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Associations between untargeted plasma metabolomic signatures and gut microbiota composition in the *Milieu Intérieur* population of healthy adults

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Abbreviations: Carr-Purcell-Meiboom-Gill, CPMG; False discovery rate, FDR; Mass spectrometry, MS; Milieu Intérieur, MI; Multivariate analyses with linear models, MaAsLin; Nuclear magnetic resonance, NMR; ¹H ¹D NMR pulse sequence Nuclear Overhauser Effect Spectroscopy, NOESY1D; Operational taxonomic units, OTU; Permutational analysis of variance, PERMANOVA; Polymerase chain reaction, PCR; Principal Component Partial R-square, PC-PR2; Standard deviation, SD; Total correlated spectroscopy, TOCSY; Trimethylamine, TMA; Trimethylamine N-oxide, TMAO.

ABSTRACT

Host-microbial co-metabolism products are being increasingly recognized to play important roles in physiological processes. However, studies undertaking a comprehensive approach to consider host-microbial metabolic relationships remain scarce. Metabolomic analysis yielding detailed information regarding metabolites found in a given biological compartment holds promise for such an approach. This work aimed to explore the associations between host plasma metabolomic signatures and gut microbiota composition in healthy adults of the *Milieu Intérieur* study. For 846 subjects, gut microbiota composition was profiled through sequencing of the 16S rRNA gene in stools. Metabolomic signatures were generated through proton nuclear magnetic resonance analysis of plasma. The associations between metabolomic variables and α - and β -diversity indexes and relative taxa abundances were tested using multi-adjusted partial Spearman correlations, PERMANOVAs, and MaAsLins, respectively. A Multiple testing correction was applied (Benjamini-Hochberg, 10%-FDR). Microbial richness was negatively associated with lipid-related signals and positively associated with amino acids, choline, creatinine, glucose, and citrate ($-0.133 \leq \text{Spearman's } \rho \leq 0.126$). Specific associations between metabolomic signals and abundances of taxa were detected (25 at the genus level and 19 at the species level): notably, numerous associations were observed for creatinine (positively associated with 11 species, and negatively associated with *Faecalibacterium prausnitzii*). This large-scale population-based study highlights metabolites associated with gut microbial features and provides new insights into the understanding of complex host-gut microbiota metabolic relationships. In particular, our results support the implication of a “gut-kidney axis”. More studies providing a detailed exploration of these complex interactions, and their implications for host health are needed.

INTRODUCTION

The gut metagenome has been estimated to be 150 to 400 times larger than the human genome ^(1,2), yielding a colossal metabolic potential ⁽³⁾. Products of the microbial metabolism comprise a vast range of molecules involved in a variety of biological functions ^(4,5) and integrated in proposed “host-microbial metabolic axes”. These molecules complement the host metabolism and subsequently influence the host health through potential beneficial or harmful effects ^(5–9). Consequently, the gut microbiota is now fully considered as an endocrine pseudo-organ within the mammalian holobiont superorganism ^(6,10).

Specific studies investigating targeted molecules – such as short-chain fatty acids, bile acids, choline metabolites, etc. – have considerably increased the mechanistic knowledge of microbial-human co-metabolism ^(4,11–13). However, studies addressing more comprehensively the highly complex microbial-host metabolic interactions remain scarce ⁽⁹⁾. Metabolomics – the systematic identification and quantification of the low-molecular weight metabolic products of a biological system at a specific point in time – enables the simultaneous detection and relative quantification of hundreds of molecules, and thus holds promise to elucidate microbial-host interactions ⁽¹⁴⁾. Studies integrating a metabolomic approach have started to expand. Notably, gut microbiota has been reported to associate with the serum metabolome. Pedersen et al. indeed showed that the serum metabolome of insulin-resistant individuals presents increased levels of branched-chain amino acids (BCAAs), which were further correlated with gut microbial genetic characteristics (namely the potential for biosynthesis of BCAAs and lack of genes encoding bacterial inward transporters BCAAs) ⁽¹⁵⁾. Additionally, comparisons of the host metabolomes (in plasma or urine) between diseased individuals ^(16–18) or subjects included in nutritional interventions ^(19–22) and controls have detected molecules stemming exclusively from microbial activity (e.g. hippurate or p-cresol) as discriminant metabolites. However, in these studies, neither the gut microbiota composition nor its activity were analyzed, and direct associations between the gut microbiota and the host metabolome were not investigated. In fact, research investigating such associations is scarce and often have been

conducted on limited sample sizes ($N \leq 100$)^(9,23–27), or focused on subjects with specific characteristics or conditions like chronic heart failure⁽²³⁾, spleen-yang-deficiency syndrome⁽²⁶⁾, obesity^(24,27), physiological and metabolic stress⁽²⁵⁾, etc. More recently, studies were performed on larger population-based samples, including several hundreds of participants and reporting associations between the composition and diversity of the gut microbiota and circulating metabolites, using metabolomics^(28–32). Following these approaches, our objective was to investigate the associations between untargeted plasma metabolomic signatures and gut microbiota composition in a large sample of healthy French subjects.

METHODS

Study population

This study was conducted in the framework of the *Milieu Intérieur* project, which enrolled 1,000 healthy French adults with primary aim to assess the determinants of immunologic variance within the general healthy population. The objectives of the present study are secondary objectives to the *Milieu Intérieur* project, for which the rationale, design, and methods have been extensively described elsewhere⁽³³⁾. Briefly, 500 females and 500 males equally distributed across 5 classes of age (from 20 to 69 years old), and of mainland French descent for at least 3 generations, were recruited in the suburban area of Rennes (Ille-et-Vilaine, Bretagne, France), between September 2012 and August 2013. Participants were considered “healthy” upon recruitment, as defined per stringent exclusion criteria comprising any chronic disease or condition involving the immune system, abnormal physical examination, or any abnormal clinical-biological analysis⁽³³⁾. Questionnaires and biological samples were administered and collected by trained medical investigators supported by a full clinical team. The study is sponsored by Institut Pasteur (Pasteur ID-RCB Number: 2012-A00238-35). It was conducted as a single center study and without any investigational product, and was approved by Comité de Protection des Personnes – Ouest 6 on 06/13/2012 (CPP Ouest 6-728/MS2) and by Agence Nationale de Sécurité du Médicament on 06/22/2012 (ID-RCB Number: 2012-A00238-35, ref. ANSM: B120239-70). The protocol, which is

registered under ClinicalTrials.gov (NCT01699893), was designed and conducted in accordance with the Declaration of Helsinki and good clinical practice as outlined in the International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use Guidelines for Good Clinical Practice.

Data collection

Covariates

During the medical visit, investigators measured body-mass index (BMI) using standardized methods, and collected information related to age, sex, smoking status, educational level, employment status, occupational category, income level, and physical activity.

Gut microbiota profiling from stool samples

Gut microbiota composition was determined through the sequencing of the 16S ribosomal RNA gene in stool samples produced at home maximum 24 hours before the scheduled medical visit, collected in a double-lined sealable bag maintaining strict anaerobic conditions, and aliquoted and stored at -80°C upon reception at the clinical site. Detailed information regarding DNA preparation, barcoding Polymerase Chain Reaction (PCR) protocol, sequencing, and computation of microbial diversity indexes are provided in **Supplementary Material and Methods 1**.

Untargeted NMR metabolomics analysis in plasma samples

Fasting whole blood was collected in heparin tubes and centrifuged (300g, 10min, room temperature) maximum 6h after blood draw, and supernatants were stored at -80°C maximum 10min after centrifugation. Plasma samples underwent 2 freeze-thaw cycles before proton nuclear magnetic resonance (NMR) analysis, the additional freeze-thaw cycle being done for all samples in order to prepare internal quality control samples necessary for the study ^(34,35) and so that that all samples would go through the same process. The NMR analysis was performed blindly on samples (randomized for age and sex) according to a previously published protocol ⁽³⁶⁾. Briefly, two complementary one-dimensional NMR sequences were acquired per plasma sample, on a 500MHz-Bruker Avance III spectrometer (Bruker, Billerica, MA, USA) at 300K – namely the ¹H 1D NMR

pulse sequence Nuclear Overhauser Effect Spectroscopy (NOESY1D) with z-gradient (Bruker pulse program noesygppr1d) and the Carr-Purcell-Meiboom-Gill (CPMG) sequences. Consequently, to acquisition, all spectra were pre-processed, calibrated, and ultimately “sliced” into “buckets” with pre-defined limits so as to maximize recovery of peak entities. The buckets were then scaled to the total summed integrals for each spectrum, and the integrals of peak entities were calculated to obtain continuous NMR variables that were used in further statistical analyses. Finally, NMR signals were assigned using ad hoc literature ⁽³⁷⁾. CHENOMX software (CHENOMX, Edmonton, Alberta, Canada) and Human Metabolome Database ⁽³⁸⁾ were used to confirm the assignments using the medium spectrum (**Supplementary Figure 3**) ⁽³⁷⁾. After the statistical analyses, the assignments of the buckets that associated with the gut microbiota were newly carefully looked and confirmed to be unambiguously assigned by gathering information from S-TOCSY, additional two-dimension NMR experiments (recorded on random samples) such as 1H–1H Total Correlated Spectroscopy (TOCSY) and J-resolved experiments. The detailed NMR metabolomic analysis protocol (sample preparation, post-acquisition spectra pre-processing, and intelligent bucketing of spectra and obtaining of NMR variables) is provided in **Supplementary Material and Methods 2**.

Statistical analyses

Associations between each NMR variable and each α -diversity index were tested using non-parametric partial Spearman correlations. Associations between each NMR variable and inter-individual dissimilarities in gut microbiota composition (Bray-Curtis index of β -diversity) were tested using permutational analysis of variance (PERMANOVA) with 999 permutations. Associations between each NMR variable and the relative abundances of genera and species were tested using multivariate associations with linear models (MaAsLin). This statistical framework performs boosted, additive general linear models and is suited to test the associations between the relative abundance of microbial community members and metadata (here the NMR variables and covariates). Relative abundances are first transformed through a variance-stabilizing arcsin-square

root transformation. Genera and species were included only if their relative abundance and prevalence were higher than 0.01% and 1%, respectively.

Partial Spearman correlations, PERMANOVAs and MaAsLins were all multi-adjusted for age (20 to <30, 30 to <40, 40 to <50, 50 to <60, and 60 to <70 years old), sex, BMI (continuous variable, in kg.m^{-2}), smoking status (non-smoker, ex-smoker, current smoker), and physical activity (continuous variable, in h.wk^{-1}). These covariates were indeed reported in the literature to have a potential impact on gut microbiota composition or metabolomic signature, or significantly associated with microbial or metabolomic features in our analyses. These associations between covariates and gut microbial and metabolomic features were tested using PERMANOVAs and Principal Component Partial R-square (PC-PR2) analysis, respectively, enabling to measure the percentage of variation in microbial composition or metabolomic data explained by the factors tested. Additionally, α - and β -diversity models (partial Spearman correlations and PERMANOVAs) were adjusted for sequencing depth. P-values were adjusted for multiple testing using a Benjamini-Hochberg correction with a 10%-False Discovery Rate (FDR).

To better visualize the interindividual variation in metabolomics signatures in our study sample, Principal Component Analysis (PCA) of the metabolomic datasets were plotted and color-coded based on age, sex, and BMI.

Spearman correlations were performed using SAS 9.4; PERMANOVAs, MaAsLins, PC-PR2, and PCA representations of metabolomic datasets were performed using R 3.3.2 (packages *vegan*, *Maaslin*, *FactoMineR*, and *FactoExtra*).

RESULTS

Characteristics of the study sample

Among 1,000 participants to the *Milieu Intérieur* study, 138 were excluded from the study sample because of missing gut microbiota composition data – due to failure during PCR, insufficient number of detected reads, or insufficient quantity of stool aliquot or DNA extracted. Among the 862 participants with available gut microbiota data, 16 were further excluded because their

metabolomic signatures could not be determined – due to insufficient quantity of plasma aliquot, or failure during NMR spectra acquisition. Participant flow-chart is presented in **Supplementary Figure 1**. Characteristics of the 846 subjects are presented in **Table 1**. Mean \pm standard deviation (SD) BMI was $24.3 \pm 3.3 \text{ kg.m}^{-2}$ and mean \pm SD physical activity (including both professional and leisure physical activity) was $5.4 \pm 6.3 \text{ h.wk}^{-1}$. The proportion of smokers was 19.9%.

Characteristics of the gut microbiota in the *Milieu Intérieur* study have been previously described^(39,40). Microbiota analysis yielded sequencing depth ranging from 5,064 to 240,472 reads per sample (mean sequencing depth \pm SD: $21,427 \pm 19,180$ reads). Detected reads clustered into 8,422 OTUs classified into 11 phyla, 328 genera, and 698 species. Firmicutes (mean relative abundance \pm SD: 0.68 ± 0.13), Bacteroidetes (0.29 ± 0.13), Proteobacteria (0.02 ± 0.03), Actinobacteria (0.007 ± 0.01), and Verrucomicrobia (0.004 ± 0.008) were the 5 most abundant phyla. **Supplementary Table 1** presents the 22 genera constituting the “core microbiota” – defined as the genera shared by at least 95% of samples⁽⁴¹⁾ – in our study population. A complete description of genera and species composing the gut microbiota of our sample is available in **Supplementary Table 2**. Regarding α -diversity indexes, Simpson’s index ranged from 0.41 to 0.98 (mean \pm SD: 0.92 ± 0.05); observed richness from 56 to 346 (mean \pm SD: 193.4 ± 55.1) and Chao1 richness estimate from 62 to 1039 (mean \pm SD: 298.3 ± 117.0). Univariate associations measuring the percentage of variation in inter-individual dissimilarities in the gut microbial composition (Bray-Curtis index of β -diversity) that is explained by different covariates (age, sex, BMI, smoking status, physical activity, and sequencing depth) are shown in **Supplementary Table 3**.

NMR analysis on plasma samples generated 260 CPMG and 269 NOESY1D variables. Spectral regions most likely corresponding to noise (i.e. CPMG regions 0-0.5ppm, 4.3-5.0ppm, and 8.55-10.0ppm; and NOESY1D regions 4.3-5.0ppm, and 9.5-11.0ppm) were excluded from further analysis. A list of the resulting 424 NMR continuous variables used in our analyses (202 CPMG signals and 215 NOESY1D: spectral regions with their metabolite assignments, as well as mean and SD) is presented in **Supplementary Tables 4 and 5**. Results of the PCA performed on the CPMG

and NOESY1D metabolomic datasets are shown in **Figure 1** and **Supplementary Figure 2**, respectively. Although no clear clustering appears, grouping patterns along gradient of sex, age, and BMI can be observed. Results of the PC-PR2 analysis measuring the percentage of variation in the metabolomic dataset that is explained by the covariates (age, sex, BMI, smoking status, and physical activity) are shown in **Supplementary Table 6**.

Associations between plasma metabolomic signals and α - and β -diversity indexes

The associations between metabolomic variables and α -diversity indexes are presented in **Figure 2**. Overall, and after correcting for multiple testing with a 10%-FDR, observed richness was associated with 24 CPMG and 8 NOESY1D signals. Negative associations were observed with lipid-related signals (namely lipoproteins or unsaturated lipids) as well as with ester- and ketone-related metabolites (partial Spearman's ρ range: -0.133 to -0.097), while positive associations were detected with signals pertaining to amino acids (polar glutamine and histidine and aromatic tyrosine, as well as related metabolites) and proteins, creatinine, as well as with choline, and glycolysis-related metabolites (glucose and citrate) (partial Spearman's ρ range: 0.087 to 0.126). Similar results were obtained with Chao1 richness estimate but no association was detected with Simpson's α -diversity index. Likewise, no association was observed between NMR metabolomic variables and inter-individual dissimilarities in gut microbiota composition (Bray-Curtis β -diversity index).

Associations between plasma metabolomic signals and bacterial taxa (relative abundances)

The associations (significant at 10%-FDR) between NMR metabolomic variable and the relative abundance of gut microbiota genera and species are presented in **Table 2**. At the genus and species levels, 25 and 19 associations were detected, respectively. Associated taxa comprised genera and species with prevalence ranging from 20% to 100% of the study population. Creatinine was the metabolite associated with most taxa (16 genera and 11 species). Notably, consistent positive associations were found between the CPMG and the NOESY1D analyses for genera *Catabacter*,

Saccharofermentans, and *Oscillibacter* and a negative association was observed for *Faecalibacterium prausnitzii*.

DISCUSSION

In the present work, we investigated the associations between characteristics of the gut microbiota – namely α -diversity, inter-individual dissimilarities, and the relative abundances of genera and species – and plasma NMR metabolomic signatures of the host in a large population-based sample of 846 healthy French adults. Overall, our results suggest that gut bacterial features are associated with the systemic metabolism of the host. In particular, we found that bacterial richness was positively associated with signals pertaining to amino acids (glutamine, histidine, tyrosine), as well as with creatinine, choline and glycolysis-related metabolites, while negative associations were observed with lipid-, as well as ester-, and ketone-related signals. Finally, specific associations between metabolomic signals and bacterial members of the gut microbiota were detected.

The metabolic relevance and importance of the gut microbiota for the host are now widely admitted, and research scrutinizing host-gut microbial metabolic relationships is an active area of investigation ⁽⁴²⁾. However, studies investigating direct associations between gut microbiota composition and host blood metabolome in large population-based samples are scarce while more available studies have been conducted on a limited number of participants or focused on specific populations ^(15,23,26,27). In particular, a study by Org et al. assessed the relationships between gut microbiota composition and circulating metabolites in 531 healthy Finnish males from the METSIM cohort ⁽²⁸⁾. Consistently with their study, we reported positive correlations between bacterial richness and glutamine, histidine, and creatinine – with similar Spearman correlation coefficients. Furthermore, a study on 399 subscribers to a US Scientific Wellness program was able to explain 45% of the gut microbiota alpha-diversity with 40 plasma metabolites, among which a high frequency of phenylalanine and tyrosine metabolites (including p-cresol sulfate, a potentially uremic toxin) and hippurate ⁽³²⁾ while a study in 1529 females from the TwinsUK cohort found five metabolites that consistently correlated with gut microbiota alpha-diversity (Shannon index)

including hippurate, p-cresol sulfate, phenylacetylglutamine, 3-phenylpropionate, and hyodeoxycholate⁽³¹⁾.

In a study conducted on 893 participants from the Dutch LifeLines-DEEP cohort, Fu et al.⁽¹³⁾ measured specific lipid levels through targeted colorimetric methods, and found that triglycerides and high-density lipoproteins were respectively negatively and positively associated with both bacterial richness and diversity. Consistently, a more recent study using NMR metabolomics in 2309 individuals from the Rotterdam Study and the LifeLines-DEEP cohort observed inverse associations between the diversity of the gut microbiota and serum levels of VLDL particles, triglycerides, total fatty acids, monounsaturated fatty acids and saturated fatty acids but also glycoprotein acetyl, alanine, isoleucine, and lactate and positive associations with HDL particles. In addition, Le Chatelier et al. associated a decreased gut microbial richness with metabolic impairments such as increased fat mass and body weight, inflammation of the adipose tissue and dyslipidemia⁽⁴³⁾. In the present work, we consistently found that lipid-related metabolomic signals were negatively associated with bacterial richness. However, we were unable to further characterize these lipid-related signals and discriminate between the different lipid classes potentially hampering the detection of specific associations previously reported^(13,28). Indeed, long-chain metabolites such as fatty acids result in an overlap of signals of protons in NMR, limiting a more specific assignment⁽⁴⁴⁾. Still, it should be noted that, in our sample, NMR signals assigned to lipids demonstrated excellent correlation with blood triglycerides (Spearman's $\rho > 0.65$, p-value < 0.0001 for spectral regions 1.207-1.311ppm, 2.151-2.166ppm, and 2.166-2.167ppm), adding support to the consistency of our results with those previous findings. Furthermore, high-fat Western diet has consistently been associated with gut microbiota impairments^(45,46), and lipid-related signals in our study correlated positively with dietary intakes of processed meat (Spearman's $\rho=0.09$, p-value=0.006 for spectral regions 2.166-2.167ppm and $\rho=0.08$, p-value=0.02 for 1.207-1.311ppm and 2.151-2.166ppm) and fried products ($\rho=0.07$, p-value=0.04 for 2.166-2.167ppm), and negatively correlated with fruit ($\rho=-0.09$, p-value=0.01 for 1.207-1.311ppm and 2.166-2.167ppm and $\rho=-0.08$, p-value=0.02 for 2.151-

2.166ppm) and vegetables ($\rho = -0.09$, $p\text{-value} = 0.007$ for 2.166-2.167ppm and $\rho = -0.07$, $p\text{-value} = 0.04$ for 2.151-2.166ppm). Nonetheless, these assumptions should be considered with caution as additional exploration and targeted identification of these lipid signals using mass spectrometry (MS) or lipidomics is necessary to get more detailed insights into the associations we observed.

Our finding of a positive association between glutamine and gut bacterial richness echoes a result from De Souza et al. who suggested that glutamine might have an anti-inflammatory effect through a modulation of specific gut bacterial members following the observation that an oral supplementation with L-glutamine decreased the Firmicutes/Bacteroidetes ratio in obese and overweight adults ⁽⁴⁷⁾.

In our study, creatinine was the metabolomic signal most associated with the relative abundances of bacterial taxa. This by-product of muscle metabolism is exclusively excreted through the kidneys and a high creatinine level in blood is notably used as an indicator of kidney dysfunction ⁽⁴⁸⁾. Our finding of positive associations between plasma creatinine and the relative abundance of various representatives of the Clostridiales order and the Ruminococcaceae family matches results from Org et al. ⁽²⁸⁾. We also reported a negative association between creatinine and *Faecalibacterium prausnitzii*, a species drawing a particular attention from the research community as it has been shown to exert anti-inflammatory properties through its ability to produce anti-inflammatory metabolites (e.g. butyrate), or to inhibit of the production of pro-inflammatory cytokines ⁽⁴⁹⁾. Various studies have reported that *F. prausnitzii* is reduced in conditions such as ulcerative colitis, inflammatory bowel disease, colorectal cancer, diabetes, psoriasis, etc. ^(49–51). Consequently, this species has been proposed as a potential biomarker of gut health ⁽⁵²⁾ and is even considered as an interesting probiotic candidate ⁽⁵³⁾. In particular, a decrease in *F. prausnitzii* was previously reported in chronic kidney disease patients compared to healthy controls ^(54,55). This echoes our result of a negative association between *F. prausnitzii* and creatinine obtained in a healthy sample without renal dysfunction. Overall, the numerous associations we detected between bacterial taxa and creatinine are in support of a gut-kidney conversation ^(56,57). In this previously described bi-directional organ

axis, gut contributes to inflammation and renal injury through microbiota dysbiosis and dysregulations in microbial metabolite production. Conversely, kidney perturbations lead to dysbiosis and disruptions in tight junction function therefore promoting inflammatory conditions in the gut. Notably, Jiang et al. reported that indicators of the renal function such as serum creatinine, estimated glomerular filtration rate, and cystatin C were the most important environmental parameters to influence the overall microbial communities in end-stage renal disease patients ⁽⁵⁵⁾. In our study a link between the gut and the kidneys seemed to be already observed even in a healthy setting where individuals were free of kidney or gut impairments. However, further exploration of renal function indicators (e.g. estimated glomerular filtration rate, cystatin C, and urine creatinine) would be of utmost importance to confirm such a link. If we hypothesize a gut-kidney axis to interpret our results, alternate interpretations could also be proposed. Indeed, negative associations have also been reported between trimethylamine N-oxide (TMAO) and *F. prausnitzii* ^(28,58) and creatinine is a precursor for the gut bacterial synthesis of trimethylamine (TMA), which is further oxidized into TMAO in the liver ⁽⁴⁸⁾. Therefore, we cannot rule out that the association we observed between creatinine and *F. prausnitzii* may actually reflect the overall TMAO metabolic pathway instead of being a true association. However, because TMAO could not be detected by NMR in our study, it was not possible to verify this assumption.

An important strength of this work pertains to its design – namely the acquisition of both detailed metabolomics data and gut microbiome data in a large population-based sample of healthy adults. While individual or specific approaches consider biomolecules separately, metabolomics allows for the simultaneous detection and relative quantification of hundreds of molecules hence the monitoring of subtle system-wide metabolic interactions ⁽⁵⁹⁾. In this study we used NMR-metabolomics with standardized and optimized protocols yielding high-quality data ⁽⁶⁰⁾. This robust and stable technology has demonstrated its suitability for long-term epidemiological studies ⁽⁶¹⁾, notably by allowing to analyze large samples quickly with a high reproducibility ⁽⁶²⁾. From a statistical standpoint, the analyses implemented here allowed to explore detailed associations

between gut bacterial and metabolomic datasets, beyond the sole assessment of global connections obtained through multivariate correlation-based approaches (e.g. co-inertia or Procrustes analyses). In addition, we were able to take into account a range of factors reported to influence the gut microbiota composition or the systemic metabolism of the host in our models, thus mitigating potential confounding. Nonetheless, residual confounding cannot be ruled out. For instance, intestinal transit time was shown to be a major confounding factor of gut microbiota composition⁽⁶³⁾, and we were not able to collect this information. Another important strength and originality of this work is that it was based on a large sample from the general population including only healthy men and women selected using strict criteria. Hence, our results were not confounded by underlying disease and reflected normal range. Yet, this may also have induced less variations in plasma metabolites levels or in gut microbiota composition and therefore resulted in associations of smaller magnitude. Some limitations should also be acknowledged for this study. First, the cross-sectional design of our study, in which blood and stool samples were taken at the same time, prevents from drawing any conclusion regarding the sequential causality of events between host metabolism (e.g., creatinine levels) and gut microbiota composition (e.g., *F. prausnitzii*). Further, plasma metabolomic signatures only give a metabolic “snapshot” that results from both the endogenous and the gut microbial metabolisms, which makes it is not possible to discriminate signals from human or microbial origin. In future studies, subsequent time points with both metabolomic and microbiota data collected would be of utmost interest to investigate the stability over time and the chronology of the associations observed in the present cross-sectional work. In addition, the fact that most detected metabolites result from multiple metabolic pathways that may be related to gut microbiota or not may also explain the modest correlation coefficients observed between plasma metabolites and gut microbial richness. It should also be acknowledged that, although highly informative, the use of 16S rRNA gene sequencing and NMR metabolomics presents technical limitations. Shotgun metagenomics for instance would give a more precise characterization of microbiota features and also bring additional information regarding the functional metabolic capacity of the gut microbiota.

This would allow to go beyond the compositional approach, limited by the redundancy of gene pool and metabolic functionalities between distinct gut microbial members ^(64,65), but still relevant according to recent studies arguing that some metabolic pathways pertain to a handful of species ⁽⁶⁶⁾. Likewise, while NMR detects only the most abundant metabolites (μM to mM range), mass spectrometry (MS) has lower detection limits and higher sensitivity for signal assignment, and would therefore allow the detection of metabolites undetectable with NMR ⁽⁶⁷⁾. This technical limitation of NMR may explain why several low-concentration metabolites deriving specifically from gut microbiota were not detected in our study. As already mentioned, the precision of NMR bucket assignment was also limited for some metabolites, especially for lipids and proteins. For instance, the unsaturation corresponds to a chemical function in the aliphatic chain of various lipids, and it is impossible to differentiate among the different lipid types within the plasma sample. Hence, this limits the comparability of our results to prior studies. In addition, plasma samples underwent two freeze-thaw cycles prior to NMR analyses (necessary to prepare internal quality controls) so that the possibility of increased metabolites cleavage cannot be ruled out. Still, this would similarly affect all samples as they all went through the same process.

Although the *Milieu Intérieur* population is somewhat representative of the French Ile-et-Vilaine region ⁽³³⁾, our study sample is not representative of the whole French adult population – caution is therefore needed in the generalization of our results. However, it is important to acknowledge that from a gut microbiota composition standpoint, our study sample was consistent with other studies performed in Western settings ^(41,68), with the same 5 most abundant phyla and shared “core genera” (**Supplementary Table 1**). Finally, the results highlighted here were mostly exploratory as they were obtained from a single population. These results will need to be replicated and confirmed in the future, using independent samples and/or analytical techniques allowing a better refinement of the detected metabolites (e.g., MS, lipidomics). In addition, the hypotheses we proposed to interpret our results were based on existing literature and would need to be developed and confirmed in experimental settings *in vitro* and/or *in vivo*. In particular, detailed mechanistic studies

comprehensively elucidating the metabolic capability of gut microorganisms are needed to enlighten the associations we detected.

Overall, the present study provides interesting insights regarding the associations between gut microbiota composition and plasma metabolic signatures of the host. Our results contribute to a better understanding of the host-gut microbiota relationships, and notably highlight a possible gut-kidney axis in healthy subjects. Because the associations we found in healthy individuals were already reported in chronic kidney disease patients, this builds up to the concept that onset of chronic diseases is a lengthy process that could be detected at early stages. This appears all the more interesting that microbial-host co-metabolites were shown to be excellent prodromal markers of future divergence in metabolic and behavioral outcomes in mice ⁽⁶⁹⁾. However, our results remain to be confirmed in independent populations, underlying mechanisms to be elucidated, and causal implications for the host health to be established.

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Authors' contributions: VP, MT: designed the research; LQM, MLA, DD, OL, MT, MI consortium: conducted the research; SM: performed microbiota sequencing; VP: performed metabolomic signature acquisition; NB, PS, MNT: supervised metabolomic signatures acquisition; VP: performed statistical analysis; MT: supervised statistical analysis; VP: wrote the original draft; MDT: revised the manuscript; VP, MDT, SM, AVB, ND, LL, CBD, MJT, EKG, BC, EP, KEA, PLM, CJ, PG, SH, LQM, MLA, DD, OL, PS, MNT, MT, MI Consortium: contributed to the data interpretation and revised each draft for important intellectual content; MT had primary responsibility for final content; and LQM, MLA, DD are coordinators of the MI Consortium. All authors read and approved the final manuscript.

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Table 1. Characteristics of the study population, *Milieu Intérieur* study, France, 2012 (N=846).

Sex		
Male		433 (51.2)
Female		413 (48.8)
Age		
20 – 29 years old		159 (18.8)
30 – 39 years old		165 (19.5)
40 – 49 years old		174 (20.6)
50 – 59 years old		171 (20.2)
60 – 69 years old		177 (20.9)
Smoking status		
Non-smoker		442 (52.2)
Ex-smoker		236 (27.9)
Current smoker		168 (19.9)
BMI, kg.m⁻²		24.3 ± 3.3
Physical activity, h.wk⁻¹		5.4 ± 6.3
Simpson's index		0.92 ± 0.05
Observed richness		193.4 ± 55.1
Chao1 richness estimate		298.3 ± 117.0

Values are N (%) or mean ± SD

Table 2. Associations between NMR variables and relative abundances of taxa with a Q-value ≤ 0.1 after multiple-testing correction, using MaAsLins, *Milieu Intérieur* study, France, 2012 (N=846).

	Shift (ppm)	Signal assignment	Phylum Class Order Family	Genus	Species	MaAsLin coefficient	Coverage ¹	P-value ²	Q-value ³
CPMG	1.021-1.051	Unassigned signal	Firmicutes Negativicutes Selenomonadales Veillonellaceae	<i>Sporomusa</i> *		-68.5	35.22	<0.0001	0.07
	1.527-1.631	CH ₂ CH ₂ COOC	Firmicutes Clostridia Clostridiales Lachnospiraceae Clostridium XIVa	<i>Clostridium</i>	<i>C. bolteae</i>	74.2	99.17	<0.0001	0.06
	2.831-2.848	Citrate/=CH-CH ₂ -CH=	Firmicutes Bacilli Bacillales Bacillaceae 1	<i>Caldibacillus</i>		-198.2	35.34	<0.0001	0.07
	2.967-3.071	Creatine/albumin lysyl	Firmicutes Clostridia Clostridiales Ruminococcaceae	<i>Hydrogenoanaerobacterium</i>	<i>H. saccharovorans</i>	-31.8	90.07	<0.0001	0.07
			Firmicutes Clostridia Clostridiales Ruminococcaceae	<i>Faecalibacterium</i>		-777.4	99.41	<0.0001	0.02
			Firmicutes Clostridia Clostridiales Ruminococcaceae	<i>Faecalibacterium</i>	<i>F. prausnitzii</i>	-797.3	99.41	<0.0001	0.03
			Firmicutes Negativicutes Selenomonadales Veillonellaceae	<i>Sporomusa</i> *		31.8	35.22	<0.0001	0.06
			Firmicutes Negativicutes Selenomonadales Veillonellaceae	<i>Sporomusa</i>	<i>S. termitida</i>	31.5	34.75	<0.0001	0.06
			Tenericutes Mollicutes Acholeplasmatales Acholeplasmataceae	<i>Acholeplasma</i>		42.0	32.98	0.0001	0.09
			Firmicutes Clostridia Clostridiales Clostridiaceae 1	<i>Caloramator</i>		63.9	58.63	<0.0001	0.06
			Firmicutes Clostridia Clostridiales Catabacteriaceae	<i>Catabacter</i> §		67.0	68.56	<0.0001	0.001
			Firmicutes Clostridia Clostridiales Catabacteriaceae	<i>Catabacter</i>	<i>C. hongkongensis</i>	64.0	68.56	<0.0001	0.002
			Firmicutes Clostridia Clostridiales Defluviitaleaceae	<i>Defluviitalea</i>		70.2	81.32	<0.0001	0.07
			Firmicutes Clostridia Clostridiales Defluviitaleaceae	<i>Defluviitalea</i>	<i>D. saccharophila</i>	71.1	81.32	<0.0001	0.07
			Firmicutes Clostridia Clostridiales Peptococcaceae 2	<i>Pelotomaculum</i>		74.5	52.36	<0.0001	0.07
	3.071-3.111	Creatinine	Firmicutes Clostridia Clostridiales Peptococcaceae 2	<i>Pelotomaculum</i>	<i>P. propionicicum</i>	82.6	51.30	<0.0001	0.01
			Firmicutes Clostridia Clostridiales Christensenellaceae	<i>Christensenella</i>		138.5	93.14	<0.0001	0.06
			Firmicutes Clostridia Clostridiales Christensenellaceae	<i>Christensenella</i>	<i>C. minuta</i>	170.2	93.14	<0.0001	0.01
			Firmicutes Clostridia Clostridiales Ruminococcaceae	<i>Saccharofermentans</i> *§		187.6	65.13	<0.0001	0.005
			Firmicutes Clostridia Clostridiales Ruminococcaceae	<i>Saccharofermentans</i>	<i>S. acetigenes</i> §	189.3	65.13	<0.0001	0.01
			Firmicutes Clostridia Clostridiales Ruminococcaceae	<i>Sporobacter</i>		305.1	93.50	<0.0001	0.02
			Firmicutes Clostridia Clostridiales Ruminococcaceae	<i>Sporobacter</i>	<i>S. termitidis</i>	273.1	93.50	<0.0001	0.07
			Firmicutes Clostridia Clostridiales Ruminococcaceae	<i>Intestinimonas</i>		320.8	98.70	<0.0001	0.003
			Firmicutes Clostridia Clostridiales Ruminococcaceae	<i>Intestinimonas</i>	<i>I. butyriciproducens</i>	352.4	98.70	<0.0001	0.002
			Firmicutes Clostridia Clostridiales Ruminococcaceae	<i>Oscillibacter</i> §		396.2	99.76	<0.0001	0.03
			Firmicutes Clostridia Clostridiales Ruminococcaceae	<i>Oscillibacter</i>	<i>O. valericigenes</i>	460.2	95.98	<0.0001	0.03
			Firmicutes Clostridia Clostridiales Eubacteriaceae	<i>Eubacterium</i>		447.6	99.88	<0.0001	0.06
			Firmicutes Clostridia Clostridiales Eubacteriaceae	<i>Eubacterium</i>	<i>E. coprostanoligenes</i>	580.5	94.44	<0.0001	0.01
	3.111-3.129	Unassigned signal	Firmicutes Clostridia Clostridiales Lachnospiraceae Clostridium XIVa	<i>Clostridium</i>	<i>C. viride</i>	-133.2	90.31	<0.0001	0.03
	3.367-3.437	Unassigned signal	Firmicutes Clostridia Clostridiales Eubacteriaceae	<i>Eubacterium</i>	<i>E. rectale</i> *	266.4	98.94	<0.0001	0.1
	3.437-3.496	Unassigned signal	Firmicutes Clostridia Clostridiales Eubacteriaceae	<i>Eubacterium</i>	<i>E. rectale</i> *	-470.9	98.94	<0.0001	0.03
	3.496-3.587	Glucose	Firmicutes Clostridia Clostridiales Eubacteriaceae	<i>Alkalibaculum</i>		-20.1	74.35	<0.0001	0.07
			Firmicutes Clostridia Clostridiales Ruminococcaceae	<i>Saccharofermentans</i> *§		-219.2	65.13	<0.0001	0.07
	5.687-5.727	Urea	Firmicutes Clostridia Clostridiales Ruminococcaceae	<i>Saccharofermentans</i>	<i>S. acetigenes</i> §	-207.7	65.13	<0.0001	0.09

NOESY1D	3.071-3.111	Creatinine	Firmicutes Clostridia Clostridiales Catabacteriaceae	<i>Catabacter</i> **	79.7	68.56	<0.0001	0.01
			Firmicutes Clostridia Clostridiales Ruminococcaceae	<i>Saccharofermentans</i> *§	265.0	65.13	<0.0001	0.03
			Firmicutes Clostridia Clostridiales Ruminococcaceae	<i>Oscillibacter</i> §	567.1	99.76	<0.0001	0.09
	3.532-3.588	Glucose	Firmicutes Clostridia Clostridiales Ruminococcaceae	<i>Pseudobacteroides</i> *	28.9	19.62	<0.0001	<0.0001
			Firmicutes Clostridia Clostridiales Ruminococcaceae	<i>Pseudobacteroides</i>	31.9	19.62	<0.0001	<0.0001
			Firmicutes Clostridia Clostridiales Clostridiaceae 4	<i>Salimesophilobacter</i>	41.9	24.82	<0.0001	0.03
			Firmicutes Clostridia Clostridiales Clostridiaceae 4	<i>Salimesophilobacter</i>	44.5	24.82	<0.0001	0.03
	3.588-3.611		Firmicutes Clostridia Clostridiales Ruminococcaceae	<i>Pseudobacteroides</i> *	-21.0	19.62	<0.0001	0.0001
	9.471-9.511	Unassigned signal	Firmicutes Clostridia Clostridiales Ruminococcaceae	<i>Clostridium III</i>	1038.8	98.11	<0.0001	0.08

¹ Prevalence of bacterial taxa in the study sample.

² p-value for MaAsLin adjusted for age, sex, BMI, smoking status, and physical activity before Benjamini-Hochberg correction; computed using the Maaslin package on R.

³ Corrected p-value (Benjamini-Hochberg method, 10%-FDR). Only associations with a Q-value ≤ 0.1 are presented.

* Taxa found in association with at least 2 NMR signals; § taxa found with both CPMG and NOESY1D

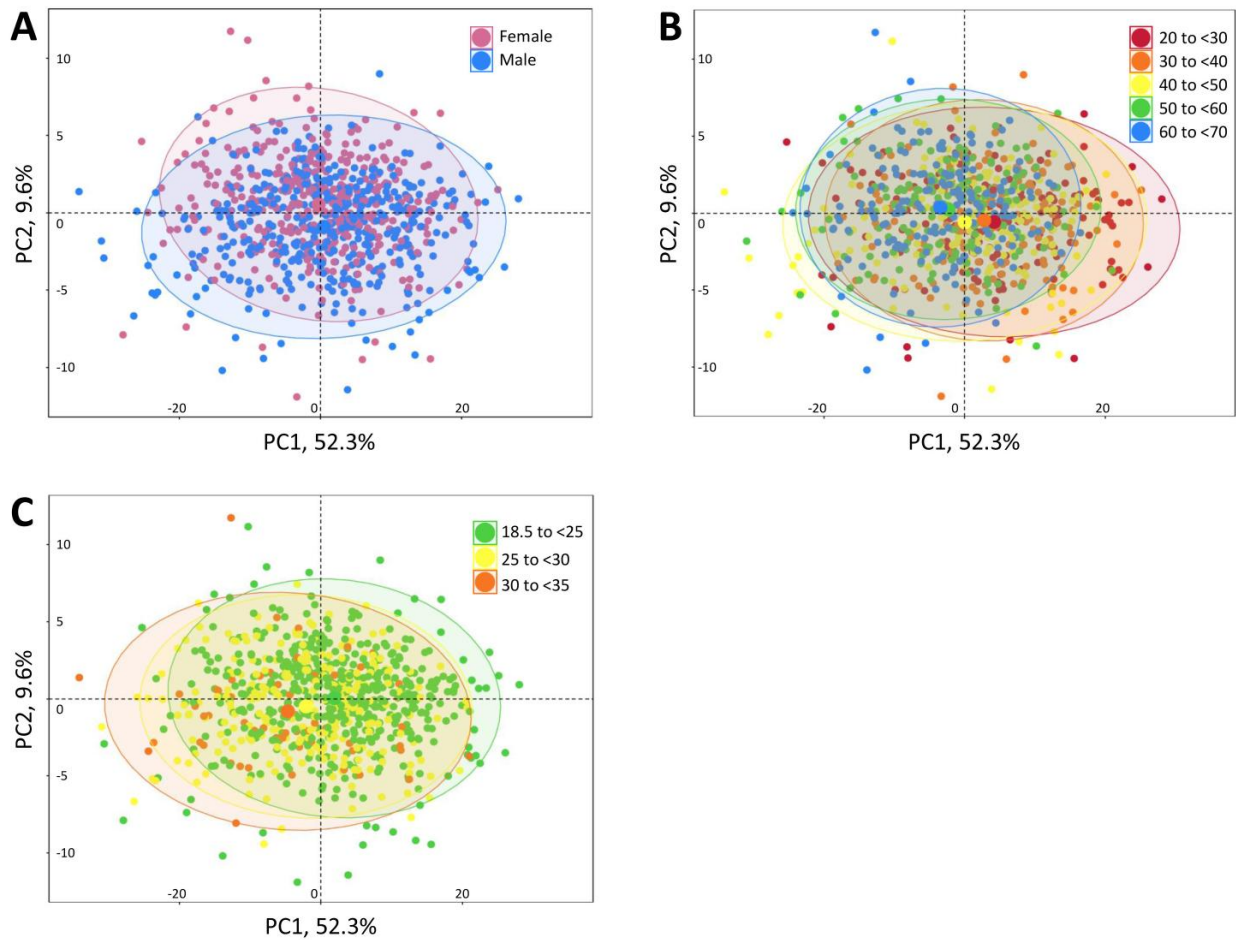


Figure 1. Interindividual variation in metabolomic signatures represented by principal components analysis of the CPMG metabolomic dataset, *Milieu Intérieur* study, France, 2012 (N=846). Each point represents an individual from the study sample. PCA was obtained via the *PCA* function (package *FactoMineR*), and plotted and color-coded based on sex (A), age (B), and BMI (C) via the *fviz_pca_ind* function (package *FactoExtra*). Concentration ellipses (95%) are shown. Percentages on the axes represent the proportion of variation explained by the two first components of the PCA.

			Richness	
Sequence	Shift (ppm)		Observed	Chao1 index
CPMG	0.567-0.607	Unassigned signal	*	-
	1.207-1.311	Lipoproteins, -CH ₂	**	*
	1.521-1.527	CH ₂ CH ₂ COOC	**	*
	2.151-2.166	Lipid, -CH ₂ =C=C-	*	*
	2.166-2.167		**	*
	2.167-2.264	Acetone, CH ₂ CO	**	*
	2.551-2.571	Methylamine	**	*
	2.831-2.848	Citrate/=CH-CH ₂ -CH=	*	-
	3.071-3.111	Creatinine	**	*
	3.167-3.269	Choline and glucose	*	-
	3.311-3.312	Glucose	*	-
	3.616-3.711	Glutamine	*	*
	5.127-5.167	Unassigned signal	**	*
	5.864-5.889		*	-
	5.889-5.969		**	-
	6.492-6.527		**	*
	6.728-6.767	Proteins	**	*
	6.857-6.943	Tyrosine	*	*
	6.912-6.943	Proteins	**	*
	6.943-6.968		**	*
	7.508-7.539	3-methylhistidine	**	-
	7.629-7.647		**	*
	7.647-7.687	1-methylhistidine	**	*
	7.687-7.772	Histidine	**	*
NOESY1D	4.191-4.214	Unassigned signal	**	**
	5.830-5.835		**	*
	5.861-5.887		**	**
	5.887-5.965		**	**
	5.965-5.966		**	**
	6.486-6.526		*	-
	6.726-6.766	Proteins	**	**
	7.646-7.686	1-methylhistidine	*	-

Spearman ρ

Figure 2. Associations between NMR variables and α -diversity indexes (observed richness and Chao1 estimate of richness) from Spearman partial correlations adjusted for age, sex, BMI, smoking status, physical activity, and sequencing depth, *Milieu Intérieur* study, France, 2012 (N=846). Q-values were obtained applying a multiple-testing correction (Benjamini-Hochberg false discovery rate method). Only associations with Q-value ≤ 0.1 for observed richness, as well as subsequent associations with Chao1 richness are presented. ‘**’ P-value ≤ 0.003 and Q-value ≤ 0.05 ; ‘*’ P-value ≤ 0.01 and Q-value ≤ 0.1 ; ‘-’ P-value ≤ 0.05 and Q-value > 0.1 . Corresponding values are shown in **Supplementary Table 7**.