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Species Variation in the Fecal Metabolome Gives Insight into Differential Gastrointestinal Function

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The metabolic composition of fecal extracts provides a window for elucidating the complex metabolic interplay between mammals and their intestinal ecosystems, and these metabolite profiles can yield information on a range of gut diseases. Here, the metabolites present in aqueous fecal extracts of humans, mice and rats were characterized using high-resolution ^{1}H NMR spectroscopy coupled with multivariate pattern recognition techniques. Additionally, the effects of sample storage and preparation methods were evaluated in order to assess the stability of fecal metabolite profiles, and to optimize information recovery from fecal samples. Finally, variations in metabolite profiles were investigated in healthy mice as a function of time. Interspecies variation was found to be greater than the variation due to either time or sample preparation. Although many fecal metabolites were common to the three species, such as short chain fatty acids and branched chain amino acids, each species generated a unique profile. Relatively higher levels of uracil, hypoxanthine, phenylacetic acid, glucose, glycine, and tyrosine amino acids were present in the rat, with β-alanine being unique to the rat, and glycerol and malonate being unique to the human. Human fecal extracts showed a greater interindividual variation than the two rodent species, reflecting the natural genetic and environmental diversity in human populations. Fecal composition in healthy mice was found to change over time, which might be explained by altered gut microbial presence or activity. The systematic characterization of fecal composition across humans, mice, and rats, together with the evaluation of inherent variation, provides a benchmark for future studies seeking to determine fecal biomarkers of disease and/or response to dietary or therapeutic interventions.

Keywords: fecal extracts • gut microbiota • ^{1}H NMR spectroscopy • multivariate statistical analysis • metabonomics • metabolomics • metabolite profiling

Introduction

There is growing awareness both of the beneficial role the gut microbiota can play in maintaining homeostasis in human health and of the adverse association of certain microbial species with diseases as diverse as cardiovascular diseases and autism.[1–6] This link has led to renewed interest in the characterization of the microbial content in the mammalian intestine. Several studies have used fecal material as a medium for assessing the presence of diseases, such as chronic pancreatitis, inflammatory bowel disease (IBD), and colon cancer.[7–11] Moreover, the potential anticarcinogenic activity of microbial metabolites present in feces, particularly short chain fatty acids (SCFAs), has been investigated by exposing cultured cells to fecal extracts.[12]

High-resolution ^{1}H NMR spectroscopy of biofluids such as urine and plasma can be used to generate diagnostic information relating to many physiological and pathological conditions, particularly when used in conjunction with pattern recognition methods.[13–17] Recent studies have shown that ^{1}H NMR spectral profiles of fecal extracts carry diagnostic information for diseases, including Crohn's disease and ulcerative colitis.[8] However, unlike urine and plasma which are relatively well-characterized in terms of their metabolite composition and extent of interspecies variation,[18,19] temporal variation and the effect of sample preparation in the fecal metabolite profile have not yet been assessed.

Here, we characterize interspecies differences between humans, mice, and rats in the content and temporal stability

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of the fecal metabolite profiles and explore the effect of different fecal sample preparation methods. Our rationale for choosing mice and rats is that these vertebrates are widely used in disease model studies. The ultimate objective of this study is to provide a baseline for future investigations applying fecal profiling to monitor the effect of dietary and pharmaceutical interventions, changes in lifestyles, and progression and regression of diseases.

Material and Methods

Fecal Sample Collection. A single stool specimen of 12 healthy humans (6 women and 6 men; age, 31.8 ± 10.2 years; range, 24–51 years) was obtained from a previous metabonomic study, cross-comparing IBD patient subgroups (e.g., Crohn's disease, ulcerative colitis) and healthy controls,[8] and measured using ^{1}H NMR spectroscopy. This study was approved by the medical ethics committee of the Cork University Hospital (Cork, Ireland), and written informed consent was obtained from each participant.

Female NMRI mice and female Wistar rats were purchased from RCC (Itingen, Switzerland) and kept in groups of 4 at the animal facilities of the Swiss Tropical Institute (Basel, Switzerland), as described elsewhere.[20] In brief, the animals were approximately 6-week-old when fecal sample collection commenced, and the average body weight of mice and rats was 25.6 and 87.4 g, respectively. Animals had free access to water and rodent food from Eberle NAFAG (Gossau, Switzerland). Fecal samples were acquired between 08:00 and 10:00 to minimize variation introduced by diurnal rhythm. The experiments were approved by the local government of Basel (permission nos. 2070 and 2081), and handling of the animals and collection of feces was according to Swiss national regulations.

A second rat strain of 8 Sprague–Dawley (SD; Crl:CD(SD)IGS BR) males (age, 7 weeks; weight, 175.5–200 g) was obtained from Charles River Laboratories (Wilmington, MA). The animals were provided with food and water *ad libitum*. All in-life studies were reviewed and approved by the Institutional Animal Care and Use Committee. This study was performed at Pfizer Global R & D (Ann Arbor, MI).

Exploration of Metabolic Variation across Species. The interspecies variation was assessed in human spectra ($n = 12$), the Wistar female rat group ($n = 10$), and finally, female NMRI mice ($n = 12$). Both rats and mice were 9-week-old at the time of sampling.

Exploration of Metabolic Variation with Age. The biochemical variation in fecal metabolite profiles was evaluated in two groups of NMRI female mice ($n_1 = 10$; $n_2 = 12$), in Wistar female rats ($n = 10$), and male SD rats ($n = 8$). The first batch of 10 mice was tested for metabolic differences between sample collection at the age of 6 weeks (baseline) and 53 days later. A more detailed time course study was performed in a second batch of 12 mice where fecal samples were obtained at 8 different time points beginning when the animals were 6 weeks of age and ending at week 11 (sampling days: 1, 3, 7, 10, 14, 21, 28, and 35). Fecal samples obtained from 10 female Wistar rats were prepared under the same conditions and assessed over a 23-day period (starting at the age of 6 weeks). Additionally, the stability of fecal composition was assessed over a 24-h time frame (consistent with a typical acclimatization period in metabolism cages) in 8 male SD rats.

Effect of Sample Preparation and Storage on the Metabolic Profile. The effects of lyophilization, sonication, and filtration on the composition and stability of the fecal metabolite profile were assessed in feces of the Wistar rat group. Full experimental details are given in the Supporting Information. Human fecal samples were compared in terms of storage, and spectra were acquired from the same fecal sample before and after freezing.

^{1}H NMR Spectroscopic Analysis of Fecal Samples. For the human samples, 200–300 mg of stool was mixed with 400 μL of phosphate-buffered saline (PBS; 1.9 mM Na_2HPO_4, 8.1 mM NaH_2PO_4, 150 mM NaCl, pH 7.4), containing 10% deuterium oxide (D_2O), which acts as a field frequency lock for the spectrometer, and 0.01% sodium 3-(trimethylsilyl) [2,2,3,3-$^2H_4$] propionate (TSP) (pH 7.4), which was used as a chemical shift reference (δ 0.0). The resulting samples were centrifuged at 14 000 g_{av} for 10 min to remove particulates. ^{1}H NMR spectra were acquired for each sample using a Bruker DRX 600 NMR spectrometer operating at 600.13 MHz for ^{1}H equipped with 5 mm broadband probe (Bruker; Rheinstetten, Germany). A standard one-dimensional (1-D) solvent-suppressed NMR spectrum was acquired for each sample using the pulse sequence [RD-90°-t_1-90°-t_m-90°-ACQ]. Water suppression was achieved by irradiation of the water peak during the recycle delay (RD = 2 s), and mixing time (t_m = 100 ms). The 90° pulse length was approximately 10 μs, and t_1 was set to 3 μs. The spectral width was 20 ppm, and a total of 64 transients were collected into 32 k data points for each spectrum.

The fecal spectral data for rats and mice were acquired with the same pulse program and parameters used for human fecal extracts, but with 128 scans, to compensate for the lower sample weight available in the rodent models (see Supporting Information for further details).

Metabolite assignments were made by reference to the literature[21–26] and by using statistical total correlation spectroscopy (STOCSY) with an in-house-developed script (Dr. O. Cloarec, Imperial College London).[27] Assignments were confirmed with standard two-dimensional (2-D) ^{1}H-^{1}H correlation spectroscopy (COSY), total correlation spectroscopy (TOCSY), ^{1}H-^{13}C heteronuclear single quantum correlation (HSQC), and heteronuclear multiple bond correlation (HMBC), performed on selected samples. The COSY and TOCSY acquisition parameters, described in detail elsewhere,[28,29] were measured on a 600 MHz NMR spectrometer, whereas the ^{1}H-^{13}C HSQC and HMBC spectra[30–32] were acquired on a Bruker Avance 800 NMR spectrometer (Rheinstetten, Germany) operating at 800.13 MHz.

Data Analysis. The NMR spectra were corrected for phase and baseline distortions using an in-house MATLAB script (Dr. T. Ebbels, Imperial College London). Spectra were normalized to total unit area (excluding the region containing the residual water resonance δ 4.7–4.9) and imported into MATLAB for multivariate analysis. Interpretation of the data was based on principal component analysis (PCA) and orthogonal projection to latent structure-discriminant analysis (O-PLS-DA)[33] of the spectra, in conjunction with visual analysis. PCA provides visualization of clustering, systematic variation (e.g., time-related changes), and outliers by projecting a swarm of coordinates into an n-dimensional hyperspace referred to as a scores plot. Each coordinate represents one individual NMR spectrum, defined by its summed spectral intensity ratio. This

is plotted on a plane defined by the highest variance of the swarm of points.[34] Each principal component (PC) is a linear combination of the original spectral descriptors. The first PC relates to the maximum variance in the data set, subsequent PCs are all orthogonal to the preceding PC and explain progressively less of the variance.

The O-PLS-DA model approach gives information about systematic differences in the data related to the biological ''class'' and allows maximum discrimination between two or more classes. As with PCA, the data can be displayed as scores plots indicating similarity between samples, but additionally, the method generates correlation coefficient plots enabling interpretation of the discriminating features in the spectral data. These coefficient plots are colored according to the significance of correlation to ''class'' (e.g., species, time, etc.) with red representing high significance and blue encoding for low significance. Back-scaling to the covariance matrix is used to maintain the original spectral structure to facilitate interpretability of the coefficient plots.[33] These methods are adapted from the standard O-PLS method defined by Trygg et al.[35]

The O-PLS-DA models were constructed using NMR spectral data as the *X*-matrix and class information as the *Y*-matrix. One orthogonal component was used to remove variation unrelated to class and a 7-fold cross validation of the data was carried out to measure the robustness of the model. The plots were performed in MATLAB with an in-house-developed script (Dr. O. Cloarec, Imperial College London).[33] The significance of the differences between two classes in O-PLS-DA was assessed by a correlation coefficient. We designated a cutoff value for the correlation coefficient of 0.53; higher coefficients indicate statistical significance ($p < 0.05$) of any given metabolite in discriminating between two classes when considering a group size of 12.

Finally, integration over selected peak regions as determined by the highest weighting coefficients in the model assessing time-related variation was performed in MATLAB using a previously published method,[36] modified by K. Veselkov. The pairwise comparison of metabolic changes at timepoints of interest was performed using a Mann–Whitney U test with Bonferroni adjustment for *p*-values in multiple comparisons.

Results

Composition of Metabolite Profiles. Fecal extracts for all three species were characterized by ^{1}H NMR spectroscopy and the metabolites identified are summarized in Table 1. Fecal spectra from the mouse, rat, and human shared several similarities in profile as the direct comparison of the 1-D ^{1}H NMR spectra in Figure 1 shows. For example, all spectra contained visible resonances from SCFAs (*n*-butyrate, propionate, and acetate), the branched chain amino acids (BCAA; i.e., valine, isoleucine, and leucine), glutamate, aspartate, tyrosine, and phenolics. An example of metabolite assignment for a mouse fecal extract is provided in a typical 2-D TOCSY spectrum in Figure 2.

Impact of Species, Age/Time, and Sample Preparation on the Metabolic Profile. Three different sources of variation (i.e., species, age/time, and sample preparation) and their impact on the fecal metabolome were considered in this study. Differences in the fecal metabolite profiles relating to sample preparation methods were minor in comparison with the variation in profile attributable to species or age/time. A detailed summary of the effect of sample preparation methods on metabolite profile variability is given in the Supporting Information. A global PCA model of the aqueous fecal extracts shows the relative contribution of species and age/time differences to the variation in the fecal profiles (Figure 3A). The most influential factor in describing the variation in the data set was species, which was differentiated in the first PC which explained 77.4% of the total variance in the data, followed by age/time which was differentiated in the second PC. Spectra of fecal samples from mice, humans, and rats formed distinct clusters. While the spectra of fecal samples from mice and rats formed relatively tight clusters in both the PCA (Figure 3A) and PLS-DA (data not shown) scores plots, the human fecal samples were distinct from the other two species and more dispersed, reflecting greater variation in the intersample metabolic composition. A closer assessment of the human group revealed some degree of differentiation between samples from men and women, due to higher levels of SCFAs (particularly acetate and *n*-butyrate) in male fecal extracts. Age did not introduce any systematic variation into the fecal profiles (see Supporting Information). Samples from humans and mice showed the clearest differentiation in the first PC with those from rats lying amid these two, in a separate cluster (Figure 3A). Thus, all three species had biochemically distinct fecal extract NMR profiles with the greatest similarity demonstrated between mice and rats, and the second closest group pairing being rats and humans. The particular metabolites which predominantly introduced variation into the spectral profile according to species or age difference are examined in the following sections.

Species Differences in Metabolic Profiles between Rat, Mouse, and Human. In addition to common metabolic features, several marked interspecies differences were observed and summarized in Table 1. Human fecal samples were characterized by lower levels of lactate and various amino acids, such as phenylalanine, alanine, threonine, glucose, and the BCAAs. Glucose resonances were only visible in 4 of the 12 human samples and were associated with the presence of uracil and fumarate, which were otherwise not detectable by visual inspection. In addition, the fecal profile in several human individuals contained glycerol (Figure 1A), which was not observed in either of the rodent species, and relatively higher concentrations of acetate and propionate (Figure 3B). Higher levels of glucose, hypoxanthine, phenylacetic acid, fumarate, tyrosine uracil, and glycine were found in rats, and resonances from β-alanine were only visible in the rat (Figure 1C).

From a direct comparison between female NMRI mice and female Wistar rats using the supernatant of sonicated slurry, higher levels of uracil, glutamate, *n*-butyrate, propionate, and acetate occurred in the rat, whereas alanine and the BCAAs were present in lower levels which is highlighted in the O-PLS-DA plot (Figure 3C). Relatively low levels of 3-hydroxy-phenylpropionic acid (3HPPA) were present in approximately 50% of the samples obtained from mice, whereas this metabolite was only observed in one human and one rat fecal sample.

The C18 axial methyl signals from bile acids were found in all three species, but the identity of the bile acids differed between the species. Rats and mice excreted higher levels of taurocholic/tauro-β-muricholic acid with the latter being relatively higher in mice as described previously,[37] whereas deoxycholic acid was the prominent bile acid prevalent in human fecal samples (Figure 3D) which is consistent with previous studies.[38,39] However, in 50% of the human fecal samples, the C18 bile acid methyl signal was obscured by a broad envelope

Table 1. List of Metabolites Found in Three Species, Human (h), Mouse (m), and Rat (r)[a]

	metabolite	proton group	δ^1H (ppm)	species
1	leucine	α-CH, γ-CH, δ-CH_3, δ-CH_3	3.72(t), 1.69(m), 0.97(d), 0.94(d)	m = r > h
2	isoleucine	δ-CH_3, β-CH_3, half γ-CH_2, half γ-CH_2, β-CH, α-CH	0.93(t), 1.00(d), 1.28(m), 1.47(m), 1.96(m), 3.68(d)	m = r > h
3	valine	α-CH, β-CH, γ-CH_3, γ-CH_3	3.59(d), 2.25(m), 0.98(d), 1.03(d)	m = r > h
4	lactate	α-CH, β-CH_3	4.11(q), 1.33(d)	m = r > h
5	threonine	α-CH, β-CH, γ-CH_3	3.60(d), 4.26(m), 1.33(d)	r > h**
6	alanine	α-CH, β-CH_3	3.81(q), 1.48(d)	m > r > h
7	lysine	α-CH, β-CH_2, γ-CH_2, δ-CH_2, ϵ-CH_2	3.77(t), 1.92(m), 1.73(m), 1.47(m), 3.05(t)	
8	arginine	α-CH, β-CH_2, γ-CH_2, δ-CH_2,	3.76(t), 1.89(m), 1.63(m), 3.23(t)	
9	acetate	CH_3	1.92(s)	h > r > m
10	proline	γ-CH_2, half β-CH_2, half β-CH_2, half δ-CH_2, half δ-CH_2, α-CH	1.99(m), 2.05(m), 2.36(m), 3.34(m), 3.45(m), 4.14(m)	r > h**
11	glutamate	α-CH, β-CH_2, γ-CH_2	3.78(m), 2.06(m), 2.36(m)	
12	methionine	α-CH, β-CH_2, γ-CH_2, δ-CH_3	3.87(m), 2.16(m), 2.65(dd), 2.15(s)	
13	glutamine	α-CH, β-CH_2, γ-CH_2,	3.78(m), 2.15(m), 2.46(m)	
14	aspartate	α-CH, half β-CH_2, half β-CH_2	3.92(m), 2.70(m), 2.81(m)	
15	asparagine	α-CH, half β-CH_2, half β-CH_2	4.01(m), 2.87(dd), 2.96(dd)	
16	ethanolamine	NH-CH_2, HO-CH_2	3.15(t), 3.78(t)	
17	glycine	CH_2	3.55(s)	r > h = m
18	uracil	CH, CH	5.81(d), 7.59(d)	r > m > h
19	tyrosine	half β-CH_2, half β-CH_2, α-CH, 3,5-CH, 2,6-CH	3.06(dd), 3.16(dd), 3.94(dd), 6.87(d), 7.20(d)	r > m > h
20	phenylalanine	3,5-CH, 4-CH, 2,6-CH, half β-CH_2, half β-CH_2, α-CH	7.44(m), 7.39(m), 7.33(m), 3.17(dd), 3.30(dd), 3.99(dd)	m > r > h
21	α-glucose	4-CH, 2-CH, 3-CH, half CH_2C6, 5-CH, half CH_2C6, 1-CH	3.42(t), 3.54(dd), 3.71(t), 3.72(dd), 3.83(dd), 3.84(m), 5.23(d)	r > m > h
	β-glucose	2-CH, 4-CH, 5-CH, half CH_2C6, 1-CH	3.24(dd), 3.40(t), 3.47(dd), 3.90(dd), 4.64(d)	r > m > h
22	formate	CH	8.46(s)	r = m > h
23	tryptophan	4-CH, 7-CH, 2-CH, 5 or 6-CH, 5 or 6-CH, 5 or 6-CH, α-CH, half β-CH_2, half β-CH_2	7.79(d), 7.56(d), 7.34(s), 7.29(t), 7.21(t), 4.06(dd), 3.49(dd), 3.31(dd)	
24	*n*-butyrate	CH_3, α-CH_2, β-CH_2	0.90(t), 2.16(t), 1.56(m)	r = h > m
25	α-ketoisocaproate	CH_2, CH, 2×CH_3	2.61(d), 2.10(m), 0.94(d)	
26	propionate	CH_2, CH_3	2.19(q), 1.06(t)	h > r > m
27	α-keto-β-methyl-*N*-valerate	CH, CH_2, CH_2, terminal-CH_3, βCCH_3	2.93(m), 1.70(m), 1.46(m), 0.90(t), 1.10(d)	
28	α-ketoisovalerate	CH, 2×CH_3	3.02(m), 1.13(d)	
29	α-hydroxyisovalerate	α-CH, β-CH, CH_3, CH_3, CH_2	3.85(d), 2.02(m), 0.97(d), 0.84(d), 1.36(s)	
30	urocanate	CHCOOH, CH(ring), 5-CH, 3-CH	6.40(d), 7.13(d), 7.41(s), 7.89(s)	
31	myoinositol	1,3-CH, 2-CH, 5-CH, 4,6-CH	3.53(dd), 4.06(t), 3.28(t), 3.63(t)	
32	fumarate	2×CH_3	6.53(s)	
33	3-hydroxyphenyl propionic acid	CH_2, CH_2COOH, ring protons	2.47(t), 2.85(t), 6.80(m)	
34	succinate	2×CH_2	2.42(s)	m > r > h**
35	glycerol	half CH_2, half CH_2, 2-CH	3.56(dd), 3.64(dd), 3.87(m)	h*
36	β-alanine	NCH_2, CH_2COOH	3.19(t), 2.56(t)	r*
37	bile acid	C18 axial methyl	0.70(m)	
38	5-aminovalerate	CH_2NH_2, CH_2COOH, 2×CH_2	3.02(t), 2.24(t), 1.65(m)	
39	phenylacetic acid	CH_2, ring protons	3.52(s), 7.29(t), 7.36(t)	r > m > h
40	hypoxanthine	ring protons	8.11(s), 8.10(s)	r > m > h
41	malonate	CH_2	3.13(s)	h*
42	trimethylamine	3×CH_3	2.88(s)	
43	methanol	CH_3	3.36(s)	

[a] Asterisk (*) denotes specific for one species; </> relatively lower/higher amount of the metabolite found in feces in species comparison; two asterisks (**) denotes a trend, which is, however, not significant at the $p < 0.05$ level.

of resonances that distorted the spectral baseline in this region, which made detailed assignment difficult.

Age-Related Metabolic Changes in Aqueous Fecal Extracts. The biochemical variation in fecal metabolite profile of the first group of 10 NMRI female mice revealed highly significant changes in the metabolic profile. In comparison with the baseline samples, those obtained 53 days later showed a marked decrease in various amino acids, including aromatic amino acids (phenylalanine and tyrosine), BCAAs (valine, leucine, and isoleucine), lysine, aspartate, glutamate, alanine, methionine, and glycine. Apart from the change in the relative amino acid concentrations, there was a significant decrease in lactate and an increase in uracil with time.

The sum of the metabolic changes over time in the second group of 12 mice was visualized in a PCA trajectory (Figure 4A) which showed substantial, time-dependent movements between days 1 and 35, particularly along the first PC. To obtain a deeper understanding of the significantly changing metabolites, an O-PLS-DA model was calculated for a series of pairwise comparisons between day 1 and days 3, 7, 21, and 35 (Figure 4B). Significantly lower levels of phenylalanine were observed at all time points when compared with day 1 (coefficient

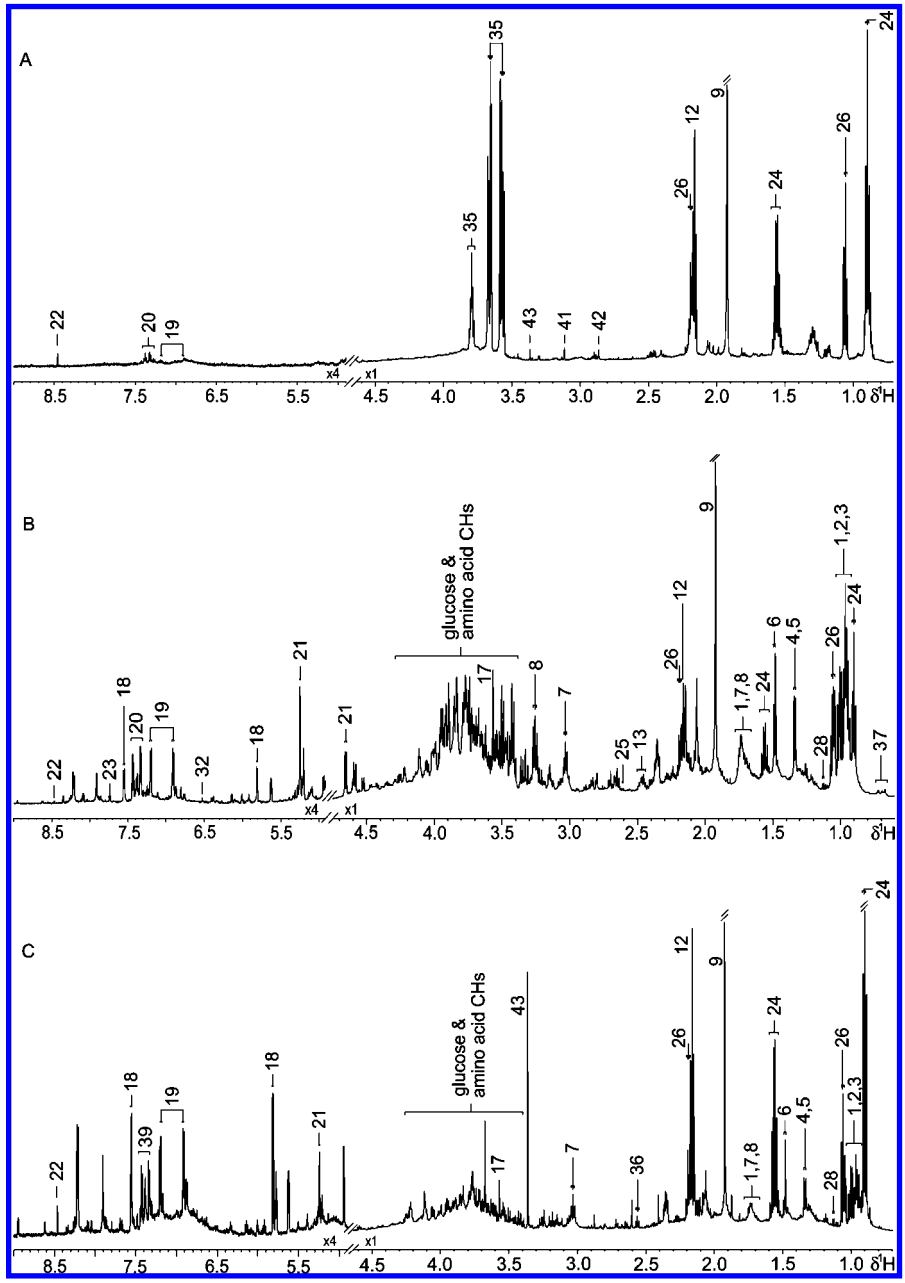

Figure 1. 1-D ^{1}H NMR spectra of (A) human, (B) mouse, and (C) rat fecal water. The numbers refer to the metabolites presented in Table 1.

0.69–0.81), while tyrosine underwent a significant decrease at days 7 (coefficient 0.67) and 21 (coefficient 0.72). Lactate was decreased from day 7 with significance increasing at later time points (coefficient 0.57–0.71). Among the metabolites which increased compared to day 1 were three SCFAs (*n*-butyrate, propionate, and acetate), which showed a significant change comparing either of the later time points with day 1. The coefficients ranged from 0.56 to 0.63 for acetate, 0.65–0.76 for propionate, and 0.64–0.81 for butyrate. Threonine and hypoxanthine were significantly differentiated by class at day 35 (coefficients 0.82 and 0.79, respectively), while threonine showed the same coefficient of 0.82 already at day 21. Uracil was significantly increased at days 3 and 35 when compared with day 1, but not at the intermediate time points (coefficients 0.62 and 0.71).

Resonances from several of the metabolites which showed time-dependent variations were integrated using an automated curve fitting program, and the relative concentration in relation to the total spectral integral was determined (Figure 4C). The *p*-values were assessed using a nonparametric 1-way analysis of variance (Mann–Whitney U) test in MATLAB. Uracil increased significantly at days 21 and 35 compared with day 1 ($p < 0.001$), similar to hypoxanthine which increased at three different days, 3, 21, and 35 compared with day 1 ($p < 0.01$). Succinate underwent a significant decrease after 35 days ($p < 0.001$), while lactate decreased already after 7 days ($p < 0.05$) and maintained its significance over the course of the experiment. An additional comparison of day 3 with days 21 and 35 also revealed statistical significant differences ($p < 0.001$). Formate decreased after 21 and 35 days ($p < 0.001$), while *n*-butyrate was increased already after day 3, compared to day 1 ($p < 0.01$) and maintained significance at all later time points. Glucose showed an inconsistent picture. Days 3 and 7 were significantly lower, compared with day 1 ($p < 0.05$), while a

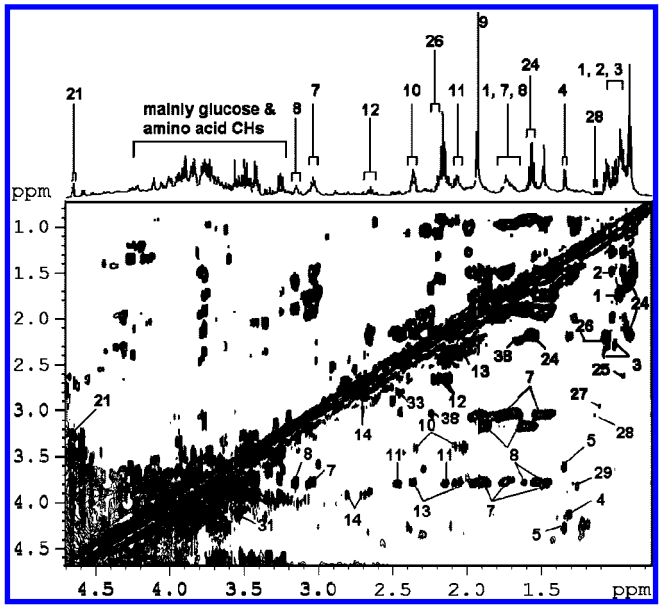

Figure 2. 600 MHz ^{1}H-^{1}H 2-D TOCSY spectra of a fecal extract from a female NMRI mouse. Keys: 1, leucine; 2, isoleucine; 3, valine; 4, lactate; 5, threonine; 7, lysine; 8, arginine; 10, proline; 11, glutamate; 12, methionine; 13, glutamine; 14, aspartate; 21, β-glucose; 24, *n*-butyrate; 25, α-ketoisocaproate; 26, propionate; 27; α-keto-β-methyl-*N*-valerate; 28, α-ketoisovalerate; 29, α-hydroxyisovalerate; 31, myoinositol.

day 1 to day 35 comparison did not differ at all. Comparing days 7 and 21 with day 35 showed statistical difference ($p <$ 0.01). The data (Figure 1) are expressed as ± 2 standard deviations of the mean.

Although time-dependent trends in increasing acetate and propionate were evident, accurate integration of these resonances was not possible due to extreme intersample variation which resulted in an inhomogeneous pattern of resonance overlap with *n*-butyrate and other metabolites in the case of propionate and acetate.

Fecal samples obtained from 10 female Wistar rats obtained at the age of 6 weeks and 23 days later were assessed and demonstrated time-related changes in the profile. A decrease in the amino acids phenylalanine, tyrosine, glycine, alanine, and the BCAAs was observed, which was consistent with the findings in mice. However, unlike the mouse model, the levels of SCFAs and uracil did not change significantly with increasing age of the rats. Rat fecal profiles proved to be stable in lyophilized samples over a short period of time, for example, 24 h.

Effect of Sample Preparation and Storage Conditions on Metabolic Profiles of Fecal Extracts. Differences in the fecal metabolite profiles relating to sample preparation methods were minor in comparison with the variation in profile attributable to species or age/time. The effects of freezing, lyophilization, and two different sterilization methods and their associated variability are detailed in the Supporting Information. In brief, the processes of freezing and lyophilization increased the BCAA levels and lyophilization decreased the SCFAs. Of the two assessed sterilization methods (sonication and filtration), only sonication had an observable effect in comparison with the untreated sample. Like lyophilization, sonication led to lower relative contribution of the SCFAs to the spectral profile.

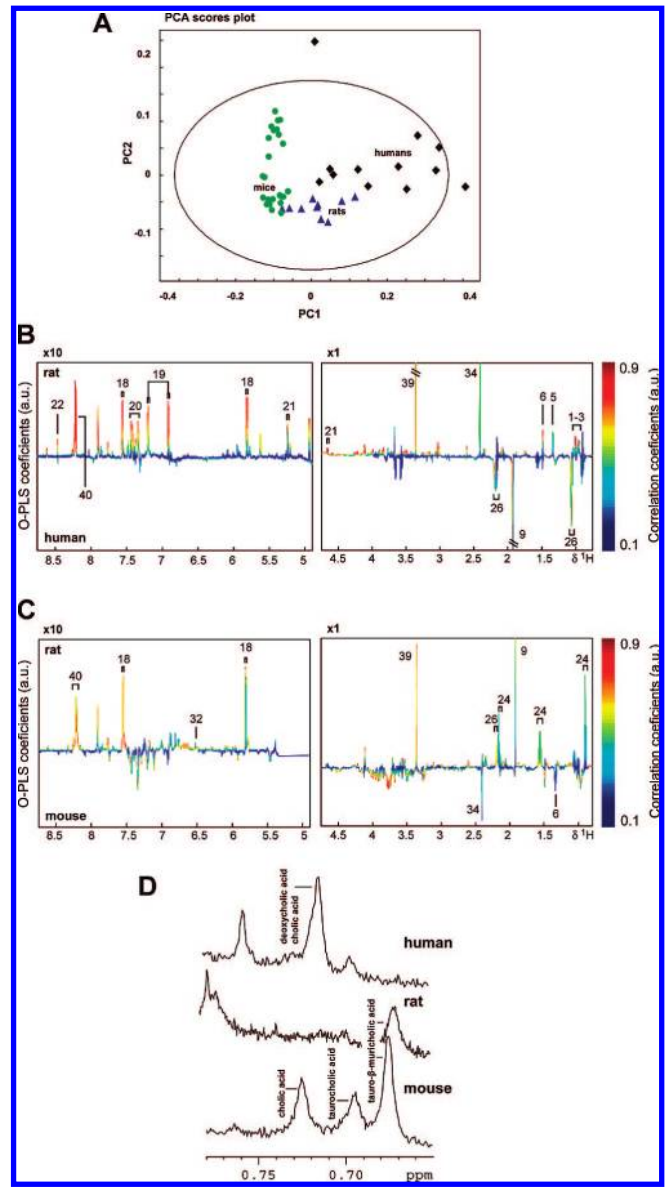

Figure 3. (A) Diagram showing the relative effects of two different sources of variation (species and age/time). PCA scores plots of fecal samples analyzed showing differentiation between species (first PC) and time (second PC). The outlier in the human group (male, 26-year-old) is due to higher levels of the SCFAs and a shift at the acetate region (δ 1.92). (B) O-PLS-DA coefficient plots of rats versus humans and (C) rats versus mice. The color scale indicates the significance of metabolite variations between the two classes, and its maximum is model-dependent. The numbers refer to the metabolites listed in Table 1. (D) Comparison of the bile acids in all three species for region δ 0.63–0.78 ppm.

Discussion

To establish their diagnostic biomarker utility, detailed examination of readily accessible biofluids that can be collected in a noninvasive fashion should be pursued. Although fecal extracts might not represent the first choice of most analyses for metabolic profiling, the intimate connection of the fecal metabolites with the gut microbiota is undeniable. Furthermore, with the growing appreciation of the importance of the gut microbes in mammalian disease,[1–6] it is now time to evaluate variations in metabolite composition between humans and widely used experimental animals.

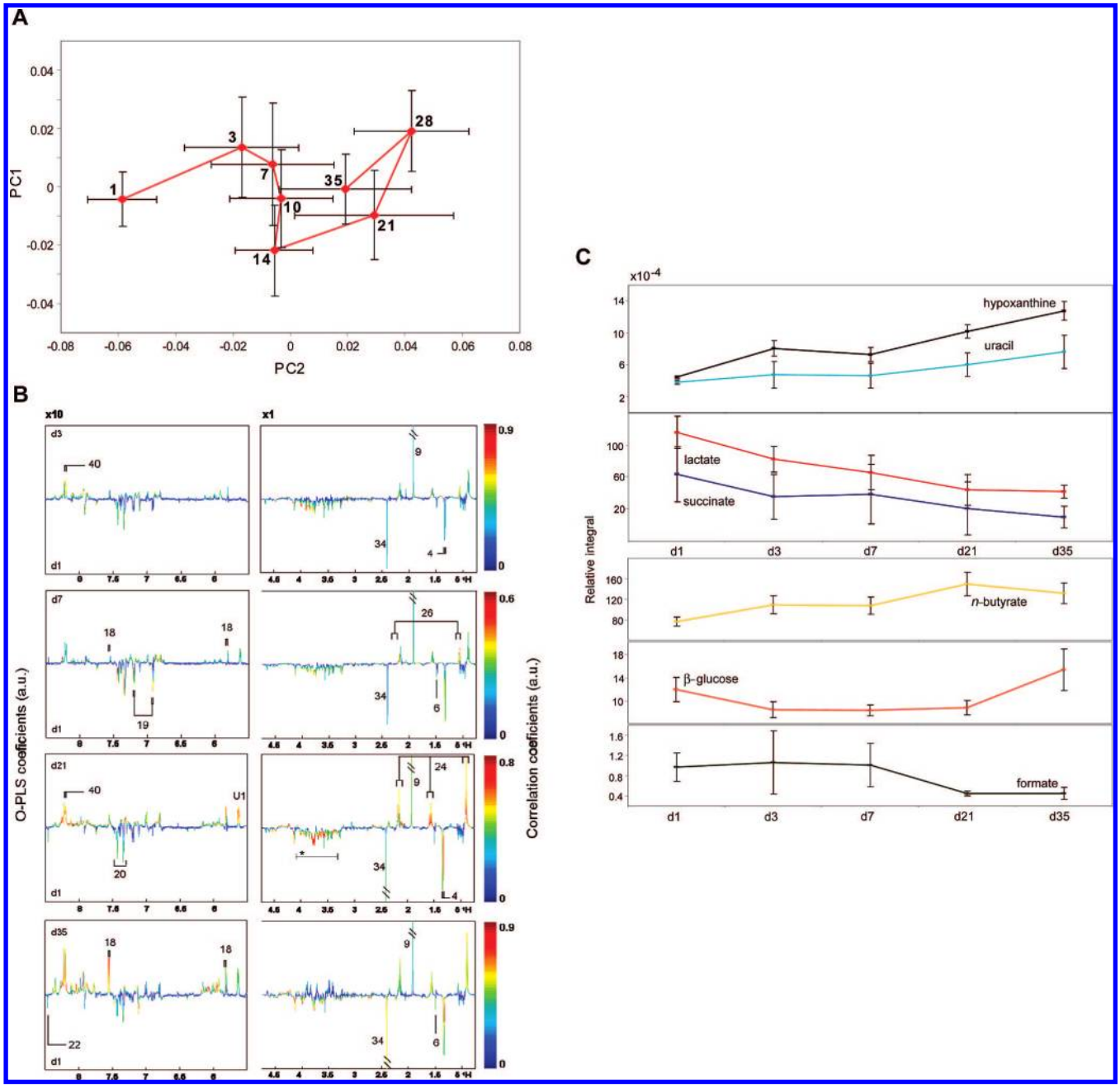

Figure 4. (A) PCA trajectory plot derived from fecal spectra obtained from the mean values for a group of 12 female NMRI mice over a 5-week study period. Mice were 6-week-old at the onset of the experiment. Each point in the plot represents average values of the first and second PC at a certain time point of fecal collection, e.g., at days 1, 3, 7, 10, 14, 21, 28, and 35. The error bars along the first PC and the second PC are expressed by 2 standard deviations of the mean. (B) O-PLS-DA coefficient plots, comparing day 1 of animal handling (class 1) with either of the later time points (class 2). The numbers refer to the metabolites in Table 1; * resonances from sugars and amino acid CH groups. (C) Normalized integrals of 5 selected spectral mean regions with corresponding metabolite identity, proportional to total concentrations of metabolites in each sample, changed significantly during the experiment. The error bars are defined by two standard deviations of the mean. Significant changes are given in the text.

Extracts of fecal water hold promise as a biological medium for diagnosing disease[8–12] and can help to characterize the symbiotic metabolic relationship between the mammalian host and its associated microbiome. However, the utility of a biofluid or tissue extract and the reliability of candidate biomarkers identified in that medium are partially dependent on the normal physiological variation in biochemical composition and on the stability of the metabolic profile. The biochemical composition in aqueous fecal extracts obtained from three different mammalian species, including temporal/age variation, and the effect of sample preparation, have been investigated in the current study with a view to assess the reliability of feces as a medium for identifying biomarkers of disease. Species, age/time, and sample preparation methods were all found to be sources of variation in the metabolic profile of fecal extracts, in decreasing order of magnitude of effect. The sample preparation method used exerted minimal influence on the spectral profile, and the magnitude of variation induced by different methods was negligible in comparison to species differences. It can therefore be assumed that information and

data relating to the identification of candidate biomarkers for pathological conditions or therapeutic interventions will be transferable across studies and laboratories.

Interspecies Differences in the Fecal Metabolite Profile. The fecal metabolite profiles of the three species investigated (humans, rats, and mice) shared some similarities but also contained metabolite patterns that were unique to each species. Metabolites that were conserved across the three species included many of the amino acids and the SCFAs (*n*-butyrate, propionate, and acetate), whereas glycerol and malonate were unique to humans, and β-alanine was specific for rats. Unsurprisingly, the variation in the fecal profiles from human samples was much greater than for either mice or rats, which were largely similar and formed tight clusters in the PCA scores plots (Figure 3A). The greater diversity in the human profiles is also reflective of exposure to greater environmental gut microbiome variation and host genetic variation than laboratory animals kept genetically uniform and under environmentally controlled conditions. The metabolic variation of the human samples is found to be influenced by the gender of the participants but not by age (range: 24–51 years). This result mirrors the difference in the relative degree of variation typically observed in urinary metabolite profiles.[19] Fecal excretion of relatively high concentrations of SCFAs, predominantly acetate, in men has not to our knowledge previously been reported. In fact, contrary to these findings, Wolever and colleagues[40] reported that serum acetate/propionate ratios did not differ between men and women. While some human metabolites like the BCAAs, lysine, asparagine, and the SCFAs (acetate, *n*-butyrate, and propionate) were found in all analyzed human samples in similar relative concentrations, other components such as the bile acids were highly variable in concentration and presence. Interestingly, uracil, glucose, and fumarate appeared to covary in the human samples, but this was not the case in either of the rodent species investigated. The glucose and pyrimidine transporters in the small intestine share a similar distribution and mode of action.[19] In addition to showing the greatest dispersion, human samples were also most divergent from the other groups, with rats and mice being the most similar to each other in profile.

Rat fecal profiles were characterized by relatively higher concentrations of uracil and tyrosine in comparison with the two other species. Particularly, high uracil levels in the rat were associated with the presence of β-alanine which was not observed in either human or mouse fecal samples. Both components are intermediates in the degradation of cytosine (Figure 5).[41] The high levels of β-alanine and cytosine in the rat fecal samples suggest a higher presence of potential inhibitors of the uracil transporters in the jejunum of the rat, which actively transports uracil from mucosa to serum. It has been shown that phenylalanine, which was also increased in the rat fecal sample, acts as a fully competitive inhibitor of the uracil transporter.[42] The degradation of uracil to β-alanine by certain intestinal bacteria may account for the relative higher levels of β-alanine found in rat feces.[43,44]

Influence of Age/Time on the Fecal Metabolite Profile. The O-PLS-DA models, differentiating samples obtained sequentially over a 5-week period on the basis of time, were significant with reasonably strong Q^2 values (0.48–0.88). These results indicate that the fecal profile was not stable over the duration of the study. The PCA trajectory (Figure 4A) also supports this observation, showing an initial deviation from the metabolic starting position in the first week of the study and then a period

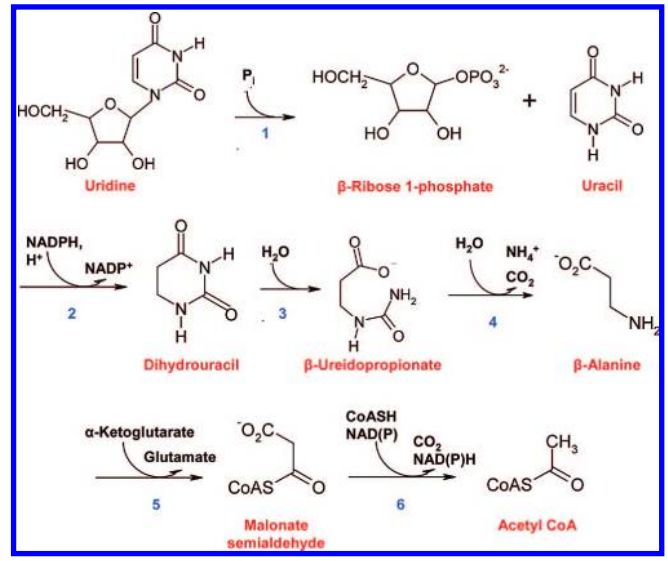

Figure 5. Degradative pathway of uracil. Key for enzymes: 1, uridine phosphorylase; 2, dihydropyrimidine dehydrogenase; 3, dihydropyrimidinase; 4, 3-ureidopropionase; 5, 3-alanine-pyruvate transaminase; 6, malonate semyaldehyde dehydrogenase.

of relative stability followed by another deviation in biochemical composition of fecal content 3 weeks after the initial sampling point. Although the global trend in time-related variation in the fecal metabolome was clear, the interanimal variation was relatively high as demonstrated by the error bars in the PCA scores plot in Figure 4A. The metabolic components predominantly associated with this temporal variation included the SCFAs (acetate, propionate, and *n*-butyrate), which increased with time along with tyrosine and uracil, whereas alanine and succinate decreased over the study duration. Both rats and mice showed similar temporal variation in the fecal metabolite profile. However, the metabolic profile was relatively stable in rats over short periods (up to 24 h), particularly when lyophilized samples were used. Since the relative contributions of the SCFAs are reduced following the lyophilization process, this is consistent with the SFCAs showing higher temporal variation than other fecal components. The mice were approximately 6-week-old at the beginning of the experiment. Female mice typically become sexually mature when aged between 6 and 7 weeks;[45–48] therefore, some of the initial metabolite changes in the first week may be related to the onset of sexual maturity since this is known to have an effect on the composition of the microbiota.[47] However, this is unlikely to account for the metabolic changes evolving after day 14 of the study (corresponding to mice aged approximately 8 weeks). Also, since the nature of changes in specific metabolites over time was largely consistent throughout the total duration of the study, with the exception of lactate, succinate, and threonine, the initial perturbations in metabolite profile changes are more likely to derive from a shift in the gut microbiota presence or activity which is independent of the onset of sexual maturity.[47,48]

As demonstrated in other publications, the variation in the biochemical composition of feces caused by age/time or interindividual differences is small compared to the variation induced by diseases such as Crohn's disease, ulcerative colitis, or bowel cancer.[7–9] The characterization of variation in the 'normal' fecal metabolome presented here, and the indication

of the more stable metabolic components of the fecal profile, should facilitate establishing the degree of confidence with which future disease-related studies can be interpreted in assessing systemic metabolic changes induced by nutritional interventions (e.g., pre- or probiotic administration), as well as lifestyle choices. Moreover, the identification of the more variable components of the metabolic signature may be key in understanding inter-species and idiosyncratic responses to drug toxicity and may also guide choices to be made in personalized healthcare.

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Supporting Information Available: Full experimental details; the effects of freezing, lyophilization, and two different sterilization methods and their associated variability are detailed in the supplementary files. This material is available free of charge via the Internet at http://pubs.acs.org.

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