RESEARCH ARTICLE

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High-Coverage Metabolome Analysis Reveals Significant Diet Effects of Autoclaved and Irradiated Feed on Mouse Fecal and Urine Metabolomics

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Scope: Using metabolomics to study the relations of nutrition and health requires stringent control of the experimental conditions used in an animal model. This work investigates the diet effects of autoclaved and irradiated feed on mouse urine and fecal metabolomics.

Methods and Results: C57BL/6 mice are fed normal-irradiation sterilized diet ($n = 9$), autoclave sterilized diet ($n = 9$), and high-irradiation sterilized diet ($n = 9$) for 4 weeks. Differential chemical isotope labeling liquid chromatography mass spectrometry is used to quantify the metabolome variations of urine and feces collected at five time points. Significant differences are observed in urine or fecal metabolomes of mice fed autoclaved diet versus mice fed high-irradiation diet or fed normal-irradiation diet, while the differences are small between the mice fed normal-irradiation and high-irradiation diet. Correlation studies of metabolite changes of diet- and aging-related biomarkers indicate a large overlap of significantly affected metabolites by the two factors.

Conclusions: Diet can be a confounding factor that needs to be carefully considered when a metabolomics study is designed and metabolomic results of a mouse model of nutritional or other biological study are interpreted. Using the same sterilized diet for a given metabolomics project is essential to control the diet effect.

1. Introduction

Rodent model, in combination with metabolome profiling of biospecimens, is increasingly used to study the relations of nutrition and health, including the roles of gut microbiota.[1–5] To ensure reproducibility and reduce variability, rodent feed is sterilized to eliminate the introduction of potentially pathogenic microorganisms that might result in clinical disease or subclinical infections that could affect physiologic responses.[6–10] Autoclave and irradiation are two main sterilization techniques available for rodent feed, and both methods can alter its chemical composition.[8–14] Irradiation led to significant changes in phytochemicals, oxidized lipids, and linoleic acid-derived products,[11,15] while autoclaving rodent feed can affect vitamins A, B1, D content, and acrylamide concentrations.[9,10,13,14] These chemical changes to dietary components in rodent feed may in turn cause metabolomic changes of biospecimens collected from mice or rats fed different diets (i.e., feed-related diet effects).

A major objective of metabolomics is to compare the metabolome differences of two or more groups of subjects or animals with different phenotypes. The extent of feed-related diet effects on the analysis of phenotype-related metabolome changes is related to the differences caused by phenotypes and diets, as well as the analytical technique used to detect the differences. A larger phenotype-induced change would diminish the contribution of a smaller diet effect. However, if a technique used to analyze the metabolome is not sufficiently sensitive or accurate to delineate the contribution of diet effect, diet effects may not be observed.

There are a growing number of reported studies in applying metabolomics to study nutritional effects of food and other diets on human health.[3,5,16,17] Among different biospecimens, urine and feces are particularly useful for investigating host and microbiome interactions. Both germ-free and conventional mice or rats have been used for metabolomics.[18–20] Currently, rodent feed is not standardized in mouse or rat model studies.[10,21,22] Most reported studies used the same diet for mice or rats subjected to different conditions to induce phenotypic changes.[23–26] However, some studies did not control the diet for comparing different groups of rodents for biological studies.[21,25,27] One

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may argue that, because of limited coverage and/or quantification accuracy by conventional analytical methods such as nuclear magnetic resonance (NMR), gas chromatography mass spectrometry (GC-MS), and liquid chromatography mass spectrometry (LC-MS), feed-related diet effects on biospecimen metabolomes may not be detected and therefore animal feed may not need to be strictly controlled.

With recent advances in more sophisticated analytical techniques, quantitative metabolome analysis with high coverage and accuracy has become possible. For example, high-performance chemical isotope labeling (CIL) LC-MS can be used to detect and quantify 8000 or more metabolites (not features) in biospecimens.[28,29] Unlike conventional LC-MS, CIL LC-MS uses rationally designed labeling reagents to concomitantly increase the LC separation efficiency and improve the MS detection sensitivity, thereby allowing the detection of many more metabolites. With differential isotope labeling, accurate relative quantification can be achieved.[28] The increased coverage and accuracy may affect the quantitative results of small molecules that are sensitive to diet effects. In this work, we report our study of a hypothesis that, when CIL LC-MS is used for metabolome analysis, we will see significant diet effects on urine and fecal metabolome. If so, we will need to control the animal model study much more stringently in future studies in order to reveal phenotype or biological effects without the confounding diet effect. To test this hypothesis, we have compared the metabolome of urine or fecal samples collected in five weekly time points from three groups of mice: mice fed normal-irradiation or normal diet (i.e., diet with low dose of irradiation) ($n = 9$), mice fed autoclave sterilized diet ($n = 9$), and mice fed high-irradiation sterilized or irradiation diet (i.e., diet with high dose of irradiation) ($n = 9$). We interrogate the relations of diet-induced metabolic changes and aging-induced changes to understand the relative contributions of diet and aging (i.e., a phenotype) on urine and fecal metabolomes.

2. Results and Discussion

2.1. Body Weight

Mouse body weight was measured weekly in all three groups and reported as mean ± SD for the 28-day measurement period (**Figure 1**A). At the beginning of the study (day 0), the average body weights of the three groups of mice were not significantly different, although the autoclaved diet group (A) had a slightly lower average weight than the irradiated diet group (I) and the normal control diet group (N). To investigate the diet effects on the growth of mice, we calculated the weight gain between two time points. As shown in Figure 1B, the average body weight gain was 1.6 ± 0.13 g/w, 0.96 ± 0.26 g/w, and 1.22 ± 0.60 g/w during the first 7 days in group N, group A, and group I, respectively. The gains were not significantly different except group A versus group N. The gain for group A was smaller than that of group N. The average weight gain for group N was 1.58 ± 0.37 g/w during day 7 to day 14, 0.34 ± 0.36 g/w during day 14 to day 21, and 0.76 ± 0.56 g/w during day 21 to day 28. The average weight gain for group A was 1.08 ± 0.47 g/w during day 7 to day 14, 0.82 ± 0.39 g/w during day 14 to day 21, and 0.58 ± 0.20 g/w during day 21 to day 28. The average weight gain for group I was 1.54 ± 0.65 g/w during day 7 to day 14, 0.52 ± 0.43 g/w during day 14 to day 21, and 0.74 ± 0.44 g/w during day 21 to day 28. All groups gained weight during the late three measurements period, and no differences in body weight gain were noted for any groups between the two measurement points. These results indicate that diet A had a significant effect in the first week, but no effect on the growth of these mice after the first week. Our results are consistent with those of a previous study which concluded that autoclaved rodent feed had no difference in body weight gain compared to irradiated feed.[9]

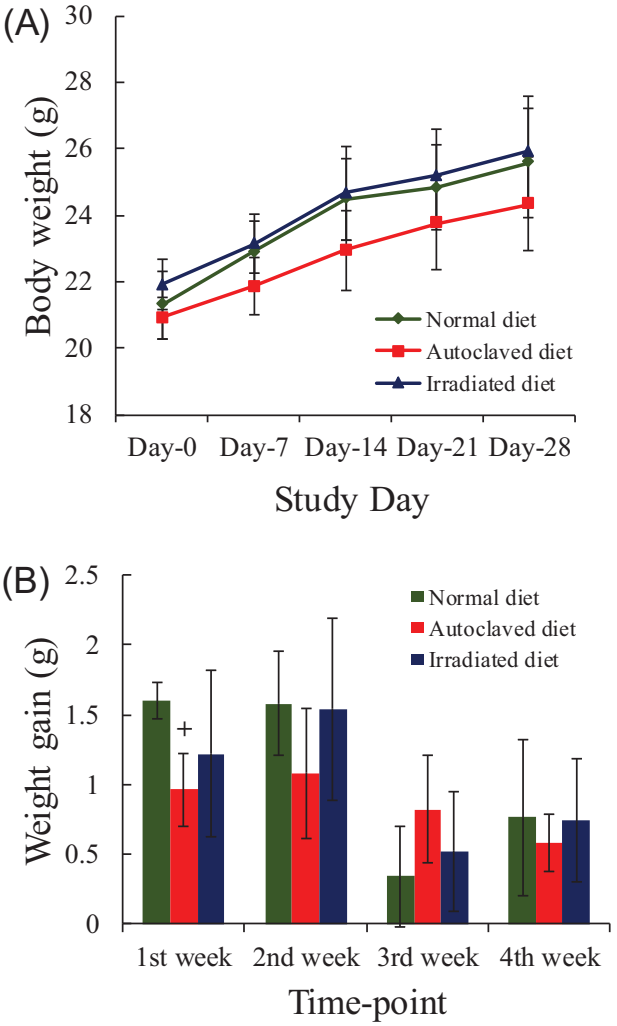

Figure 1. A) Mean body weight, and B) weight gain (g; mean ± SD) in mice fed different diets over a period of 28 days. "+" indicates a significant difference ($p < 0.05$) between diet A group and diet N group.

2.2. Metabolome Analysis

We used dansylation isotope labeling LC-MS to analyze the amine/phenol submetabolome of urine, feces and diet.[30] Amine- and phenol-containing metabolites cover about 34–45% of the chemical space of known metabolite entries in various databases.[29] While labeling chemistries targeting other functional groups such as acids,[31] carbonyls[32] and hydroxyls[33] could be used to further increase the coverage, the amine/phenol

submetabolome profiling already represents a good coverage of most known metabolic pathways.[28] In our work, for accurate relative quantification of the metabolites in comparative samples, the amount of individual samples was initially normalized based on the measurement of the UV absorbance of all the dansyl labeled metabolites.[34] The ^{13}C-labeled pool was used as a reference or a global internal standard that was mixed with a ^{12}C-dansyl labeled individual sample by equal mole amount. The same amount of mixtures prepared from all individual samples was injected into LC-MS. The ^{12}C-/^{13}C-labeled metabolite was detected as a peak pair; only protonated molecular ions, $[M+H]^+$, was retained while redundant peaks such as adduct ions and dimers were filtered out.[35] In this way, a ^{12}C-labeled metabolite in different samples of the same type (e.g., diet, urine, and feces) could be relatively quantified using the peak-intensity ratio values of the ^{12}C-labeled metabolite from individual samples and its ^{13}C-labeled counterpart from the same pool.

There were 4722, 4212, and 1769 peak pairs or metabolites detected from urine, feces, and diet, respectively. Among them, 203 urine, 231 fecal, and 200 diet metabolites were positively identified using the dansyl standard library (Tables S1A-S1C, Supporting Information), and 530 urine, 380 fecal, and 284 diet metabolites were putatively identified, with high confidence, using the Linked Identity (LI) library based on mass and predicted retention time matches (Tables S2A-S2C, Supporting Information). In addition, 712 urine, 600 feces, and 249 diet peak pairs were matched using accurate mass search to zero-reaction MyCompoundID (MCID) library, 2916 urine, 2494 feces, and 979 diet peak pairs to one-reaction MCID library, and 3446 urine, 3006 feces, and 1142 diet peak pairs to two-reaction MCID library (Tables S3A-S3C, Supporting Information). Thus, 4179 (88.5%), 3610 (85.9%), and 1626 (91.9%) pairs could be positively identified or putatively matched from urine, fecal, and diet samples, respectively. This high level of coverage for the amine/phenol submetabolome, in combination with accurate relative quantification from ^{12}C- and ^{13}C-differential labeling,[29] provided us the opportunity to examine how diet can influence the metabolome results more comprehensively, compared to conventional NMR, GC-MS and LC-MS methods.

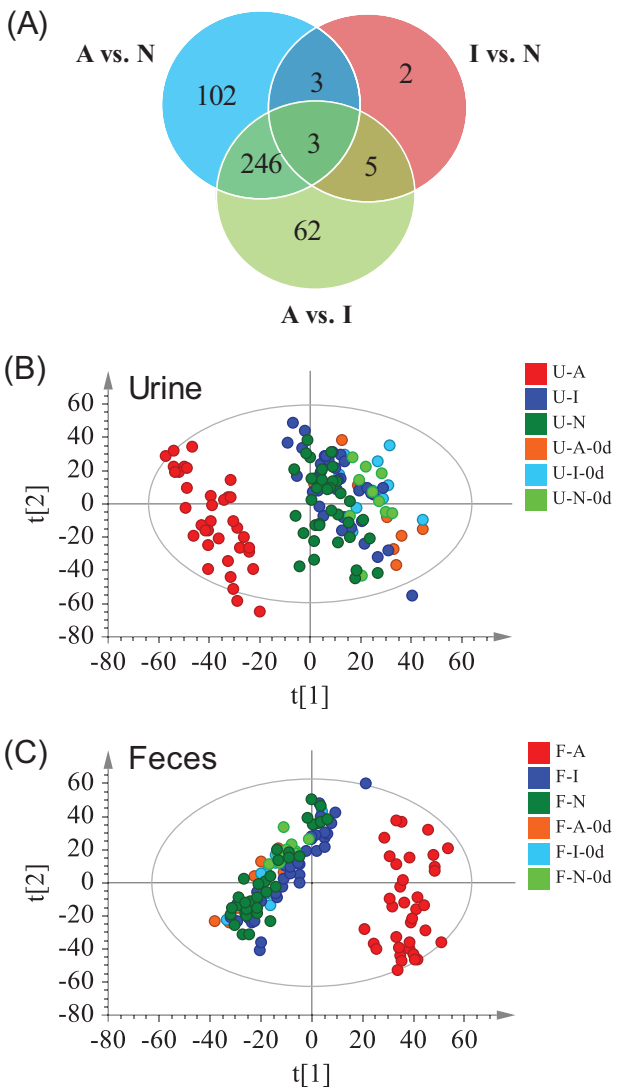

Figure 2. A) Venn diagram of the number of significant metabolites found in binary comparison of diet A versus diet N, diet I versus diet N, and diet A versus diet I using FC >2 or FC <0.5 and FDR-p <0.05. PCA plots of the amine/phenol submetabolomes from B) urine samples and C) fecal samples.

2.3. Metabolome Differences of Diets

To investigate the effects of sterilization on the chemical compositions of animal diets, the metabolome profile of each diet was determined by replicate injections of experimental triplicates of ^{12}C-/^{13}C-labeled diet mixtures ($n = 6$). Binary comparisons of the metabolomes of diet A versus diet N, diet I versus diet N, and diet A versus diet I were performed and the number of significantly different metabolites [>2-fold change (FC) and false discovery rate (FDR)-adjust p-value or q-value <0.05] was found to be 354, 13, and 316, respectively (Table S4, Supporting Information). **Figure 2**A shows the Venn diagram for comparing the number of significant metabolites found in these three comparisons. It is clear that there are great metabolome differences between the autoclaved diet and the normal control diet or irradiated diet. However, there is very little difference between the normal control diet and the irradiated diet. Furthermore, there are more significant metabolites found in the comparison of diet A versus diet N than in the comparison of diet A versus diet I. Many significant metabolites are unique to the comparisons of diet A versus diet N and diet A versus diet I. These results indicate that autoclave treatment alters the diet metabolome much more significantly than high-dose-irradiation treatment. The high temperature and pressure during the autoclave process cause the changes of some metabolites via degradation and other possible reactions. The irradiation process kills pathogens, bacteria, and other microorganisms that might be present in diet, but does not significantly change the vast majority of the diet metabolites.

We note that, among the significantly different metabolites related to autoclave sterilization, they include the vitamin B_6 group (pyridoxine, pyridoxal and pyridoxamine). It was found that the concentrations of pyridoxal and pyridoxamine were decreased during sterilization, and irradiation affected less than autoclave

(Figure S1, Supporting Information). Our results are consistent with those of previous studies which demonstrated loss of vitamins during autoclaving.[10,13]

2.4. Metabolome Differences of Urine and Feces

Knowing that great metabolome differences exist in autoclave and irradiation treated diets, we investigated the diet effects on mouse urine and fecal metabolomes. We applied multivariate statistical analysis to the urine or fecal metabolome data set collected from mice on day 0, 7, 14, 21, and 28 fed with three different diets. The principal component analysis (PCA) plots shown in Figure S2, Supporting Information show that the quality control (QC) samples cluster together closely, indicating that the instrument performance was good and stable during the data collection period. Figure 2B,C shows the PCA plots of the metabolome data generated from urine and fecal samples without QC samples. Urine or fecal samples collected from mice fed autoclaved diet are clearly separated from the other two diets. The PCA plots of the metabolome data of three groups from the urine and fecal samples collected on different days are shown in **Figure 3**. Figure S3, Supporting Information shows the corresponding PCA plots including the QC samples. The QC samples cluster together tightly and thus excellent analytical reproducibility was achieved during the process of running the samples. As it is shown in Figure 3, on day 0, the separation of urine or fecal samples from different diets is not very clear, which is expected since the three groups of mice were grown under the same conditions and were randomly picked. There are some separations between group A with other two groups from day 7 to day 28, while the separations between group N and I are not clear.

To examine the data more closely, we performed binary comparisons of the metabolomes of diet A versus diet N, diet I versus diet N, and diet A versus diet I at five time points using volcano plots. The numbers of significant metabolites using the criteria of FC >2 or <0.5 and q-value <0.05 separating the three groups in each time point are plotted in **Figure 4**. For feces, there is no significant metabolite in three binary comparisons of day 0 samples. This observation is not surprising, considering that three groups of mice were grown under the same condition with normal diet. From day 7 to day 28, there are 578 (day 7), 610 (day 14), 504 (day 21), and 529 (day 28) significant metabolites in the comparisons of diet A versus diet N, 6 (day 7), 40 (day 14), 31 (day 21), and 39 (day 28) significant metabolites in the comparisons of diet I versus diet N, and 461 (day 7), 327 (day 14), 532 (day 21), and 559 (day 28) significant metabolites in the comparisons of diet A versus diet I. Thus, for the later four time points, group A is significantly different from group N or group I in the fecal metabolome data, indicating a strong diet effect caused by diet A. However, the differences between group N and group I are small. Similar findings are shown in the urine metabolome data (Figure 4, blue bars).

Taken together, the results of PCA and volcano plot analyses clearly show that the use of autoclave-treated feed can affect much more significantly in both urine and fecal metabolomes than using irradiation-treated feed. The question is whether the diet effect will interfere with the detection of phenotype-induced metabolome changes and, if so, whether CIL LC-MS has the sufficient sensitivity and metabolite coverage to reveal the interferences. We addressed this question by examining the co-factors, diet and growth time or aging, on metabolome changes, as described below.

2.5. Impact of Diet and Time on Urine and Fecal Metabolomes

Mouse model is widely used to study a phenotype over an extended period. In our study of mice fed with different diets, we can monitor the longitudinal changes of individual metabolites. These changes are related to aging; however, the type of aging related metabolites (aging biomarkers) and the extent of their changes might be dependent on the diet. We used two-factor analysis of variance (ANOVA) to compare the metabolome data obtained from group A and group I. FDR adjustment was used to correct for multiple testing errors to control the false discovery rate, and the null hypothesis (no effect) was rejected at the level of probability of 0.05. **Figure** 5A shows the Venn diagram of the number of significant metabolites related to the diet effect, time effect, and diet∗time effect on the urine metabolome. There are a total of 3204 metabolites that are related to diet, time, and diet*time interaction. Among them, there are 2456 metabolites mainly affected by diet, 1260 metabolites by aging, 722 metabolites commonly affected by diet and aging effect, and 735 metabolites affected by the interaction of the two effects. For the fecal samples, there are 2786 metabolites related to diet, time, and interaction. There are 2139, 1468, 838, and 240 metabolites related to diet effect, time effect, diet and time effect, and diet∗time effect, respectively (Figure 5B).

The results shown in Figure 5 indicate that many more urine or fecal metabolites are affected by diet than aging. There are more diet*time interacted metabolites in urine than in feces. Only 17 interacted metabolites are unique in feces, compared to 210 interacted metabolites in urine. We used ANOVA simultaneous component analysis (ASCA) to explore major changing patterns of metabolites affected by diet, time and/or both. The results are shown in Figure S4, Supporting Information. The metabolites that follow the trends (well modeled) as well as clearly deviate (outliers) are shown in Figure S5, Supporting Information. All models passed the 100-permutations test except for diet*time effect model (Figure S6, Supporting Information). ASCA revealed a significant diet, time, and diet*time effect for 290, 295, and 173 metabolites in urine (Tables S5A–S5C, Supporting Information), and 146, 291, 185 metabolites in feces, respectively (Tables S6A-S6C, Supporting Information). These 295 and 291 metabolites were selected to produce heat maps to show concentration differences in two diets as a function of aging in urine and fecal samples (Figure S7, Supporting Information). These metabolites show common trends in diet A and diet I groups with gradual increase or decrease in their concentrations in four time points. These metabolites may be viewed as aging related biomarkers. Two examples of urine and fecal aging biomarkers are shown in **Figure** 6A–D, respectively. The same trends of concentration changes from day 7 to day 28 are displayed in mice fed diet A and diet I.

However, there are 173 and 185 significant metabolites related to the diet*time effect from the urine and fecal metabolome, respectively. Some metabolites were found to be aging-dependent

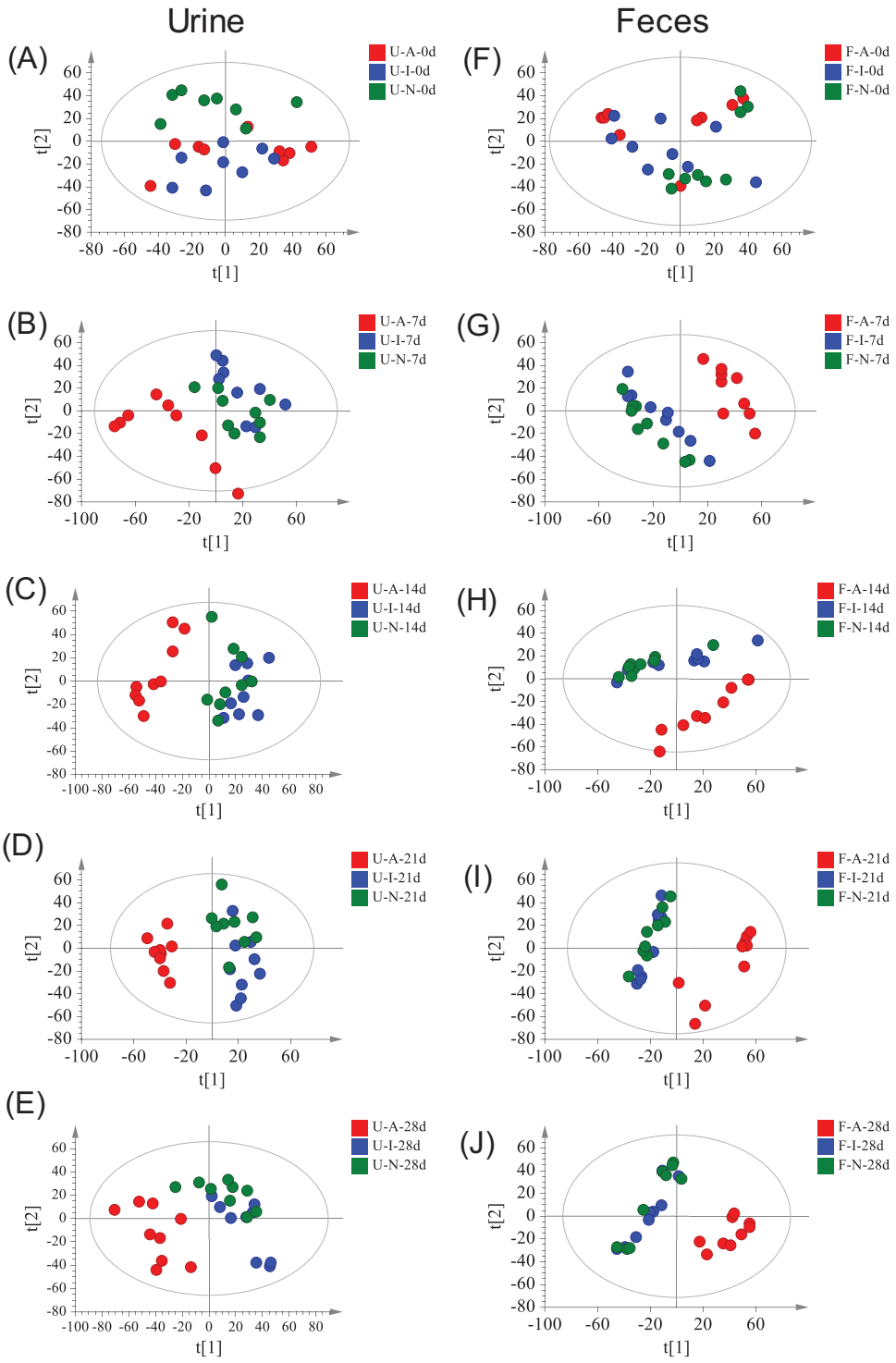

Figure 3. PCA plots of mouse metabolome data from A) urine on day 0, B) urine on day 7, C) urine on day 14, D) urine on day 21, E) urine on day 28, F) feces on day 0, G) feces on day 7, H) feces on day 14, I) feces on day 21, and J) feces on day 28.

(e.g., methionine,[36] hypotaurine,[37] and tyrosine,[38]), i.e., potentially aging biomarkers, in one diet. However, when a different diet was used, they were no longer significant. Thus, these aging biomarkers are diet-dependent. Figure 6E shows an example of fecal metabolite that shows a trend of concentration changes (gradual increase) in diet A group, but no significant change in diet I group except day 28 having higher concentrations than the other days. Figure 6F shows a urine metabolite with no significant concentration changes in diet A group, while a trend of decreasing concentrations is shown in diet I group.

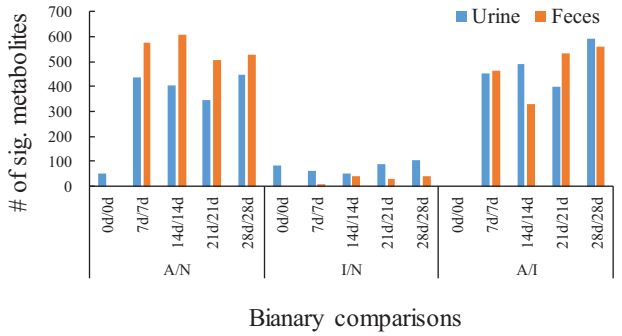

Figure 4. Number of significant metabolites found in various binary comparisons using FC >2 or FC <0.5 and FDR-p <0.05 in urine and fecal samples.

2.6. Biological Significance of Changed Metabolites

While studying the biological mechanisms of aging- and diet-induced metabolic changes is not the focus of this study, biological significance of some representative metabolites are briefly described below.

Among the 295 urine and 291 fecal metabolites related to aging, 19 and 49 can be identified in tiers 1 and 2. They include hypotaurine, an important sulfur-containing and nonpeptidic amino acid, which is a precursor of taurine and an antioxidant. It has been reported to play an important role in the hepatoprotective effect against oxidative stress-mediated liver injuries in rat.[39] Furthermore, it can quench oxidants released by human neutrophils, inhibit lipid peroxidation, and prevent the inactivation of superoxide dismutase by hydrogen peroxide.[37] Hypotaurine was also found to be decreased with age in *Caenorhabditis elegans*.[40] Although the molecular mechanism of hypotaurine delays aging is not well understood, a recent study showed that increasing hypotaurine level could significantly extend the lifespan of *C. elegans*. This was attributed to hypotaurine's antioxidant property by activating the stress-related transcription factors, DAF-16/FOXO and SKN-1/ NRF2, and regulating several age-related signaling pathways, including insulin/insulin-like growth factor-1 signaling (IIS), reproductive signaling and dietary restriction (DR)-like signaling.[37]

Among the 290 urine and 146 fecal metabolites related to diet, 36 and 12 can be identified in tiers 1 and 2. They include isomer of aminoadipic acid (AAA). Aminoadipic acid was found to have significant high levels in the mice fed with autoclave sterilized diet. AAA is an intermediate metabolite in the metabolism (i.e., breakdown or degradation) of lysine and saccharopine, and a potential small-molecule marker of oxidative stress.[41] AAA was reported to be a modulator of insulin secretion and a predictive biomarker for diabetes risk, and found to play an important role in regulating glycolipid metabolism.[42,43] In a study of the dietary effect on metabolic differences, AAA was measured in human plasma along with several other metabolites and found to be higher after beef consumption than after herring meal. [44]

Among the 173 urine and 185 fecal metabolites related to both diet and time, 29 and 30 can be identified in tiers 1 and 2. One example is isomer of saccharopine, which is formed by condensation of lysine and alpha-ketoglutate. It can be oxidized to α-aminoadipic semialdehyde, which ultimately leads to generation of acetyl-CoA that enters the tricarboxylic acid cycle. Some studies showed that an abnormal increase in both lysine and saccharopine in the blood or urine might be associated with developmental retardation, intellectual disability, and spastic diplegia. [45] Zhou et al.[45] demonstrated that saccharopine was a mitochondrial toxin detrimental to mitochondrial dynamics and function both in *Caenorhbditis elegans* and mice. In a study of the impact of maternal and post-weaning metabolic status on the adult male offspring's metabolome in liver, hypothalamus and olfactory bulb three tissues involved in energy homeostasis,

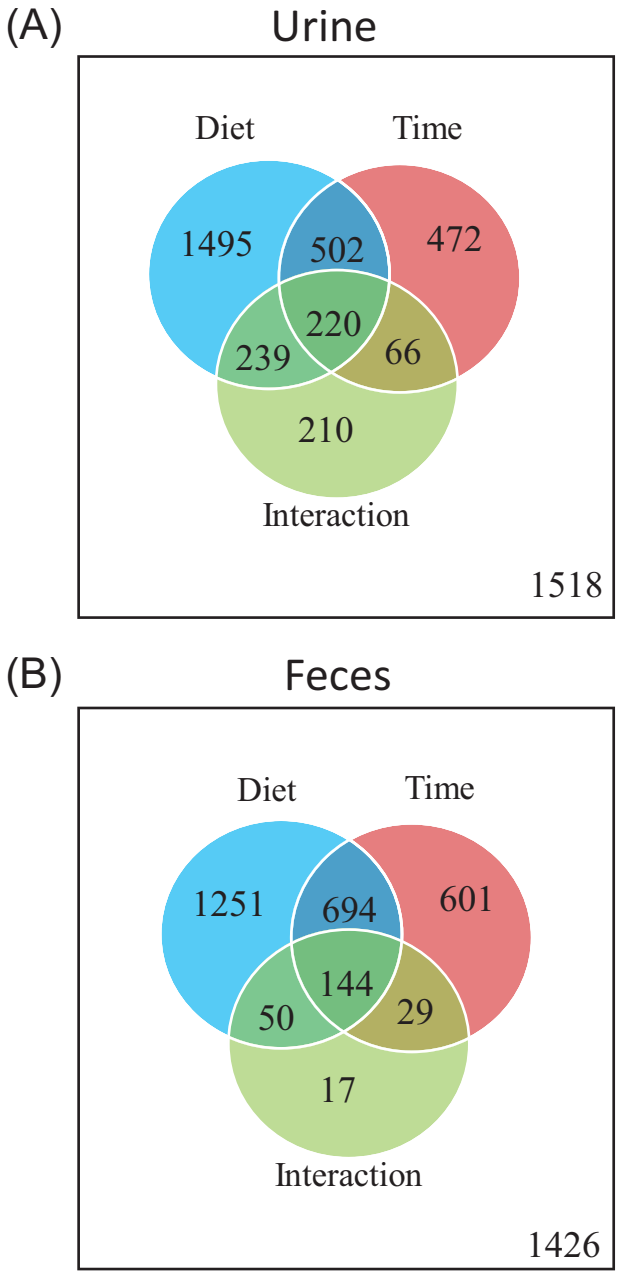

Figure 5. Venn diagram of the number of effected metabolites from two-way ANOVA of diet A and diet I in A) urine samples and B) feces samples. The interaction metabolites from diet*time refer to those affected by both diet and time, which were determined by two-way ANOVA.

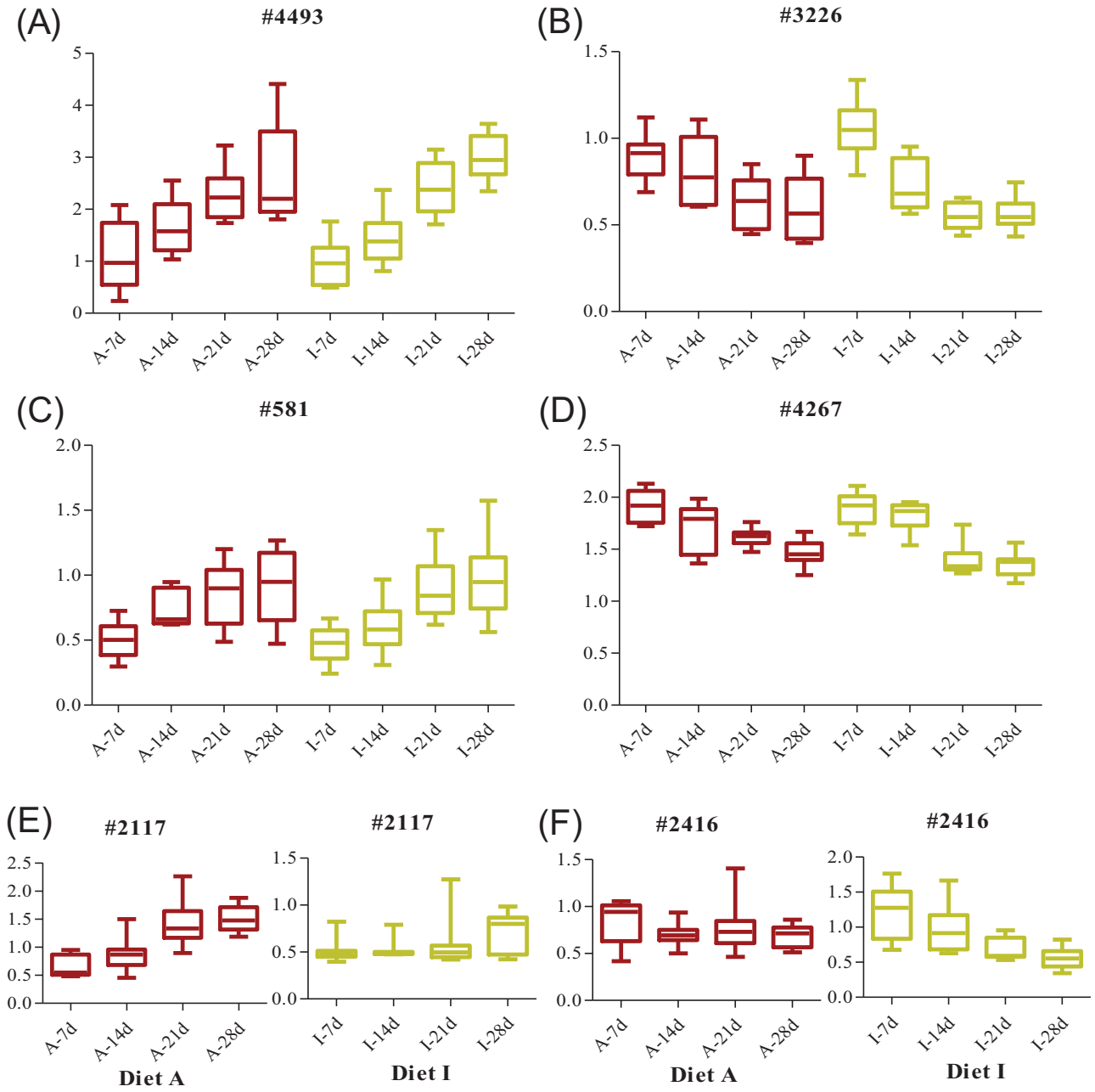

Figure 6. Box plots of representative examples to show the concentration change trends in urine and fecal samples. A) #4493 and B) #3226 from urine samples. C) #581 and D) #4267 from feces samples. Box plots of the same metabolite (#2117 or #2416) with its concentration level in diet A and in diet I from E) urine samples and F) fecal samples.

saccharopine, and other two metabolites were found to be affected by the post-weaning diet in all three tissues, and the abundance of saccharopine was significantly lower in mice fed a high-fat diet than a control diet. [46]

2.7. Controlling Diet Effect

Our study clearly demonstrates that diet effect from the rodent feed sterilized using autoclave and irradiation can be observed using the high-coverage CIL LC-MS technique and diet effect can have a significant impact on the determination of phenotype-induced metabolite changes (e.g., aging biomarkers). Thus, the use of the same diet for different groups of rodents for biological study is critical. If mice of different phenotypes are fed different diets, the diet effect can interfere with the study of the biological effect. Some of the biological findings published in studies without diet control need to be interpreted with great care. The reported phenotype-induced metabolite changes might be due to diet effect.

While keeping the same diet for a given metabolomics project within a study can be easily done, a big question is how the results obtained from two studies with different diets (e.g., reports from different labs) can be compared. Currently, diet is not standardized and there are many suppliers of animal feed. One possible route to address this issue is to use two or more diets to examine the diet effect within a metabolomics study and, if possible, delineate the biological and diet effects. However, this would add extra time and cost in an animal model study. In the absence of any information on diet effect, comparison of results found in animal models with different diets needs to be carefully done. Hopefully, in the future, when more metabolomics studies of diet effects become available, a growing list of metabolites that can be affected by diet will be determined, which can serve as a diet-related reference. In any case, for reporting a metabolomics study, at the minimum, compositional and nutritional information of animal feed should be presented. This information needs to be as detailed as possible, which requires manufacturers to characterize their animal feed more extensively than those currently provided.

3. Conclusions

We have examined two sterilization methods (autoclave and irradiation) to evaluate the effects of them on urine and fecal metabolome. By comparing the metabolomic data of urine and feces generated from CIL LC-MS, we observed clear separation of the mice fed diet A and diet N or diet I, but little separation of mice fed diet N and diet I. Furthermore, the diet can affect some potentially aging biomarkers. This study demonstrates that, in order to reveal the biological effects on urine and fecal metabolome, it is critical to use the same sterilized diet in a given study. In future work, to study the diet effect in finding the biomarkers of other phenotypes such as a disease, we may use a similar approach of feeding the mice with two different diets to determine if disease-biomarkers are diet dependent or not.

While this study focuses on analyzing urine and fecal samples, we believe that investigating the diet effects on metabolomics of other types of biospecimens, such as serum/plasma, using a global metabolome analysis approach is warranted. In addition, this study does not cover the lipidome; global lipidome analysis requires a different analytical platform to provide high accuracy and high coverage lipid analysis. Understanding the diet effects on global lipidomics of biospecimens is clearly needed. Finally, the diet effects shown herein are related to the mouse model. For human studies, strict control of diet is difficult and diet may affect concentration changes associated with a phenotype for a greater number of metabolites. There is a need to use high-coverage quantitative metabolome methods to examine how diet can affect the human metabolome in order to understand better the diet effects on human samples.

4. Experimental Section

Sample Collection and Processing: All animal experiments were conducted according to a protocol that was approved by the Animal Care Ethics Committee of the 1st Affiliated Hospital of Zhejiang University. Six-week-old specific pathogen-free (SPF) male C57BL/6 mice purchased from SLAC Laboratory Animal Co., Ltd. (Shanghai, China) were used in these experiments. The pelleted diet sterilized by Cobalt 60 gamma rays exposed to a dose of 25kGy (P110F-25, SLACOM, Shanghai, China) was used as the standard rodent feed (i.e., normal diet). Supplemental Note S1 lists the feed's composition and nutritional values.

Mice were bred in barrier facilities and maintained in individually ventilated cages (IVC) in a room at 24 °C, relative humidity of 50%, 12 h light/dark. Upon arrival in the laboratory at the age of 7 weeks, all mice were fed normal diet during their 7-d acclimation period. Then animals were randomized into three groups of nine mice each. One group was maintained on normal diet to serve as reference (marked as N), another group was fed normal diet autoclaved at 110 °C (marked as A), and the third group was fed normal diet sterilized by Co-irradiation at a dose of 50kGy (marked as I). Mouse body weight was measured weekly during the experiment. Urine and feces were collected weekly from each animal over the duration of the experiment (i.e., on day D0, D7, D14, D21, D28). Fresh feces were collected by lifting the mouse from the cage using one hand to grab the end of tail.[47] Mouse defecated quickly once held properly and the feces could be collected into a 2.0 mL Eppendorf tube. Urine collection was performed by gently pressing the abdomen.[47] The tubes containing the freshly collected urine and feces were placed on ice and then transferred to a –80 °C freezer.

After the stored samples were thawed, metabolites were extracted for CIL LC-MS analysis. For fecal metabolite extraction, fecal samples were subjected to sequential solvent extraction by water and acetonitrile (ACN) as descried before.[48] Briefly, 400 µL water was added into each sample for the first extraction, followed by using 400 µL ACN for the 2nd extraction. The supernatants from the two extractions were combined and dried with a SpeedVac and then stored at –80 °C for further use.

Derivatization, Normalization, and Mixing: The light-reagent, ^{12}C-dansyl chloride (Dns-Cl), was purchased from Sigma–Aldrich, St. Louis, MO and the heavy-chain reagent, ^{13}C-Dns-Cl, was from Nova Medical Testing, Inc. (Edmonton, Canada; www.novamt.com). Dansylation labeling of fecal and urine samples was done as detailed in Supplemental Note S2-1. The individual samples were labeled separately using ^{12}C-dansyl chloride and quantified by LC-UV based on absorption at 338 nm (Note S2-2, Supporting Information) for sample normalization. A pooled fecal or urine sample was prepared by mixing the same amount of aliquot from each of the 150 extracts. A portion of the fecal-pool or urine-pool was taken and labeled by ^{13}C-dansylation. An aliquot of the ^{13}C-labeled pool was mixed with a ^{12}C-labeled individual sample in 1:1 mole ratio to produce a mixture for LC-MS analysis.

LC-MS: An UltiMate 3000 UHPLC system (Thermo Scientific, MA) with an Waters ACQUITY UPLC BEH C18 column (2.1 mm × 15 cm, 1.7 µm particle size, 130 Å pore size) connected to the Impact II Quadrupole Time-of-Flight (QTOF) mass spectrometer (Bruker, Billerica, MA) was used for LC-MS analysis. The running conditions are listed in Supplemental Note S2-3. All samples were arranged in a random sequence during the LC-MS runs. Only positive ion detection was used as dansyl labeled metabolites are preferentially ionized in the positive mode. The pooled quality control (QC) sample was prepared by mixing 1:1 ^{12}C-/^{13}C-dansyl-labeled pools. A QC run was done every 20 sample runs.

Data Processing and Analysis: In CIL LC-MS, the ^{12}C- and ^{13}C-dansyl labeled metabolites are detected in the form of peak pairs. Metabolite peak pair extraction, sample-wise alignment, and missing value retrieval were conducted by using IsoMS Pro.[29,35] Peak pairs without peak ratios present in at least 80% of samples in any group were removed. The remaining peak pairs with peak ratio RSD of less than 30% among QCs were retained and exported to SIMCA-P+ 12.0 software (Umetrics, Umeå, Sweden) and MetaboAnalyst (www.metaboanalyst.ca) for statistical analyses. Finally, metabolite identification was done according to the three-tier approach as reported previously.[29] Briefly, In tier 1, peak pairs were searched against a labeled metabolite library (CIL Library) based on accurate mass and retention time. The CIL Library (i.e., dansyl amines and phenols) contains 711 experimental entries, including metabolites and dipeptides. In tier 2, linked identity library (LI Library) was used for identification of the remaining peak pairs. The LI Library includes metabolic-pathway-related metabolites (more than 7000 entries extracted from the

KEGG database), providing high-confidence putative identification results based on accurate mass and predicted retention time matches. In tier 3, the remaining peak pairs were searched, based on accurate mass match, against the MyCompoundID (MCID) library composed of 8021 known human endogenous metabolites (zero-reaction library) and their predicted metabolic products from one metabolic reaction (375809 compounds) (one-reaction library) and two metabolic reactions (10583901 compounds) (two-reaction library).[49]

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

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Conflict of Interest

The authors declare no conflict of interest.

Author Contributions

All authors were involved in designing the research study. X.S. and J.Y. conducted experiments. X.S., D.C., and W.H. analyzed data. X.S. and L.L. wrote the manuscript. All authors edited and approved the manuscript.

Data Availability Statement

The metabolomics data and metadata files are available upon request from the corresponding authors.

Keywords

diet effects, LC-MS, metabolomics, mouse feed, urine and feces

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