in all functional food treated samples, all of amino acids, nitrogenous compounds and sugars are reduced with time (except for sumac treated sample with regard to sugar abundance 24 h post incubation). (B) Schematic diagram outlining the metabolic pathways adopted by microbiota to convert amino acids and sugars into SCFA through either Carbon metabolism or Nitrogen metabolism and explaining the part A findings of the Radar chart.
Compliance with ethics requirements

This article does not contain any studies with human or animal subjects.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary material

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