Title: NMR-based metabonomic analysis of physiological responses to starvation and refeeding in the rat

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Abstract

Starvation is a postabsorptive condition derived from a limitation on food resources by external factors. Energy homeostasis is maintained under this condition by using sources other than glucose via adaptive mechanisms. After refeeding, when food is available, other adaptive processes are linked to energy balance. However, less has been reported about the physiological mechanisms present as a result of these conditions, considering the rat as a supraorganism. Metabolic profiling by nuclear magnetic resonance spectroscopy was used to elucidate the physiological metabolic differences in urine specimens collected under starved, refed and recovered conditions. In addition, since starvation induced lack of faecal production and not all animals produced faeces during refeeding, 24-h pooled faecal water samples were also analysed. Urinary metabolites upregulated by starvation included 2-butanamidoacetate, 3-hydroxyisovalerate, ketoleucine, methylmalonate, *p*-cresyl glucuronide, *p*-cresyl sulfate, phenylacetyl glycine, pseudouridine, creatinine, taurine and N-acetyl glycoprotein, which were related to renal and skeletal muscle function, β -oxidation, turnover of proteins and RNA, and host-microbial interactions. Food-derived metabolites, including gut microbial co-metabolites, and tricarboxylic acid cycle intermediates were upregulated under refed and recovered conditions, which characterised anabolic urinary metabolotypes. The upregulation of creatine and pantothenate indicated an absorptive state after refeeding. Fecal short chain fatty acids, 3-(3-hydroxyphenyl)propionate, lactate and acetoin provided additional information about the combinatorial metabolism between the host and gut microbiota. This investigation contributes to allow a deeper understanding of physiological responses associated with starvation and refeeding.

1. Introduction

The complexity of metabolic and physiological interactions of the host–microbiota can be studied with a non-destructive and non-invasive analytical method, NMR-based metabonomics used in conjunction with chemometrics and statistical spectroscopy. This methodology can assess the metabolic profile of urine and faeces by simultaneously identifying a wide range of structurally diverse metabolites in a single experiment with little sample preparation and highly reproducible results. In this way the time-related metabolic effects of different conditions and treatments can be determined.¹⁻⁴

Many NMR-detectable metabolites that integrate the metabonome represent the sum of interactions of all the individual metabolomes and their products within a complex organism. The gut microbiota, a virtual organ that forms part of this supraorganism and contributes to the metabonome, interacts with its host in a constant bidirectional communication that maintains homeostasis through the so-called gut-microbiota-brain-liver-immune system axis. The disruption of this interaction can be observed through metabotype patterns related to biochemical pathways.^{2,3,5-8} During the pathogenesis of neurological, cardiovascular, renal and gastrointestinal disorders, there are alterations in the complex interactions of the supraorganism that have been observed as changes in gut microbial-host co-metabolites.^{6,7,9}

Starvation or fasting represents a suitable model that can be used to standardize tests for physiological, pathophysiological, nutritional, toxicological and pharmacological purposes.¹⁰⁻¹⁵ Starvation refers to a postabsorptive or steady-state resulting from some extrinsic limitation on food resources, and fasting to the same condition derived from an intrinsic mechanism (foregoing an opportunity to eat even when food is available).¹⁰

The physiological adaptive mechanisms carried out by the host under starvation are well known,^{10,15-17} but less has been reported about the normal changes occurring in the supraorganism as a result of this condition. Since the host and the gut microbiota exhibit numerous mutually beneficial and cooperative interactions involved in energy homeostasis that are related to health and disease,^{7,8,18,19} it is important to know more about this interaction under the condition of starvation and refeeding. It has been observed that starvation disrupts the composition and function of the gut microbiota as a result of changes in the architecture of the gastrointestinal tract produced by food deprivation.²⁰⁻²²

Although metabolites in urine and faeces represent waste or toxins, changes in their composition can give important information about the state of homeostasis of a supraorganism under physiological, pharmacological, toxicological and pathological conditions. Thus, it is useful to monitor the outcome of the continuous bidirectional communication between the host and the gut microbiota under these conditions. By analysing the metabolome of urine and faecal samples with non-invasive methodologies, physiological changes can be differentiated from those occurring under abnormal conditions, which allows for accurate biological interpretations.

The aim of the present study was to demonstrate the effect of starvation and refeeding on the urinary metabotype in order to understand the dynamic adaptation of the supraorganism in response to such conditions. This adaptive mechanism was evidenced by changes in several urinary metabolites and co-metabolites related to energy metabolism and host-microbial interactions. Such knowledge will contribute to defining the aetiology and pathology of disease, and to elucidate mechanisms of action of a drug or toxin in clinical or preclinical trials when starvation/fasting is included in the experimental design.

2. Experimental section

2.1. Animal handling and sample collection

Animal experiments were carried out in accordance with the Mexican norms provided in the Seventh Title of the Regulations of the General Law of Health in regard to health research, and the Official Standard (NOM-082-ZOO-1999) with respect to the care and use of laboratory animals. Thirty adult female Wistar rats weighing 270-280 g were acclimatized for one week under environmentally stable conditions (22-24 °C, 50-55% relative humidity, and a 12:12 h light/dark cycle with lights on at 7 AM). Animals were fed with a standard rodent diet (PMI Nutrition International, LLC. rodent laboratory chow 5001, Brentwood, MO, US) and water was available *ad libitum*. Only female rats were housed in the vivarium.

In order to gather biological samples at the end of a starvation period of 20h, animals starved for 16h were individually housed in separate metabolic cages designed to preclude contamination of the urine, separate it from faeces, and collect it in tubes (3M12D100/3700M071, Tecniplast, Buguggiate, Va, Italy), and food was returned 4h later.

At the beginning of the sample collection period (t_0), the bladders of animals were emptied by gentle compression of the abdomen and the voided urine was discarded, which is a

routine procedure for collection of 24h urine specimens. Animals had access to water *ad libitum* throughout the study. Urine samples were collected at intervals of 6h ($t_1=0700$ to 1300, $t_2=1300$ to 1900) and 12h ($t_3=1900$ to 0700). Faecal samples were collected after 24 h (7:00 AM to 7:00 AM). Urine volumes and faecal pellet weights were recorded at each time point. All samples were stored at -80°C prior to NMR analysis.

2.2. Chemicals and sample preparation for NMR spectroscopic analysis

The urine samples were thawed, vortexed, and allowed to stand for 10 min at room temperature prior to NMR analysis. In order to reduce pH variability, to 440 μL of rat urine were added 220 μL of a 0.2 M phosphate buffer (pH 7.4) containing 0.1 % (w/v) of sodium azide (Sigma Aldrich), and then the mixture was centrifuged at 15,600 g and room temperature for 10 min. An aliquot of 540 μL of the supernatant was added to 60 μL of TSP (3-trimethylsilyl-[2,2,3,3- H_4]-propionic acid sodium salt, Sigma Aldrich) in D_2O (99.9% in D, Sigma Aldrich) to give a final TSP concentration of 1 mM.¹

The faecal samples were homogenized with 0.2 M phosphate buffer (as aforementioned, 5 mL of buffer per gram of stool). The homogenate was subjected to 10 cycles of sonication-vortex-break (10 s per step), and then centrifuged at 15,600 g and room temperature for 10 min.⁴ Finally, 540 μL of the supernatant was added to 60 μL of a TSP/ D_2O solution to give a final TSP concentration of 1 mM.¹ All prepared samples were placed in 5 mm NMR tubes.

2.3. NMR spectroscopy analysis of urine and faecal water

One-dimensional (1D) ^1H NMR spectra of prepared urine and faecal water were acquired at 298 K on a Varian NMR system 500 spectrometer operating at 499.8 MHz (now Agilent Technologies, Santa Clara, CA, US). A standard one-dimensional pulse sequence NOESYPR was used (recycle delay- 90° - t_1 - 90° - t_m - 90° -acquisition), where t_1 represented the first increment in the NOESY experiment and was set to 3 μs . Water presaturation was used during both the recycle delay (1s) and mixing time (t_m , 100 ms), providing an acquisition time of 4s. For each sample, 128 transients (32 dummy scans) were collected into 64k data points over a 20 ppm spectral width. The FIDs were multiplied by an exponential weighting function corresponding to a line broadening of 0.3 Hz, and data were zero-filled to 64k data points prior to Fourier transformation (FT).¹

Two-dimensional (2D) homo- and heteronuclear NMR spectra were acquired to confirm the presence of metabolites. ^1H -J-resolved spectroscopy (JRES), ^1H - ^1H total correlation spectroscopy (TOCSY), and ^1H - ^{13}C heteronuclear multiple quantum correlation (HMQC)

were acquired for selected samples (of both urine and faecal water). Parameters for acquisition and processing are described in the Supporting Information (SI).

2.4. Data processing of 1D ^1H NMR spectra

The 1D ^1H NMR spectra were manually phased, baseline corrected and referenced to TSP at δ 0.0 using Agilent VnmrJ 4.2. Full resolution 1D ^1H NMR spectra (~20 k and ~22 k data points for urine and faecal water, respectively) were imported into MatLab (R2014a, The MathWorks Inc., Natick, MA). For urine spectra ($n = 90$), the spectral region for residual water and urea resonances (δ 4.12-6.47 ppm) was removed prior to normalisation. For faecal water spectra ($n = 30$), the region containing the water resonance (δ 4.07-5.75 ppm) was removed. For both compartments, the region corresponding to TSP (δ 0.20–0.50 ppm) was removed and normalised using the probabilistic quotient method.²³

2.5. Identification of metabolites

The structural identification of metabolites in urine and faecal water was achieved by 2D NMR experiments and statistical total correlation spectroscopy (STOCSY) on 1D spectra.²⁴ Literature^{1,2,4,24–29} or databases, such as the Human Metabolome Data Base (HMDB; <http://www.hmdb.ca/>) or the Biological Magnetic Resonance Data Bank (BMRB; <http://www.bmrwisc.edu>), along with Chenomx NMR Suite 8.0 (Chenomx Inc., Edmonton, Alberta, Canada), were used for confirmation of assignments.

2.6. Multivariate data analysis

Multivariate data analysis (MVA) was performed using SIMCA software (v. 13.0; Umetrics, Sweden). Principal component analysis (PCA) and orthogonal projection to latent structure discriminant analysis (OPLS-DA) were applied to the processed Pareto-scaled NMR data. The models were validated by both a 7-fold cross-validation and CV-ANOVA testing. The regression coefficients from the OPLS-DA models were divided by the jack-knife interval standard error to give an estimate of the t-statistic. Variables with a $|t\text{-statistic}| \geq 1.96$ (z-score, corresponding to the 97.5 percentile) were considered significant. The corresponding loadings were back-transformed in Excel (Microsoft, USA) and plotted with the colour-coded value of the t-statistic of the variables in MatLab. Statistical changes were supported by visual examination of the spectra.

2.7. Semi-targeted approach

The integration was obtained for each identified metabolite in urine and faecal water, using the equation

$$I = \sum_{k=1}^n \left(\int_{j_k^h}^{j_k^l} I(x) dx \right)$$

where I is the sum of the intensities of the resonance signals that comprise the fingerprint and relative concentration of a metabolite, k corresponds to a spectral region peak, and j_k^h and j_k^l are the high-field and low-field borders, respectively, of the intensities $I(x)$, which correspond to the chemical shift assignments that match with the structure of a given metabolite (Tables S1 and S2). These data were used to construct a new matrix ($\mathbf{X}$), with m variables (columns) and n observations (rows). To tackle the problem of metabolites with overlapped and shifted peaks (i.e., citrate, creatinine, creatine and succinate), peak intensities were identified by an extensive and careful manual inspection/peak-picking procedure for all spectra, and the integral of a respective NMR spectral region was very similar for all samples.

2.7.1. Urinary metabolite patterns

In order to identify patterns of urinary metabolites in accordance with time, hierarchical cluster analysis (HCA) was performed on data set $\mathbf{X}$ using the Euclidean distance measurement and Ward's method.

2.7.2. Metabolite-metabolite correlation analysis

The Pearson correlation coefficient (r) is a measure of the strength and direction of a linear association between two variables (i.e., metabolites). From the matrix $\mathbf{X}$, pairwise correlation matrices ($\mathbf{Cs}$) were obtained, which were comprised of elements with the Pearson correlation coefficients computed after comparing all the variables. The pairwise comparison was performed as follows: 6 vs 12, 6 vs 24 and 12 vs 24. Furthermore, from the faecal water data set (pooled time-series of 24-h collections), an autocorrelation matrix was obtained ($\mathbf{ACM}$). A cut off of $|r| \geq 0.7$ with $P < 0.05$ was used to indicate a significant correlation.

In addition, a bi-compartmental correlation was carried out in order to observe correlations between the urinary and faecal water metabolotypes. A determination was made of the average of the peak areas from each urinary metabolite at the three points in time (comparable to the pooled time-series of 24-hour urine samples), and the resulting matrix was correlated with the metabolite peak areas from the faecal water data set. A threshold of $|r| \geq 0.65$ with $P < 0.05$ was considered for a significant correlation.

2.7.3. Univariate data analysis

A Kruskal-Wallis test was performed for each metabolite integration (*I*) by comparing the three points in time. With the aim of adjusting multiple comparisons and determining significance, the Bonferroni correction was used, with thresholds of $P \leq 1.66 \times 10^{-2}$ ($P \leq 0.05/3$), $P \leq 3.33 \times 10^{-3}$ ($P \leq 0.01/3$), and $P \leq 3.33 \times 10^{-4}$ ($P \leq 0.001/3$). In order to express time dependent changes in relative concentrations of urinary metabolites, the binary logarithm of their ratios was used:

$$\log_2(\text{ratio}) = \log_2\left(\frac{I_a}{I_b}\right)$$

where *I* is the metabolite relative concentration in terms of integration (as aforementioned) before and after a given time point (i.e., $I_b = \{6,12\}$; $I_a = \{12,24\}$). Since the behaviour of this transformation is symmetrical, a metabolite that increases by a factor of 2 has a $\log_2(\text{ratio})$ of 1, a metabolite that decreases by a factor of 2 has a $\log_2(\text{ratio})$ of -1, and a metabolite without change (with a ratio of 1) has a $\log_2(\text{ratio})$ equal to zero.

The semi-targeted analysis was conducted using MatLab (R2014a, The MathWorks Inc., Natick, MA).

2.7.4. Venn diagram

In order to show the degree of inter-compartmental overlap of metabolic profiles between urine and faeces, a Venn diagram was constructed using Venny software (v. 2.0), available online (<http://bioinfogp.cnb.csic.es/tools/venny/>).

3. Results

3.1. Pattern of urinary metabolites

An overview of the adaptive changes reflected in the urinary metabotype in response to starvation and refeeding is shown by plotting the PCA scores. A group clustering trend can be appreciated at the three points in time evaluated (Figure 1A). The cluster found with the urine samples collected at h6 is the most separated from each of the other two clusters, collected at h12 and h24. By using the untargeted approach, 38 and 25 metabolites were identified in urine and faecal water, respectively (Figures S1-S2 and Tables S1-S3), of which seven are common to both compartments (as depicted in the Venn diagram; Figure 1C).

Increased levels of 2-butanamidoacetate (2-BAA), 3-hydroxyisovalerate (3-HIV), ketoleucine, methylmalonate (MM) and glycoprotein 3 (NAC3) were observed exclusively in the *t*₁ metabotype. The increased levels found of creatinine, *p*-cresyl glucuronide (*p*-

251 CG), *p*-cresyl sulfate (*p*-CS) and pseudouridine (PSU) were highest at h6, and
252 subsequently were higher at h12 than at h24. Glycoproteins 1 & 2 (NACs 1&2) and
253 phenylacetylglycine (PAG) showed the lowest levels at h24 (Figures 2-4 A, B (left)). All
254 these metabolites comprised the first cluster (Figure 1B).

255 In addition, the metabolites that constructed the sixth cluster were 2-hydroxyisobutyrate (2-
256 HIB), 2-oxoglutarate (2-OG), carnitine, citrate, dimethylglycine (DMG), formate, glycine,
257 proline betaine (PB), succinate, *trans*-aconitate, trigonelline and U2, whose urinary levels
258 were higher in the metabotypes at h12 and h24 than that at h6 (Figures 2-4 A, B (left)).

259 The urinary levels of 3-(3-Hydroxyphenyl)propionate (*m*HPPA) and U3 (both members of
260 the fifth cluster) increased over the course of the experiment, having a low point at h6 and
261 reaching a peak at h24. Acetate and trimethylamine-N-oxide (TMAO) (from the same
262 cluster) and 5-hydroxy-1-methylhydantoin (5-HMH, from cluster 6) showed special
263 patterns, being the only metabolites that did not correlate with each other and with the rest
264 of the urinary metabolites. TMAO levels only showed an increase at h24 compared to h6,
265 while 5-HMH levels were greater at h12 vs h6. On the other hand, acetate levels had the
266 same pattern as hippurate and methylamine (MA), increasing over time and reaching the
267 highest concentration at h24. These three metabolites are in cluster 5 (Figures 1B, 2-4B
268 (left)).

269 The pattern observed by hierarchical cluster analysis shows that the urinary metabotypes
270 are significantly different over time, which is attributed to the distinct states represented by
271 starvation, refeeding and recovery as mainly depicted in the clusters 1, 5 and 6.
272 Accordingly, metabolites from the first cluster that showed correlations had an inverse
273 relationship with those from the fifth and sixth clusters, which are related to dietary intake.
274 Therefore, the cluster 1 was linked to starvation, and clusters 5 and 6 to the condition of
275 refeeding and recovery (Figures 1B, 2-4B (right), and Tables S4-S7).

276 Alanine, *cis*-aconitate, creatine, lactate, pantothenate, taurine and U1 formed the second
277 cluster, while 3-indoxylsulfate (3-IS), dimethylsulfone (DMS) and 1-methylnicotinamide
278 (MND) formed the third cluster (Figure 2B). Although these metabolites did not correlate
279 with each other or with the rest of urinary metabolites, they showed a pattern of
280 absence/presence throughout the experiment. For instance, the only increase in the level
281 of creatine was found at h12 compared to h24, while alanine and dimethylsulfone levels
282 were only higher at h24 compared to h6, and taurine and U1 showed the lowest urinary
283 levels at h24. Contrarily, the urinary levels of *cis*-aconitate, lactate, 3-indoxylsulfate, 1-

methylnicotinamide and dimethylamine (DMA) showed no changes throughout the experiment (Figures 2-4).

Cluster 4 was integrated by DMA and unassigned spectral region (USR). Considering the pairwise comparison between h6 and h12 (Figures 1B, 2-4, Table S5), only USR showed negative correlations with metabolites from cluster 5 (e.g., 2-HIB, carnitine, formate, PB, *trans*-aconitate and trigonelline) and positive correlations with metabolites from cluster 1 (e.g., 2-BAA, 3-HIV, ketoleucine, MM and NAC3). Regarding h12 vs h24, USR only had a direct relationship with MA and an inverse relationship with PAG. Contrary to the pattern of pantothenate, USR (consisting of one or two metabolites) showed the lowest concentration at h12. Therefore, the metabolites involved in this unassigned region were related to the state of starvation.

Statistical filtering was performed to improve the biological interpretation of the results and to reveal important changes in the urinary metabotype derived from physiological events in response to starvation and refeeding. This technique consisted of selecting the metabolites detected by OPLS-DA (with a $|t\text{-statistic}| \geq 1.96$) as well as identifying significant metabolite-metabolite correlations ($|r| \geq 0.7$ with $P < 0.05$), univariate statistical significance ($P \leq 1.66 \times 10^{-2}$; $P \leq 3.33 \times 10^{-3}$ and $P \leq 3.33 \times 10^{-4}$, Kruskal-Wallis test) and fold change (with $|\text{ratio}| \geq 1.2$ ($|\log_2(\text{ratio})| \geq 0.26$)), as summarized in Table 1. Moreover, there were positive correlations among significantly upregulated metabolites and negative correlations between these and significantly downregulated metabolites, in regard to either t_1 or t_2 (h6 or h12, respectively) in pairwise comparison with t_3 (h24). These patterns can also be observed via HCA, which classified these metabolites into different clusters according to the physiological conditions at each time-point evaluated in the present investigation.

In this context, since at h6 the urinary metabolome was comprised of upregulated metabolites derived from catabolic pathways and downregulated metabolites related to food consumption, and at this time point the urinary flow rate (UFR) was found to be increased (Figure S3), the t_1 metabotype reflected the starved condition. Conversely, the t_2 metabotype was comprised of upregulated food-derived metabolites and downregulated metabolites related to catabolic pathways, and at this time point (12h) the lowest UFR was observed. Likewise, the t_3 metabotype showed the same pattern but was defined by the highest urinary levels of TCA intermediates and food-derived metabolites. Therefore, the t_2 metabotype may reflect an absorptive condition after refeeding and the t_3 metabotype a

recovered state. Moreover, urine samples collected at the third point in time can be considered as a control group, since in this period the animals were under normal experimental conditions (food and water provided *ad libitum*) in a complete scotophase (dark period), in which rodents are more active and have energy homeostasis of matching energy intake to energy expenditure over long intervals of time during the normal fasting-feeding cycle. Regarding UFR, the increase in this parameter comparing h24 with h12 was not significant, although there was a tendency for it to be higher at the former time point.

Additionally, the intensities of *pCG* and *pCS* were compared in order to observe which metabolite was more abundant in urine samples. The comparisons were carried out in two stages, at first only with the t_1 data set, in which the observed signals for both metabolites were very intense. Then all data sets were included, finding that *pCG* was significantly more abundant than *pCS* in both comparisons ($P = 2.402 \times 10^{-6}$ and $P = 1.085 \times 10^{-4}$, respectively).

4. Discussion

4.1. Metabolomics/metabonomics analysis

Within the context of the NMR-based metabolomics/metabonomics approach, rats (like humans) are considered as a supraorganism. The metabolome/metabonome is the final outcome of homeostasis, whether derived from normal or altered conditions. The present study aimed to determine the adaptive changes that take place under the condition of starvation and refeeding. Many metabolites detected by NMR are involved in the major metabolic pathways of a supraorganism and represent the current state of homeostasis, thus proving to be highly informative of relative pathway activity.^{2,3} That is, their patterns, directions and relationships with other metabolites are of interest rather than their absolute concentration², which in conjunction are a powerful hypothesis-generating scenario. Furthermore, NMR spectroscopy has a detection limit in the sub-micromolar range.³

The resonance signals define the fingerprint of a metabolite and its relative concentration, by using a semi-targeted analysis via the metabolite correlation matrix along with the $\log_2$ ratio (fold-change) relative to the peak areas of each metabolite in a pairwise comparison, it is possible to obtain information about the dynamic system of a biological organism. Therefore, it is possible to generate hypotheses about physiological or pathophysiological changes over time. This so-called metabolite correlation matrix employs the sum of peak areas that are matched with the structure of a metabolite to view the degree of covariation with the rest of metabolites contained in the data matrix. Furthermore, the unassigned

spectral regions/metabolites were also included in the metabolite correlation matrix in order to observe their relationship with identified urinary metabolites. Therefore, this semi-targeted technique provides important information about the similarities in molecular structure between metabolites, their biological source or biochemical pathways (i.e., starvation, refeeding, diet, TCA, cometabolism), and the increase or decrease (fold-change) in relative concentration of a particular metabolite over time.

It is important to mention the problem with urine dilution resulting from food deprivation. To make accurate comparisons, PQN normalization was carried out to compensate for the differences in the overall concentrations of all samples that derived from physiological mechanisms of urine concentration and water uptake behaviour. Additionally, this normalization considers the dilution factor used in sample preparation.²³

4.2. Starvation

Under normal conditions, energy homeostasis makes it possible to match energy intake to energy expenditure over long intervals of time, and thereby ensure stability in the amount of body energy stored or that used to sustain life during periods of high energy demand. In response to energy deprivation, peripheral tissues and CNS neurocircuits initiate an adaptive mechanism whose priority function is to restore euglycaemia and the supply of energy to the brain, erythrocytes and vital organs. This adaptive mechanism is influenced by humoral mediators such as leptin, catecholamines, corticosterone, cortisol, insulin, glucagon, peptide YY, thyroxine (T4), triiodothyronine (T3), glucagon peptide 1 (GLP1) and cholecystikinin (CCK). Since the gut microbiota also influences this adaptation through bidirectional communication with the host, it is an integral part of the energy homeostasis system under normal conditions and starvation.^{14,15,22}

In this context, rats have a characteristic nocturnal pattern, typically being more active and eating more in the scotophase than in the photophase (light period). Accordingly, during the scotophase rats have a high energy-demand that must correlate with energy intake. They achieve this balance by consuming food until reaching euglycaemia and satiety.^{15,20,21} Afterwards, the rat postprandial period (absorptive or non-steady state) can take place,³⁰ with its subsequent postabsorptive period or fasting-feeding cycle. This metabolic cycle controls the composition and function of the gut microbiota even during food deprivation.^{20,21}

With energy deprivation, glycolysis is increased and glycogenolysis is promoted. During the course of starvation, energy demand increases at the time that hepatic glycogen stores

are depleted. Concomitantly, the supraorganism displays adaptive responses, based on alternative energy sources other than glucose, in order to maintain homeostasis and sustain life. These adaptations take place via catabolic pathways such as lipolysis, glycogenolysis, gluconeogenesis, β -oxidation, ketogenesis and skeletal muscle protein turnover. Accordingly, gluconeogenesis and increased lactate blood levels inhibit the glycolytic pathway and vice versa, processes that are exclusive to the liver and kidney.^{10,15} These responses in the host are evidenced by the downregulation of TCA intermediates in the t₁ urinary metabotype, which can be related to the high rate of anaplerosis³¹ (i.e., β -oxidation) and reduction in glycolysis under the starved condition. Therefore, the urinary excretion of these metabolites is reduced.

Starvation stimulates protein breakdown, thereby increasing the concentrations of branched-chain amino acids (BCAAs, isoleucine, leucine and valine) in adipose and muscle cells. The catabolism of BCAAs as well as the Cori and glucose-alanine cycles restore glycaemia via gluconeogenesis^{10,14,15,17,32} or produce acetyl and succinyl CoA for use in the TCA cycle³³ in the skeletal muscle-liver-brain axis. The BCAAs catabolic pathway occurs at the highest rates in skeletal muscle, in which leucine is metabolized via branched-chain α -keto acid dehydrogenase (BCKD) to yield ketoleucine, NADH and FADH₂ which are involved in ATP biosynthesis. However, there is only 1 dehydrogenase enzyme for the three BCAAs, all three α -keto acids produced can be accumulated and/or excreted in the urine.^{33,34} Furthermore, when protein turnover is increased, the activity of α -ketoisocaproate dioxygenase (KICD) also rises. This enzyme converts ketoleucine to 3-hydroxyisovaleric acid in rat and human liver^{35,36} and pancreas.¹⁶ It has been suggested that 3-HIV may inhibit muscle proteolysis,³⁷ and that KICD possibly functions as a *safety valve* to prevent excessive accumulation of ketoleucine, which is quite toxic. This mechanism could also protect against the consumption of over 50% of proteins, which is related to death.^{35,36} Furthermore, the production of 3-HIV can be altered by fasting and refeeding, since the distal colon is a carbohydrate- and energy-deficient environment where colon microbiota via oxidative deamination of BCAAs can produce branched-chain fatty acids (BCFAs) such as isovalerate, isobutyrate and isocaproate, whose biosynthesis is reduced in the presence of carbohydrate sources.¹⁸ In this context, when β -oxidation is highly active during the state of starvation, isovalerate may reach the liver mitochondria to undergo this process. However, because of being a tertiary alcohol, 3-HIV is not a suitable substrate for completing this oxidation and it can therefore be exported to the cytosol with a previous hydrolysis that releases CoA-SH into the mitochondria.

Additional information about energy homeostasis was the increase in creatinine urinary levels in the t_1 metabotype, which may indicate that energy stores in skeletal muscles (e.g., creatine phosphate) are depleted during starvation and/or the creatinine observed at t_1 was synthesized *de novo* due to food deprivation. Creatinine is also regarded as a renal biomarker of homeostasis and some intestinal bacteria can produce it or degrade it into methylamine.³⁸ The pattern observed in the urinary levels of creatinine may also be informative about the renal functional responses to starvation, since alterations in glomerular filtration rate and polyuria are induced in starved animals.^{31,39} Therefore, the increased levels of creatinine at t_1 may have derived not only from the skeletal muscle protein turnover, but also from changes in renal filtration induced by starvation, as this osmolyte is neither secreted nor reabsorbed by the renal tubule in the female rat.⁴⁰ Accordingly, NAC levels were higher in the t_1 than in the t_2 or t_3 metabotype, which may have resulted from protein or peptide mobilization during starvation, since the presence of urinary proteins is a response to the stress produced by food deprivation.⁴¹ Regarding the excess of urinary creatine after refeeding in t_2 , it may derived from either by intestinal absorption of dietary creatine or by *de novo* creatine biosynthesis via kidney-liver-skeletal muscle axis.³⁸

In addition, the amino acid taurine is involved in skeletal muscle homeostasis and several physiological functions have been described for it, as conjugating agent for bile acids, osmoregulator, modulator of calcium homeostasis and signalling, endogenous antioxidant and anti-inflammatory compound in various tissues. The liver tightly regulates its intracellular cysteine pool addressing 2 opposing homeostatic requirements, the need to have adequate levels to meet the production of other essential molecules (e.g. glutathione, coenzyme A, taurine, and inorganic sulfur), and the need to keep cysteine concentrations below the threshold of oxidative stress and cytotoxicity.^{42,43} The upregulation of taurine under starved condition may be related to protein turnover, skeletal muscle and energy homeostasis whereby the integration of cysteine and coenzyme A pathways are involved in taurine biosynthesis.

Upregulation of RNA catabolites in urine has been related to protein turnover and perturbations in RNA metabolism. Pseudouridine is one of the three main RNA catabolites, its excretion reflects whole-body RNA turnover, and therefore whole-body protein metabolism, which is sensitive to food deprivation.^{2,44} Accordingly, these catabolic pathways are active under starvation and reflected in the t_1 urinary metabotype, which is

characterised by the upregulation not only of pseudouridine but also of metabolites related to protein turnover, which are positively correlated.

The methylmalonyl-CoA mutase (MCM) plays a key role in the degradation of valine, isoleucine, methionine, threonine, odd-chain fatty acids, and cholesterol, in order to yield succinyl-CoA, a TCA intermediate. At the first step succinyl-CoA is produced which subsequently is converted into D-methylmalonyl-CoA and then racemized and isomerized to produce succinyl-CoA via MCM. This reaction is irreversible and does not proceed when vitamin B₁₂ is deficient, as is the case with starvation, MM is deported to the cytosol and then excreted in urine, denoting a vitamin B₁₂ deficiency.^{45,46}

The gut microbiota is also affected by starvation, which is reflected in the cometabolism of some aromatic amino acids that can yield *p*-cresyl glucuronide (*p*CG) and *p*-cresyl sulfate (*p*CS). This virtual organ biosynthesizes *p*-cresol using tyrosine (Tyr) as starting material, an amino acid that can be derived from either *p*-aromatic hydroxylation of phenylalanine (Phe) or the protein-amino acid pool, or both. Once absorbed, *p*-cresol is conjugated with glucuronide and/or sulfate in the liver to yield *p*-cresol glucuronide and *p*-cresol sulfate, which are excreted in urine.^{5,9} Therefore, the increased urinary levels of *p*CG and *p*CS in the t₁ metabotype may have derived from the increased protein turnover produced during starvation, thus supplying the amino acid pool with the aforementioned aromatic amino acids that undergo combinatorial metabolism between the host and the gut microbiota. In addition, the formation of *p*CG was more favorable than that of *p*CS under the condition of starvation, perhaps because sulfation is a saturable reaction limited by the availability of PAPS (3'-phosphoadenosine 5'-phosphosulfate), which can be reduced by food deprivation. Furthermore, sulfation requires more energy than glucuronidation (overall, 2ATPs vs 1 UTP), which is not saturable, and *p*-cresol may compete for sulfation with indole to yield 3-IS, another cometabolite.^{5,6} In fact, hepatic sulfation in rats has been considered as a high-affinity, low capacity conjugation reaction, whereas glucuronidation is a low-affinity, high capacity conjugation reaction, and both are competing pathways in biotransformation reactions.⁴⁷⁻⁴⁹ Therefore, the depuration of *p*-cresol, a gut-derived uremic toxin,⁶ is a survival mechanism, because its excretion as a phase II type biotransformation metabolite requires energy, and it is increased even during starvation. Likewise, the upregulation of PAG in the t₁ metabotype was observed. This cometabolite, derived from the liver catabolism and/or microbial fermentation of phenylalanine, yields phenylacetate and this in turn conjugates with glycine.^{9,50} Its biosynthesis, not strictly limited to diet sources, can be carried out using endobiotic intermediates, such as phenylalanine. One

source of this α -amino acid may be the protein turnover that takes place under starvation.^{51,52} In summary, the pattern observed in the phase II drug-like biotransformation co-metabolites PAG, *p*CG and *p*CS suggests the use of amino acids from not dietary resources. Thus, the proteins or amino acids released by skeletal muscle proteins catabolism may reached the colon and be fermented by the gut microbiota to produce phenylacetate and *p*-cresol which in turn reach the liver and undergo biotransformation, which requires energy to proceed. Furthermore, phenylalanine that derived from protein turnover can reach the liver, where PAG is produced *in situ*.

On the other hand, the combinatorial metabolism between the host and the gut microbiota as well as the functional interactions within microbial members are dynamic, complex and vary according to community composition. The colonic mucus layer is a very challenging habitat, whereby a major determinant of microbiota composition and cometabolism with the host is related to the availability of nutrients, intestinal motility and secretions, and the functional competition for resources to survive within gut microbes under the dynamic and rapid renewal of the mucus layer secreted by the host.^{22,53} Accordingly, under starved condition the gut microbiota may use glycoproteins from the mucus layer as an alternative carbon source, from which may derive the requested building blocks to produce PAG, *p*CG and *p*CS. Therefore, protein turnover was reflected not only by the upregulation of these co-metabolites in the t_1 urinary metabotype but also by their downregulation even after refeeding and recovery, when dietary sources for their biosynthesis were available. This also implies that *p*-cresol detoxification is important for the maintenance of homeostasis, since it is nephrotoxic.

Conversely, after ad lib feeding, urinary levels of hippurate start to increase because the pathway of this cometabolite starts with the production of benzoic acid from bacterial fermentation of dietary polyphenols and/or aromatic amino acids (e.g., chlorogenic acid, catechin, Phe and Tyr), or it is simply ingested directly from food. Afterwards, benzoic acid is conjugated with glycine in the liver and to a lesser extent in the kidney, at the expense of ATP and CoA-SH.⁵⁰⁻⁵² Therefore, the high demand of energy for the biosynthesis of hippurate may cause a reduction in this process during starvation due to the priority of using energy for survival mechanisms, which in turn can explain the downregulation of hippuric acid at t_1 . The lower, but not absent, urinary levels of hippurate during starvation may be originated from phenylalanine, which can yield phenylpropionate via microbial fermentation that undergo β -oxidation by the host to produce benzoate and acetyl-CoA.

Since β -oxidation is active during starvation and has a common compartmental location with glycine conjugation, it is likely that hippurate is produced by non-dietary precursors.^{5,51}

The inverse pattern observed between hippurate and PAG may also be related to the so-called deportation system, which is a vital homeostatic mechanism to prevent harm in the central nervous system by removing the excess of glycine or nitrogen via glycine conjugation. Accordingly, when glycine and benzoate are available after refeeding, it is that hippurate is yielded. During food deprivation, conversely, glycine and phenylacetate may be derived from protein turnover. Moreover, under this condition glycine can be synthesized *de novo* from CO_2 and NH_4^+ , the latter being an end product of protein oxidation.^{11,17,52} This process uses benzoate or phenylacetate as a carrier for glycine deportation, leading to its irreversible excretion in the form of hippurate or PAG, respectively.^{9,52} Furthermore, since mitochondrial fatty acid oxidation is highly active under the starved state, butyryl-CoA can be accumulated. This electrophilic form of butyrate can undergo glycine conjugation to yield 2-butanamidoacetate, which is then excreted in the urine. Hence, the latter metabolite is upregulated when overproduced during starvation.⁵⁴ Moreover, the relationship between anaplerotic pathways is supported by the observation of a bi-compartmental correlation, the positive correlation between 2-BAA and glutamate, aspartate and BCAAs (Figure S4). These amino acids are produced via catabolism of proteins of skeletal muscle. Upon reaching the small intestine, they are used as an alternative energy source via anaplerosis.^{11,16,33,34} In this respect, since glycine conjugation of mitochondrial acyl-CoAs is an important metabolic pathway responsible for maintaining adequate levels of free coenzyme A (CoASH), this pathway can influence gluconeogenesis, β -oxidation, and the electron transport chain.⁵¹ Hence, the upregulation of PAG and 2-BAA during starvation may be derived from a trial effect: detoxification of NH_4^+ , and regulation of mitochondrial energy homeostasis by avoiding accumulation of phenylacetyl-CoA and butyryl-CoA, and maintaining CoA-SH in adequate levels.^{51,52} In addition to this glycine deportation, the excess of this amino acid, most likely derived from diet, was excreted in urine at the last two points in time.

Therefore, in the present model of starvation comprising a complete scotophase as well as 4 h before and after it, catabolic pathways were active due to the imbalance between energy intake and energy expenditure. This condition characterised the urinary metabotype and could be noted in the specimens collected at the first point in time (h6). Furthermore, 2 hours of refeeding were not enough to reverse 20 h of starvation, as is evidenced by considerable changes in the t_1 urinary metabotype. These findings are

consistent with previous studies, in which rats starved for 20 h showed significant depletion of glycogen in liver,¹² and while rats fasted for 1 day showed increased muscle proteolysis.^{13,14} Moreover, the pattern observed in t_1 urinary metabotype was similar to rat urine specimens collected during a 16-h period of starvation. For instance, urinary levels of hippurate, DMG, citrate, 2-oxoglutarate and methylamine decreased, while PAG, taurine and creatinine increased. Also, polyuria was observed in starved animals.³¹ Likewise, a fasting-refeeding kinetic study in mice indicated that caloric restriction maintains higher rates of gluconeogenesis and protein catabolism, even during a few hours after refeeding.¹¹

4.3. Refeeding

After refeeding, dietary resources start to reach the places where they are metabolized or cometabolized in order to maintain homeostasis by balancing the intake/expenditure of energy. Moreover, as negative feedback during the postprandial and postabsorptive state, insulin is released into the bloodstream to lower glucose levels, enhance membrane transport of glucose into fat and muscle cells, and inhibit glycogenolysis and gluconeogenesis. Contrary to the case of food deprivation, which involves catabolism to produce energy from sources other than glucose, with normal feeding and a constant diet, anabolism is prominent.^{20,21} Thus, in the latter case the metabolic profile of urine reflects mainly compensatory mechanisms developed during the refeeding-postprandial period (t_2) and postabsorptive state along with the normal fasting-feeding cycle (that comprised the recovered or normal condition, t_3). Contrary to the t_1 metabotype, in t_2 and t_3 the match between food intake and energy expenditure can be appreciated by observing the increased levels of TCA intermediates as well as metabolites derived from the diet, such as trigonelline, *trans*-aconitate and PB, which are no longer completely utilized by the supraorganism due to their abundance. The excess of TCA intermediates is regulated by the excretion of what is not used, as metabolism through its anaplerosis and cataplerosis pathways maintains constant quantities of anaplerotic substrates, to avoid an override of normal control of energy homeostasis.¹⁶

Trigonelline and PB are reportedly contained in alfalfa and citrus.^{27,55} In addition, *trans*-aconitate may be derived from the isomerization of *cis*-aconitate, a TCA intermediate (KEGG database, <http://www.genome.jp/kegg/>), or from the diet, as it is present in cane molasses.⁵⁶ Since alfalfa and cane molasses are ingredients of the food provided to the rats, trigonelline, *trans*-aconitate and PB are likely not metabolized/cometabolized without

structural modifications. In the event that there were metabolites from them, they would likely be present in quantities not detectable by NMR.

Compared to the other metabotypes, pantothenate showed the highest urinary levels at t_2 . This vitamin plays an important role in the tight regulation of hepatic coenzyme A metabolism, which is involved in the TCA cycle, ketogenesis and fatty acid metabolism.^{16,43,57} After refeeding, during the absorptive state, dietary pantothenate can reach the glomerular filtrate, from where it is cleared by renal excretion due to its hydrosolubility.

The cometabolite *m*HPPA is an intermediate in the hippurate pathway, which is carried out by the gut microbiota.^{9,50} Since dietary precursors of hippurate, and therefore of *m*HPPA, were supplied after refeeding and sustained until the end of the present study, these gut microbial co-metabolites were part of the urinary metabotype under refed and recovered conditions.

Another cometabolite originating from dietary sources is 2-HIB, which derives from the hepatic aliphatic hydroxylation of isobutyrate, a BCFA produced by the gut microbial fermentation of BCAAs.^{2,18} From dietary non-digestible fibre, the gut microbiota produces formate and acetate (short fatty acids, SCFAs) whose urinary levels increase after refeeding.^{7,19}

The behaviour of carnitine, TMAO, MA, DMG and glycine denotes that they are derived from dietary sources. Carnitine, TMAO and MA are related to the gut microbial metabolism of choline and are involved in the metabolism of fatty acids.⁸ DMG and glycine are produced during the host metabolism of choline, which is related to cholinergic neurotransmission that activates muscles in the peripheral nervous system.³²

According to the pattern of the unknown assignments, USR and U1 may be metabolites related to the condition of starvation, and U2 and U3 derived from dietary sources.

Since metabolic functions and energy balance are regulated by the gut microbiota as well as by the host,⁸ the adaptation mechanisms triggered by food deprivation and refeeding involve continuous bidirectional communication between the symbiotic parts of the supraorganism. Under such conditions, the absence of necessary nutrients and the physical remodelling of the gastrointestinal tract (e.g, the luminal mucus layer or lumen where the commensal gut bacteria reside) have an impact on the composition and function of gut microbiota, whose adaptive mechanism is dynamic in response to new conditions of

the epithelial mucus it faces.¹⁰ For instance, the gut microbiota is able to use host glycans present in mucus and on the surface of the gut epithelial cells as a source of energy when dietary polysaccharides are limited,^{22,53} and *p*-cresol has been associated with differences in composition of the gut bacterial community derived from changes in the gastrointestinal tract.⁸ Conversely, in healthy animals fed on a consistent diet, populations of symbionts are stabilized through interspecific competition and resource partitioning,¹⁰ leading to definite normal levels of metabolites and co-metabolites in the urinary metabotype, as in *t*₃. The metabolic pathways related to changes of the most important urinary metabolites under starvation, refeeding and recovery are integrated and summarized in Figure 5.

4.4. Faecal water metabotype

Since starvation induced lack of faecal production and not all animals produced faeces during refeeding, 24-h pooled faecal water samples were also analysed in order to complement the information obtained by the urinary metabotypes. Although it was a 24h-pooled sample, the information obtained provides positive autocorrelations between metabolites, meaning that metabolites correlating with each other are structural analogues and/or have the same biochemical pathway or origin (Figure S5). For instance, the correlation between lactate and acetoin may be due to the fact that both are produced in pyruvate metabolism and/or by the gut microbiota,^{19,58} and because both metabolites contain an α -hydroxy ketone group in their molecular structure. Moreover, the gut microbiota fermentation of non-digestible carbohydrates produces SCFAs such as acetate, propionate and butyrate, which can be used by the host or excreted in faeces. The proton NMR signals of SCFAs in faecal water samples characterised the spectra, as they are highly correlated. Propionate can be used by the host for gluconeogenesis, while butyrate is an energy source used by colonocytes. SCFAs, on the other hand, play an important role in the modulation of the immune response by reducing intestinal permeability.^{7,8,19}

Branched-chain amino acids correlated with alanine, aspartate, glutamate, methionine, phenylalanine and tyrosine. Overall, the amino acids found in faecal water that correlated with each other seem to have similar origin. Accordingly, the unabsorbed proteins (released from the gastrointestinal mucus gel), peptides or free amino acids that escape assimilation in the small intestine eventually reach the colon, where they are either fermented by the gut microbiota or remain intact, to be excreted in faeces, and some amino acids can be released from the lysis of bacteria during the preparation of samples.^{7,8,18,19,53}

Regarding the correlation between acetate and *m*HPPA, it is known that both are sym-xenobiotic co-metabolites. Concerning *m*HPPA, some phenol compounds (essential and non-essential aromatic amino acids and/or secondary metabolites of dietary sources) reach the colon, where they can be fermented by resident bacteria and then excreted in faeces.^{9,50}

It was also found that xanthine correlated with uracil. Despite their different molecular structure, they are products of purine and pyrimidine catabolism, respectively, which converge in the DNA and RNA metabolic pathway (KEGG database, <http://www.genome.jp/kegg/>). In addition, uracil is used in *de novo* biosynthesis of pantothenate, which is exclusive for bacteria and other prokaryotes, and may provide an alternative source of this vitamin that complements its presence in the diet or during starvation.⁵⁷

5. Conclusions

This study demonstrated that physiological adaptations in response to food deprivation and refeeding involve the continuous bidirectional communication between the symbiotic parts of the supraorganism, which is related to the homeostatic control of energy balance. The present NMR-based metabolic profiling revealed a catabolic metabotype produced by food deprivation, whereby upregulated metabolites were related to renal and skeletal muscle function, catabolic pathways such as β -oxidation, turnover of proteins and RNA, and host-microbial interactions. After refeeding, food-derived metabolites, including gut microbial co-metabolites, and tricarboxylic acid cycle intermediates were upregulated under refed and recovered conditions, in which the upregulation of creatine and pantothenate indicated an absorptive state after refeeding. In the 24-h faecal water metabotype was also observed the presence of gut microbial–host co-metabolites.

The current work provided the basis for differentiating non-physiological and pathological changes from normal physiological responses related to energy metabolism and host-microbial interactions. As starvation and refeeding are considered a convenient procedure for animal models that are used to assess the pharmacological or toxicological effect of compounds, or to evaluate disease and treatment, this information can be used for improving biological interpretation of data in future research.

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7. Figure captions

Figure 1. (A) 3D principal component analysis (PCA) scores plot of the urinary metabolotypes. Color code: green (h6), blue (h12), red (h24). (B) The hierarchical clustering shows the patterns of urinary metabolites. (C) Venn diagram demonstrating the number of unique and shared metabolites in urine and faeces. Key as indicated in Supplementary Table S2.

Figure 2. (A) OPLS-DA cross-validated scores (left) and loading plots (right) derived from 1D ^1H NMR spectra of urine, indicating the differentiation between h6 (green) and h12 (blue) metabolotypes. Significant variables are coloured based on their t-statistic. (B) Correlations of urinary metabolite NMR peak areas with $|r| \geq 0.7$ and $P < 0.05$ (right). Blue denotes a positive correlation and red a negative correlation. Heat-map summarizing metabolic changes (left). Green represents higher relative concentration and red lower relative concentration versus h6. $*P \leq 1.66 \times 10^{-2}$; $***P \leq 3.33 \times 10^{-4}$ (Kruskal-Wallis with Bonferroni post hoc test). Key as indicated in Supplementary Table S2.

Figure 3. (A) OPLS-DA cross-validated scores (left) and loading plots (right) derived from 1D ^1H NMR spectra of urine, indicating the differentiation between h6 (green) and h24 (red) metabolotypes. Significant variables are coloured based on their t-statistic. (B) Correlations of urinary metabolite NMR peak areas with $|r| \geq 0.7$ and $P < 0.05$ (right). Blue denotes a positive correlation and red a negative correlation. Heat-map summarizing metabolic changes (left). Green represents higher relative concentration and red lower relative concentration versus h6. $*P \leq 1.66 \times 10^{-2}$; $**P \leq 3.33 \times 10^{-3}$; $***P \leq 3.33 \times 10^{-4}$ (Kruskal-Wallis with Bonferroni post hoc test). Key as indicated in Supplementary Table S2.

Figure 4. (A) OPLS-DA cross-validated scores (left) and loading plots (right) derived from 1D ^1H NMR spectra of urine, indicating the differentiation between h12 (blue) and h24 (red) metabolotypes. Significant variables are coloured based on their t-statistic. (B) Correlations of urinary metabolite NMR peak areas with $|r| \geq 0.7$ and $P < 0.05$ (right). Blue denotes a positive correlation and red a negative correlation. Heat-map summarizing metabolic changes (left). Green represents higher relative concentration and red lower relative concentration versus h12. $*P \leq 1.66 \times 10^{-2}$; $**P \leq 3.33 \times 10^{-3}$; $***P \leq 3.33 \times 10^{-4}$ (Kruskal-Wallis with Bonferroni post hoc test). Key as indicated in Supplementary Table S2.

Figure 5. Partial visualization of the metabolic pathways related to starvation, refeeding and recovered state. *p*-Cresyl glucuronide is more abundant than *p*-cresyl sulfate. **Creatine** and **pantothenate** were indicative of absorptive state after refeeding. Key: Phe, phenylalanine; Tyr, tyrosine; SULT, phenol sulfotransferase; UGT, UDP-glucuronosyl-transferase; CDO, cysteine dioxygenase; CS, cysteinesulfinate; HT, hypotaurine; CoA, coenzyme A; CA, cysteamine; CK, creatine kinase; GAA, guanidinoacetate; AGAT, arginine:glycine amidinotransferase; GAMT, S-adenosyl-methionine:N-guanidinoacetate methyltransferase; BCKD, branched-chain α -keto acid dehydrogenase; KICD, α -ketoisocaproate dioxygenase, the *safety valve*; Val, valine; Ile, isoleucine; Met, methionine; Thr, threonine; OCFA, odd chain fatty acids; MCM, methylmalonyl-CoA mutase; NAC, N-acetyl glycoprotein; *m*HPPA, 3-(3-hydroxyphenyl)propionate; BCAAs, branched-chain amino acids; 2-HIB, 2-hydroxyisobutyrate; DMG, dimethylglycine; TMA, trimethylamine; FMO, flavin-containing monooxygenase; TMAO, trimethylamine-N-oxide; MA, methylamine.

Figure 1

Figure 2

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Figure 4

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980 Figure 5

Comparison	No. ^f	Metabolite ^g	ratio	log ₂ (ratio)	Cluster number
6vs12 ^d	20	***Ketoleucine	-1.63	-0.70	1
	1	***2-BAA	-1.60	-0.68	
	28	***pCG	-1.42	-0.50	
	33	***PSU	-1.40	-0.49	
	13	***Creatinine	-1.38	-0.46	
	24	***MM	-1.28	-0.36	
	29	*pCS	-1.25	-0.32	
	4	***3-HIV	-1.24	-0.31	
	27	***NAC3	-1.24	-0.31	
	42	***USR	-1.37	-0.46	4
	41	***U3	1.40	0.50	5
	22	*mHPPA	1.61	0.69	
	18	***Glycine	1.32	0.40	6
	40	***U2	1.40	0.49	
	6	***5-HMH	1.52	0.61	
	3	***2-OG	1.61	0.69	
	34	***Succinate	1.72	0.78	
	32	***PB	1.92	0.94	
	10	***Carnitine	2.00	1.00	
	11	***Citrate	2.08	1.06	
	2	***2-HIB	2.18	1.13	
	15	***DMG	2.53	1.34	
	17	***Formate	3.15	1.66	
	37	***Trigonelline	4.41	2.14	
	35	***trans-Ac	7.22	2.85	
6vs24 ^d	28	***pCG	-2.03	-1.02	1
	13	***Creatinine	-1.90	-0.93	
	33	***PSU	-1.86	-0.9	
	31	***PAG	-1.84	-0.88	
	20	***Ketoleucine	-1.78	-0.83	
	29	***pCS	-1.75	-0.81	
	1	***2-BAA	-1.73	-0.79	
	27	***NAC3	-1.35	-0.44	
	4	***3-HIV	-1.33	-0.41	
	24	***MM	-1.30	-0.38	
	36	**Taurine	-1.37	-0.45	2
	23	***MA	1.33	0.42	5
	7	***Acetate	1.43	0.52	
	38	*TMAO	1.66	0.73	
	19	***Hippurate	1.77	0.82	
	41	***U3	1.85	0.89	

	22	*** <i>mHPPA</i>	2.85	1.51	
	18	*** <i>Glycine</i>	1.48	0.57	6
	40	***U2	1.64	0.71	
	34	*** <i>Succinate</i>	1.66	0.73	
	11	***Citrate	1.77	0.82	
	3	***2-OG	1.93	0.95	
	32	***PB	2.21	1.14	
	2	***2-HIB	2.28	1.19	
	10	***Carnitine	2.29	1.2	
	15	***DMG	2.88	1.53	
	17	*** <i>Formate</i>	3.05	1.61	
	37	***Trigonelline	5.48	2.45	
	35	*** <i>trans</i> -Ac	7.03	2.81	
12vs24 ^e	12	**Creatine	-2.03	-1.02	1
	31	***PAG	-1.56	-0.64	
	28	*** <i>pCG</i>	-1.43	-0.52	
	29	*** <i>pCS</i>	-1.40	-0.49	
	13	**Creatinine	-1.38	-0.46	
	33	**PSU	-1.33	-0.41	
	36	**Taurine	-1.45	-0.54	2
	30	***Pantothenate	-1.27	-0.35	
	39	***U1	-1.24	-0.31	
	42	***USR	1.39	0.48	4
	7	** <i>Acetate</i>	1.23	0.30	5
	41	*U3	1.31	0.39	
	23	***MA	1.36	0.45	
	19	*** <i>Hippurate</i>	1.59	0.67	
	22	*** <i>mHPPA</i>	1.76	0.82	

^aKruskal-Wallis with Bonferroni post hoc test (* $P \leq 1.66 \times 10^{-2}$; ** $P \leq 3.33 \times 10^{-3}$; *** $P \leq 3.33 \times 10^{-4}$).

^bOPLS-DA model (|t-statistic| ≥ 1.96). ^cLog₂ ratio (fold-change), positive sign indicates up-regulated metabolites and negative sign indicates down-regulated metabolites. ^d↑ Above or ↓ below h6. ^e↑ Above or ↓ below h12. ^fID number. ^gKey as indicated in Supplementary Table S2. *Gut microbial-host cometabolite*. *Metabolites* and *Co-metabolites* shared between urine and faecal water.

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