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ORIGINAL ARTICLE

High fat diet reveals sex-specific fecal and liver metabolic alterations in C57BL/6J obese mice

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Abstract
Obesity is a major health concern that poses significant risks for many other diseases, including diabetes, cardiovascular disease, and cancer. Prevalence of these diseases varies by biological sex. This study utilizes a mouse (C57BL/6J) model of obesity to analyze liver and fecal metabolic profiles at various time points of dietary exposure: 5, 9, and 12 months in control or high fat diet (HFD)-exposed mice. Our study discovered that the female HFD group has a more discernable perturbation and set of significant changes in metabolic profiles than the male HFD group. In the female mice, HFD fecal metabolites including pyruvate, aspartate, and glutamate were lower than control diet-exposed mice after both 9th and 12th month exposure time points, while lactate and alanine were significantly downregulated only at the 12th month. Perturbations of liver metabolic profiles were observed in both male and female HFD groups, compared to controls at the 12th month. Overall, the female HFD group showed higher lactate and glutathione levels compared to controls, while the male HFD group showed higher levels of glutamine and taurine compared to controls. These metabolite-based findings in both fecal and liver samples for a diet-induced effect of obesity may help guide future pioneering discoveries relating to the analysis and prevention of obesity in people, especially for females.

Keywords Metabolites · High fat diet · Liver · Fecal · Metabolomics · Mouse model · Preclinical model · Obesity · Sex differences

1 Introduction

Rates of adult obesity in the United States have worsened and the national obesity rate has reached 40%. According to the National Health and Nutrition Examination Survey, in 2022, 41.9% of adults and 20% of chilren aged 2–19 years are obese in the United States. Obesity contributes to many health issues, including diabetes, heart disease, stroke, and some cancers (*Adult Obesity Causes & Consequences | Overweight & Obesity | CDC*, 2021; Hales et al., 2015; Piché et al., 2020). The healthcare cost of obesity and its related diseases has risen to 190.2 billion dollars in 2019 (Ramasamy et al., 2019).

Obesity is a complex disease contributed from genetic, environmental, and lifestyle factors (Heianza & Qi, 2017; Pataky et al., 2010). Diet is one of the most important modifiable environmental factors that influence human health. Western diets high in fat and calories have contributed to the obesity epidemic in the United States (Jéquier, 2002; Rakhra et al., 2020). Obesity is characterized by increased

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adipose tissue and body weight. Adipose tissue secretes a wide range of hormones and cytokines that play important roles in glucose and lipid metabolism. Many organs such as the liver, muscle, and brain are negatively affected by obesity, and their metabolism is also altered (Hajer et al., 2008; Yang et al., 2018). Metabolites from various organs in obese models are used to analyze the progression of obesity. Therefore, understanding the metabolic changes caused by obesity can help to elucidate important pathways relevant for obesity and associated comorbidities.

Metabolomics is a powerful tool that has significantly advanced in recent years and can be used to identify key metabolites in metabolic pathways (Ivanisevic & Thomas, 2018). Metabolites are small chemicals that represent the downstream products from genomic, transcriptomic, and proteomic variability (Ivanisevic & Thomas, 2018; Newgard, 2017). The study of metabolites and metabolic profiles can illustrate potential mechanisms of diet-induced physiological dysregulation, trace the pharmacological effects of drug therapies, and provide a highly integrated profile of biological status in cells, tissues, or organs (Newgard, 2017).

Metabolomic studies enable precise monitoring of metabolites in obesity research by providing detailed characterization of metabolites (Meiliana et al., 2021; Nicholson et al., 1999). Metabolomic profiling of high fat diet (HFD) induced obese mice has identified alteration in key metabolites, including glycolysis, tricarboxylic acid (TCA) cycle, lipid metabolism, choline metabolism, amino acid metabolism, and creatine metabolism (Broadfield et al., 2021; Xie et al., 2012; Yang et al., 2021). Moreover, HFD induced insulin-resistant rats are observed to downregulate nicotinamide adenine dinucleotide hydride (NADH) levels in nicotinate and nicotinamide metabolism, reduce taurine, and alter glutathione (GSH) metabolism (Le et al., 2016). Recent metabolomic studies in HFD mice show a strong link between gut microbial composition and obesity, where gut microbiota influence host hepatic lipid metabolism (Aron-Wisnewsky et al., 2021; Cuevas-Sierra et al., 2019; Druart et al., 2015). These studies have shown that, metabolomics is a powerful tool to illustrate potential mechanisms of nutrient metabolism in obesity and provide biomarkers for obesity and associated diseases.

Biological sex plays an important role in energy metabolism, adipose tissue storage, energy partitioning, and balance. Sex-specific regulation most likely influences the development of obesity and response to pharmacological intervention (Jacobs et al., 2019; Katz et al., 2013; Mauvais-Jarvis, 2015). A study performed in Mexican adolescents (n = 352) showed sex-specific pathways associated with changes in adiposity (e.g. BMI, fat percentage, body composition), and changes of muscle mass (Rodríguez-Carmona et al., 2022). A human study showed a sex-specific metabolomic analysis of male and female subjects with central obesity. The differential expression in 94 out of 177 metabolites was observed and these metabolites include branch chain amino acids (BCAAs), acetate, 3-hydroxybutyrate, lysophosphatidylcholine (LPCs), and phosphatidylcholine (PCs) (Szymańska et al., 2012). Another human study investigating serum metabolic profiles in obese and lean subjects showed sex-specific changes in BCAAs, fatty acid, tryptophan, and the metabolites in bile acid metabolism and the TCA cycle(Xie et al., 2014). Among these metabolites, BCAAs were differentially expressed in obese men, but not in obese women (Xie et al., 2014). In the rodent models, leptin deficient obese mice showed sex-specific metabolic alterations. Taurine, glycine, citrate, and urea levels in urine were significantly altered in obese female mice, while pyruvate and serum glucose were significantly altered in obese male mice (Gómez-Zorita et al., 2019; Mayengbam et al., 2019; Won et al., 2013). However, more comparisons of sex-specific metabolomic profiles are needed to discover the physiological mechanisms of sex-specific development of obesity.

There are also limited studies observing sex-specific metabolic changes through growth and development. Many studies described above were done at a single time point. Thus, there is a need for longitudinal metabolomic studies to track the progression of obesity. This study investigates the longitudinal sex-specific metabolic alterations in mice exposed to long-term HFD. The results can identify biomarkers at various stages of obesity, illustrate physiological pathways, and provide pro-diagnostic strategies for obesity intervention.

2 Materials and methods

2.1 Animals and diets

One-month-old male and female C57BL/6J mice were obtained from Jackson Laboratories and housed in individual cages in an animal facility maintained on a 12-hour light (7 am-7 pm) /dark (7 pm-7 am) cycle, and the temperature was kept at 24–26 °C. Mice were fed *ad libitum* for twelve months with either a control or an HFD diet purchased from Research Diets Inc. (New Brunswick, NJ, Catalog# D12450H, Catalog#D12451 respectively) (n = 6–10 per sex per dietary group). The control diet had a 10% kcal/g diet, while HFD had a 45% kcal/g diet. The primary source of fat in the food was lard. Food consumption and body weights were measured weekly for all animals. Fecal samples were collected at the 5th, 9th, and 12th months of dietary exposure. All the animals were sacrificed at the end of the 12th

month of dietary exposure. Liver samples were harvested and snap-frozen in liquid nitrogen and stored at -80^0 C until analysis. The animal protocol (#18 – 006) was approved by the Institutional Animal Care and Use Committee at the North Carolina Agricultural and Technical State University.

2.2 Fecal sample preparation

Dry, frozen fecal samples (6–10 per sex per dietary exposure group per time point) were extracted using water with a previously established method (Gratton et al., 2016). A phosphate buffer of D2O was added to the extracted samples, and the final samples contained 10% of D2O with 0.1 M phosphate buffer (pH 7.4) and 0.5 mM trimethylsilyl propanoic acid (TSP). The samples were transferred to 5 mm NMR tubes after being centrifuged for further nuclear magnetic resonance (NMR) acquisition.

2.3 Liver tissue sample preparation

Liver tissues were slowly thawed and 100 mg of tissue used for metabolomic analysis. Liver tissue was extracted by using a two-step method including, the homogenization of tissue in cold 2.5:1 methanol-water solvent followed by the addition of ice-cold chloroform and water solvent (Wu et al., 2008). After centrifugation, the upper polar phase was dried and reconstituted in a phosphate buffer. The reconstituted tissue extracts were transferred to 5 mm NMR tubes for further NMR analysis.

2.4 NMR analysis

A Bruker Ascend 400 MHz high-resolution NMR with a sample Xpress autosampler was applied in this study and all the experiments were carried out using ICON-NMR software (Bruker Biospin) and controlled by ICON-NMR. A 1D NOESY experiment with water suppression (noesygppr1d) was carried out with 32k increments, and 64 transients. All the spectra were carefully phased and calibrated to TSP in Bruker Topspin 4.06 (Bruker Biospin). The metabolites identification was carried out using Chenomx 8.6 (Chenomx Inc).

Table 1 Physiological data

	Male Mice		Female Mice	
	Control (n = 9)	HFD (n = 9)	Control (n = 8)	HFD (n = 9)
body weight (g)				
5th month	30.87 ± 3.55	41.39 ± 6.94**	24.30 ± 2.69	29.45 ± 8.21*
9th month	42.6 ± 3.23	53.25 ± 2.81**	29.53 ± 4.39	37.03 ± 4.39*
12th month	45.25 ± 4.03	53.64 ± 5.27**	31.88 ± 3.67	45.22 ± 3.67**
weekly average diet consumption (g)				
5th month	21.10 ± 1.33	20.74 ± 2.51	18.85 ± 1.57	17.76 ± 3.0
9th month	23.55 ± 1.67	23.27 ± 2.29	19.79 ± 1.79	18.75 ± 2.36
12th month	26.48 ± 2.08	25.72 ± 4.54	22.05 ± 1.40	23.49 ± 2.80

Data expressed as mean ± SD **p < 0.01, *p < 0.05 as compared with control

2.5 Metabolic pathway analysis

The metabolic pathway analysis was carried out using the KEGG database and MetaboAnalyst 4.0.

2.6 Data analysis

The NMR spectra were obtained using Amix 4.0 (Bruker BioSpin) and the NMR spectra were bucketed using a previously reported automatic method (Wang et al., 2020). The processed data were normalized to the total peak intensity. Metabolite identification was carried out using Chenomx 8.6 (Chenomx Inc). The Principal Component Analysis (PCA) was carried out using PLS toolbox (Eigenvector Research). Metabolic pathway analysis, the global test, and the random forest study were carried out using MetaboAnalyst 4.0.

Key metabolites were analyzed using repeated measures three-way ANOVA (factors: gender, treatment, and time) followed by Sidak's multiple comparisons. The analysis used GraphPad Prism software version 9.0.0 for Windows (GraphPad Software, San Diego, CA). A p-value less than 0.05 ($p < 0.05$) was considered statistically significant.

3 Results

As shown in Table 1, HFD fed male and female mice gained significantly more weight compared to their control groups at all the time points. However, the diet consumption of HFD fed mice in both the male and female groups was similar to their respective control groups.

3.1 Fecal samples at the 5th month

NMR profiling of the fecal samples during the 5th month of dietary exposure was conducted to investigate early signs of metabolic perturbations in male and female mice (Table 2). Clues of metabolic changes were first observed in the female HFD group compared to the female control group; in contrast, the male groups showed fewer alterations in metabolites at this time point. Metabolites such as lactate, 3-methyl-2-oxovalerate, and taurine were significantly altered, while the total bile acid and propionate exhibited an increasing trend in the female

Table 2 The fecal metabolites analyzed 5th month on male and female samples. The fold change (FC) was calculated using HFD vs. Control. False discovery rate (FDR) is expressed as a q value

	Male			Female		
Metabolites	p value	FDR (q)	FC (HFD/Control)	p value	FDR (q)	FC (HFD/ Control)
2-Oxoglutarate	2.84E-01	4.97E-01	1.74	1.20E-01	5.60E-01	0.86
2-Oxoisocaproate	8.31E-01	5.13E-01	1.02	8.04E-01	1.06E+00	1.02
3-Methyl-2-oxovalerate	7.25E-01	5.25E-01	1.06	2.88E-02	6.05E-01	1.69
Acetate	2.98E-01	4.47E-01	1.39	5.89E-01	1.03E+00	1.12
Acetoin	6.03E-01	5.07E-01	0.97	9.65E-01	1.04E+00	1
Adenine	4.87E-02	5.11E-01	0.79	1.48E-01	5.65E-01	0.85
Alanine	1.86E-01	7.81E-01	0.82	6.14E-01	1.03E+00	1.03
Asparagine	3.74E-01	4.91E-01	1.07	6.22E-01	9.68E-01	0.93
Aspartate	4.66E-01	4.89E-01	1.36	9.65E-02	5.07E-01	0.74
Butyrate	7.07E-01	5.30E-01	1.04	9.37E-01	1.06E+00	0.99
Cholate	2.35E-01	4.94E-01	0.88	2.68E-01	7.50E-01	1.12
Choline	1.36E-01	9.52E-01	1.19	4.50E-01	9.00E-01	1.07
Ethanol	9.37E-01	4.92E-01	1.01	3.53E-02	4.94E-01	1.3
Ethanolamine	2.70E-01	5.15E-01	1.16	8.21E-01	1.04E+00	0.93
Formate	9.97E-01	4.99E-01	1.00	1.52E-01	5.32E-01	0.57
Fumarate	2.19E-01	5.11E-01	1.74	8.04E-01	1.09E+00	1.13
Glucose	8.11E-01	5.16E-01	1.03	7.58E-01	1.06E+00	0.9
Glutamate	4.70E-01	4.70E-01	0.94	6.36E-01	9.54E-01	1.04
Glutamine	7.27E-01	5.09E-01	0.98	7.13E-01	1.03E+00	1.02
Glycine	3.78E-01	4.41E-01	0.80	3.54E-01	7.83E-01	0.92
Hypoxanthine	3.09E-02	6.49E-01	0.80	1.47E-01	6.17E-01	0.84
Isoleucine	5.37E-01	5.13E-01	0.94	1.83E-01	5.91E-01	1.19
Lactate	8.34E-01	5.00E-01	0.99	4.01E-02	3.37E-01	0.82
Lysine	3.60E-01	5.04E-01	1.27	3.21E-01	7.49E-01	1.07
Methionine	9.32E-01	5.02E-01	0.99	6.18E-01	9.98E-01	1.03
N,N-Dimethylformamide	1.86E-01	6.51E-01	0.42	2.44E-02	1.02E+00	0.55
Phenylalanine	7.62E-01	5.16E-01	1.03	9.69E-01	1.02E+00	1
Proline	9.64E-01	4.94E-01	1.00	4.07E-01	8.55E-01	0.89
Propionate	9.30E-01	5.14E-01	0.99	7.91E-02	5.54E-01	1.27
Pyruvate	6.57E-01	5.31E-01	1.04	9.88E-01	9.88E-01	1
Sarcosine	8.96E-01	5.23E-01	1.01	2.94E-01	7.72E-01	1.09
Serine	3.75E-01	4.63E-01	0.88	1.91E-01	5.73E-01	0.84
Succinate	5.88E-01	5.15E-01	1.15	4.55E-01	8.31E-01	0.88
Sucrose	2.04E-01	6.12E-01	1.59	9.59E-01	1.06E+00	1.03
Tartrate	7.88E-01	5.17E-01	1.09	3.17E-01	7.83E-01	0.77
Taurine	5.60E-01	5.11E-01	1.24	3.77E-02	3.96E-01	0.46
total bile acid	9.06E-01	5.14E-01	1.03	7.95E-02	4.77E-01	1.59
Typtophan	2.92E-01	4.72E-01	1.10	9.88E-01	1.01E+00	1
Tyrosine	4.10E-01	4.53E-01	1.08	9.01E-01	1.08E+00	0.99
Uracil	7.06E-01	5.49E-01	1.05	8.52E-01	1.05E+00	0.96
Valine	2.06E-01	5.41E-01	0.87	4.51E-01	8.61E-01	1.05
Xanthine	1.62E-01	8.51E-01	0.85	9.34E-01	1.09E+00	0.99+AA2:G45

HFD group compared to the female control group ($p < 0.05$). In the male HFD group, hypoxanthine and adenine were decreased compared to control male mice ($p < 0.05$). PCA was carried out to visualize the differences between the exposure groups and sexes. However, the PCA plot did not show a distinct separation between both dietary treatment groups and sexes **(Fig.** 1).

3.2 Fecal samples in the 9th month

The combinational metabolites distributions in the PCA score plot **(Fig.** 2A) indicated a much weaker difference between dietary exposure groups in male mice. The male HFD group, in comparison to the control group, showed a significant increase in isoleucine, 3-methyl-2-oxovalerate, and total bile

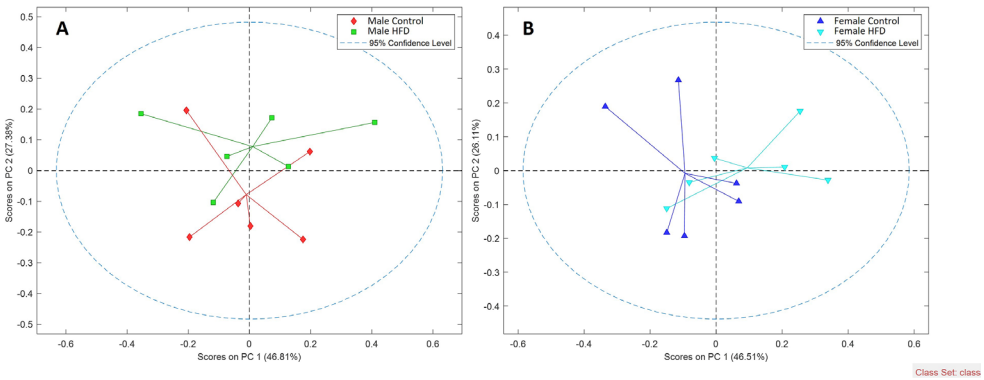


Fig. 1 PCA studies on the fecal samples collected in the 5th month. A, the PCA score plot of the male samples for control vs. the HFD. B, the PCA score plot of the female samples for control vs. the HFD

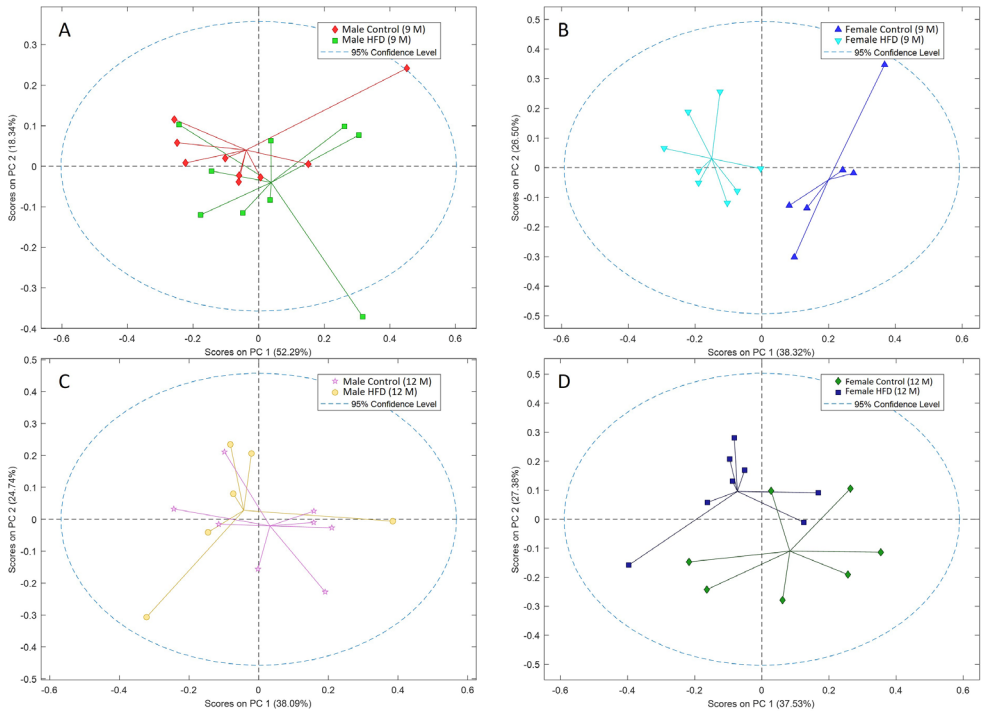


Fig. 2 PCA score plots on the fecal samples. **A**, the 9th -month male samples (Control vs. the HFD). **B**, the 9th -month female samples (Control vs. the HFD). **C**, the 12th -month male samples (Control vs. the HFD). D, the 12th -month female samples (Control vs. the HFD)

acid, in contrast to a decrease in succinate (Table 3, $p < 0.05$). The glucose concentration was significantly decreased, which was not shown in the female group ($p < 0.05$). The metabolites at the 9th month of dietary exposure showed high perturbation in the female groups. Fecal metabolites such as cholate, glycocholate, isoleucine, 3-methyl-2-oxovalerate, and total bile acids were significantly increased, while acetate, glutamate, hypoxanthine, pyruvate, propionate, and succinate were significantly decreased in the female HFD group compared to the female control group (Table 3, $p < 0.05$). The metabolite separation between control and HFD fed female mice was also supported by the PCA score plot **(Fig.** 2B).

3.3 Fecal samples at the 12th month

For the male groups, serine, threonine, and tryptophan were significantly downregulated (Table 3, $p < 0.05$). The PCA score plot showed a similar mixed distribution between control and HFD male mice as the 9th -month data **(Fig.** 2C). The female HFD group still showed high perturbation with cholate, isoleucine, total bile acid, and valine significantly increased, while alanine, glutamate, hypoxanthine, lactate, 2-oxoglutarate, 2-oxoisocaproate, and pyruvate were significantly decreased (Table 3, $p < 0.05$). The changes of cholate, glutamate, isoleucine, pyruvate, and total bile acid were consistent with the 9th month. The PCA score plot showed a distinct separation between the control and the HFD female mice with slight overlap **(Fig.** 2D).

We used three-way ANOVA to examine the effect of diet, sex, and time on key metabolites. Results showed that the main effect of time is significant for total bile ($p = 0.0001$), glutamate ($p = 0.0382$), glutamine ($p = 0.0034$), glycine ($p = 0.0148$), methionine ($p = 0.0053$), and glucose ($p = 0.0192$) (Fig. 3). In the female group, there is a significant difference between total bile levels in the control and HFD groups at both 9 months ($p < 0.001$) and 12th month ($p < 0.05$) (Fig. 3A). ANOVA

Table 3 Fecal Metabolites analyzed at 9th month and 12th month on both male and female samples. The fold change (FC) was calculated using HFD vs. Control. False discovery rate (FDR) is expressed as q value

	9th month						12th month					
	Male			Female			Male			Female		
Metabolites	P value	FDR (q)	FC	P value	FDR (q)	FC	p value	FDR (q)	FC	p value	FDR (q)	FC
2-Oxoglutarate	7.85E-01	9.34E-01	1.02	6.86E-02	1.78E-01	0.81	8.63E-02	4.75E-01	0.79	7.07E-03	1.56E-01	0.76
2-Oxoisocaproate	4.73E-01	8.32E-01	0.92	6.68E-01	8.64E-01	0.94	1.17E-01	3.96E-01	0.84	3.03E-02	1.11E-01	0.75
3-Methyl-2-oxovalerate	1.39E-02	6.12E-01	1.21	1.46E-03	2.14E-02	1.32	9.22E-01	9.66E-01	1.01	5.98E-01	8.22E-01	1.08
Acetate	5.00E-01	7.86E-01	1.14	4.02E-03	2.95E-02	0.55	8.86E-01	9.75E-01	0.97	3.18E-01	6.08E-01	0.79
Acetoin	4.05E-01	8.10E-01	1.05	1.27E-02	6.21E-02	1.18	5.23E-01	7.67E-01	1.05	4.51E-01	7.35E-01	1.09
Alanine	7.86E-01	9.10E-01	0.97	2.45E-01	4.90E-01	0.81	1.20E-01	3.77E-01	0.74	9.24E-03	6.78E-02	0.79
Asparagine	4.45E-01	8.16E-01	0.91	8.23E-01	8.83E-01	1.03	1.68E-01	4.35E-01	0.84	5.56E-01	8.15E-01	0.92
Aspartate	5.89E-02	3.70E-01	0.66	5.06E-02	1.59E-01	0.60	2.86E-01	5.24E-01	0.85	1.92E-02	1.06E-01	0.72
Butyrate	8.45E-01	9.07E-01	1.02	2.52E-01	4.82E-01	0.87	1.35E-01	3.96E-01	1.15	1.35E-01	3.30E-01	0.84
Cholate	5.02E-01	7.62E-01	0.96	4.11E-04	9.04E-03	1.45	4.27E-01	6.71E-01	1.08	7.08E-03	1.04E-01	1.37
Choline	3.25E-01	7.94E-01	0.90	4.73E-01	6.94E-01	0.88	6.63E-01	8.33E-01	0.95	8.10E-02	2.23E-01	0.86
Ethanol	1.23E-01	6.01E-01	1.22	3.01E-01	5.30E-01	1.13	5.74E-01	7.89E-01	1.05	6.75E-01	8.49E-01	1.06
Ethanolamine	4.41E-02	3.23E-01	0.85	5.20E-02	1.53E-01	0.80	3.24E-01	5.70E-01	1.35	6.52E-01	8.69E-01	1.14
Formate	1.68E-01	6.72E-01	0.73	7.62E-01	8.38E-01	1.08	9.72E-02	4.28E-01	1.67	9.04E-01	9.70E-01	0.96
Fumarate	3.68E-01	8.10E-01	0.69	1.31E-01	3.03E-01	0.15	7.32E-01	8.26E-01	0.84	1.51E-01	3.50E-01	0.53
Glucose	1.54E-02	2.26E-01	0.78	3.10E-01	5.25E-01	0.88	3.27E-01	5.33E-01	0.88	5.34E-01	8.10E-01	0.91
Glutamate	8.31E-01	9.38E-01	1.02	1.40E-02	6.16E-02	0.72	2.03E-01	4.70E-01	0.89	2.39E-02	9.56E-02	0.88
Glutamine	2.66E-01	7.80E-01	1.05	7.14E-01	8.49E-01	1.02	7.20E-01	8.34E-01	0.97	5.72E-01	8.12E-01	1.06
Glycine	6.39E-01	8.79E-01	0.96	5.60E-01	7.70E-01	0.94	3.24E-01	5.48E-01	1.16	9.97E-01	9.97E-01	1.00
Glycocholate	9.15E-01	9.59E-01	1.01	1.62E-04	7.13E-03	1.88	6.64E-01	8.12E-01	1.08	2.54E-01	5.08E-01	1.28
Histidine	9.34E-01	9.34E-01	0.99	2.56E-01	4.69E-01	0.82	9.58E-01	9.80E-01	1.01	7.46E-01	9.12E-01	1.04
Hypoxanthine	2.37E-01	7.45E-01	0.84	2.91E-03	2.56E-02	0.56	5.85E-02	6.44E-01	0.81	1.03E-02	6.47E-02	0.75
Isoleucine	3.76E-02	3.31E-01	1.14	2.51E-03	2.76E-02	1.58	2.17E-01	4.55E-01	1.18	7.37E-03	8.11E-02	1.51
Lactate	6.08E-01	8.63E-01	1.07	6.54E-01	8.72E-01	1.11	1.78E-01	4.35E-01	0.77	7.73E-03	6.80E-02	0.68
Lysine	6.47E-01	8.63E-01	0.95	3.52E-01	5.74E-01	0.91	1.07E-01	3.92E-01	0.79	8.87E-01	1.00E + 00	0.99
Methionine	9.27E-01	9.49E-01	0.99	6.55E-02	1.80E-01	0.87	5.76E-01	7.68E-01	0.95	8.25E-01	9.81E-01	0.98
N,N-Dimethylformamide	3.95E-01	8.28E-01	0.89	1.63E-01	3.42E-01	0.78	8.14E-02	5.12E-01	0.78	1.60E-01	3.35E-01	0.81
Phenylalanine	6.67E-01	8.63E-01	1.07	8.78E-01	8.98E-01	0.97	2.75E-01	5.26E-01	0.82	1.11E-01	2.87E-01	1.26
Proline	3.16E-01	8.18E-01	0.89	9.33E-01	9.33E-01	0.99	8.96E-01	9.62E-01	0.97	6.62E-01	8.57E-01	0.92
Propionate	3.55E-01	8.22E-01	1.04	2.45E-02	8.98E-02	0.82	5.66E-01	8.03E-01	0.93	6.21E-02	2.10E-01	0.60
Pyruvate	7.15E-01	8.99E-01	0.96	5.29E-03	3.33E-02	0.63	1.07E-01	4.28E-01	0.73	6.27E-03	2.76E-01	0.68
Sarcosine	8.42E-01	9.26E-01	0.98	7.05E-01	8.86E-01	1.04	5.86E-01	7.58E-01	0.96	8.74E-01	1.01E + 00	0.98
Serine	3.14E-01	8.64E-01	0.90	1.30E-01	3.18E-01	0.77	2.24E-02	4.93E-01	0.86	4.16E-01	7.04E-01	0.94
Succinate	3.69E-02	4.06E-01	0.81	1.80E-02	7.20E-02	0.72	9.20E-02	4.50E-01	0.85	6.96E-02	2.04E-01	0.84
Sucrose	1.46E-01	6.42E-01	-2.47	7.20E-01	8.34E-01	0.61	1.53E-01	4.21E-01	3.61	4.87E-01	7.65E-01	2.10
Tartrate	2.14E-01	7.24E-01	0.81	7.12E-01	8.70E-01	0.93	6.91E-01	8.22E-01	0.96	3.82E-01	7.00E-01	1.10
Taurine	4.93E-01	8.03E-01	1.12	8.72E-01	9.14E-01	1.04	5.05E-01	7.66E-01	0.92	6.86E-02	2.16E-01	1.45

Table 3 (continued)

	9th month						12th month					
	Male			Female			Male			Female		
Threonine	1.86E-01	6.82E-01	0.85	4.45E-01	6.99E-01	0.89	1.33E-02	5.85E-01	0.85	8.97E-01	9.87E-01	1.01
total bile acid	1.49E-02	3.28E-01	1.33	9.96E-03	5.48E-02	1.88	2.07E-01	4.55E-01	1.35	2.06E-02	1.01E-01	1.81
Tryptophan	4.12E-01	7.88E-01	0.91	5.35E-01	7.59E-01	0.90	2.91E-02	4.27E-01	0.73	9.85E-01	1.01E+00	1.00
Tyrosine	7.55E-01	9.23E-01	0.95	7.42E-01	8.37E-01	0.93	6.27E-02	5.52E-01	0.68	9.58E-01	1.00E+00	0.99
Uracil	5.06E-01	7.42E-01	0.46	4.54E-01	6.89E-01	0.51	2.51E-01	5.02E-01	2.01	3.89E-01	6.85E-01	2.42
Valine	4.78E-01	8.09E-01	1.03	1.34E-01	2.95E-01	1.24	9.74E-01	9.74E-01	1.00	2.24E-02	9.86E-02	1.28
Xanthine	1.06E-01	5.83E-01	0.82	4.04E-02	1.37E-01	0.67	7.28E-02	5.34E-01	0.81	1.55E-01	3.41E-01	0.85

results also exhibited the significant main effect of sex on lactate ($p=0.0121$), alanine ($p=0.0394$), valine ($p<0.0001$), cholate ($p<0.0001$), total bile ($p=0.0030$) and hypoxanthine ($p=0.0019$) (Fig. 4A). The interaction of time and sex was significant for glutamate and taurine ($p<0.05$). A significant difference in total bile levels was observed in the 9th month male and female HFD group (Fig. 4E). Similarly, a significant difference was also observed between male and female control groups for valine and cholate ($p<0.001$); however, this difference disappeared in HFD groups (Fig. 4C, D).

3.4 Liver metabolites at the 12th month

In the liver extracts, 33 metabolites were detected (Table 4), and both the male and female groups showed significant perturbation in many metabolites. The PCA score plots showed that both the male **(Fig.** 5A) and the female groups **(Fig.** 5B) had a clear difference between the control and the HFD groups with a slight overlap. The male group showed significant increase in glutamine, glutamate, methionine, pyruvate, and taurine after HFD ($p<0.01$, Table 4). The female group showed a similar significant increase in metabolites, including acetate, azelate, choline, glutamate, glutathione, lactate, methionine, and pyruvate ($p<0.05$, Table 4). Within these metabolites, only glutamate, methionine, and pyruvate are common for both male and female HFD mice. Many metabolites had a low level of significant changes, so a pathway analysis was carried out on all the altered fecal and liver metabolites.

3.5 Metabolic pathway analysis

The fecal metabolites in female mice showed significant alterations in glycine, serine, and threonine metabolism at the 9th month of dietary exposure, while the alanine, aspartate, and glutamate metabolic pathways showed significant changes at the 12th month ($p<0.05$). All these metabolic pathways in the male mice remained unaltered at all-time points **(Fig.** 6A, C). D-glutamine and D-glutamate pathways were significantly altered at the 9th month and 12th month time points in female mice ($p<0.05$). However, similar perturbation was not observed in male mice **(Fig.** 6B, D).

Liver metabolic pathway analysis, both male and female hepatic metabolites showed an increase of D-glutamine, D-glutamate, and glutathione metabolisms after HFD treatment. Furthermore, the male HFD group was also observed with altered glutathione, hypotaurine, and taurine metabolisms. A random forest analysis of the important metabolites for the male group was further carried out; glutamine, glutamate, methionine, pyruvate and taurine were found as the most important metabolites **(Fig.** 7A, B, C). In the female HFD group, glycine, pyruvate, serine, and threonine metabolism pathways were major contributors of metabolic dysfunction,

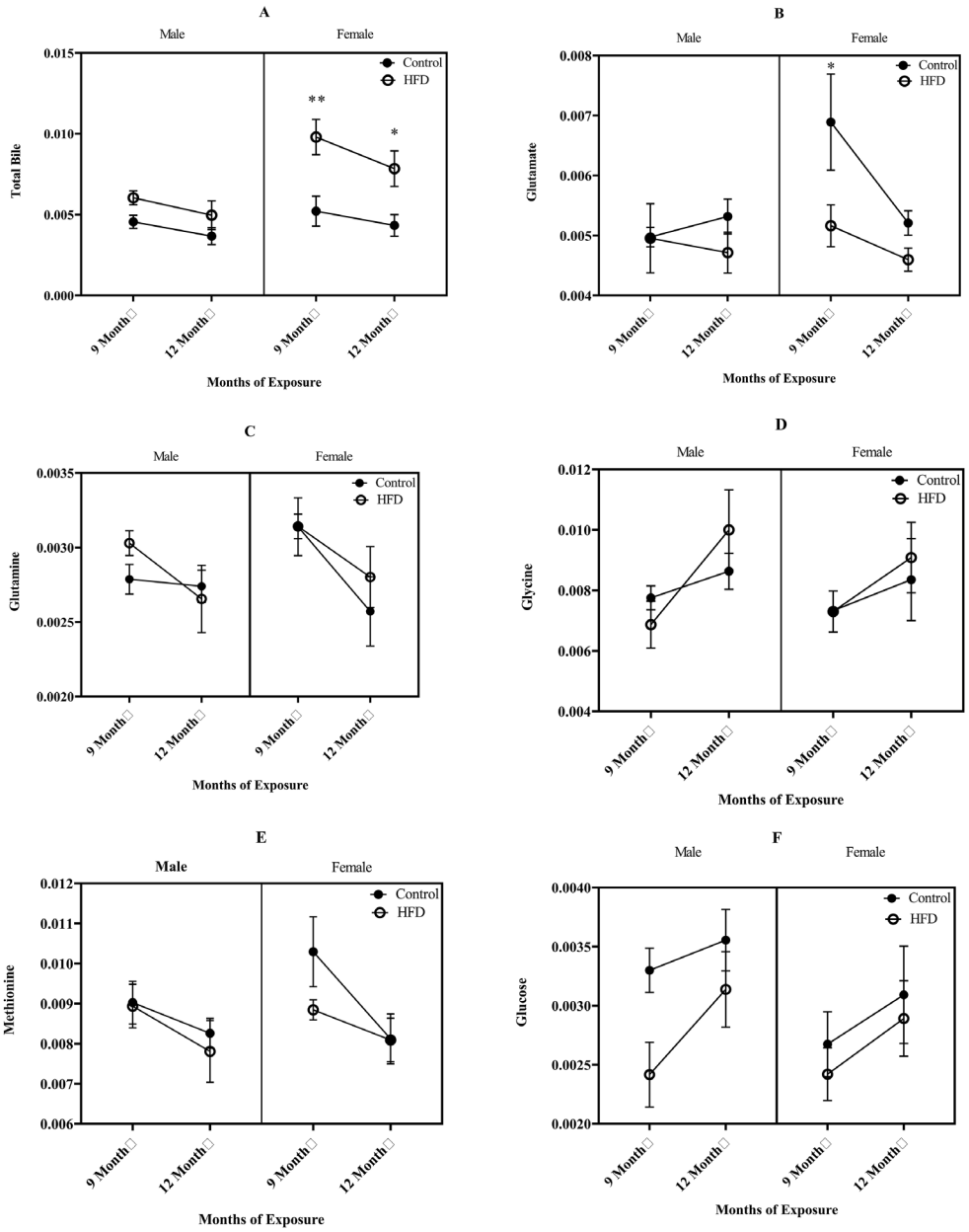


Fig. 3 The Effect of dietary treatment on key metabolites in male and female fecal samples. Data expressed are mean ± SEM **p < 0.01, *p < 0.05

while azelate, choline, and glutamate were found as important metabolites **(Fig.** 7D, E, F). In summary, both male and female mice had significant hepatic metabolic perturbation, but only the female groups showed significant fecal metabolic changes, which points towards a sex-specific metabolic response to the HFD exposure.

4 Discussion

The present study investigates the longitudinal sex-specific alterations in fecal metabolites and endpoint liver metabolites in HFD exposed C57BL/6J mice. A recent study by Hubbard and colleagues found that a prolonged HFD diet worsens sexually dimorphic obesity (Hubbard et al., 2019). The goal is to compare the sex difference in metabolites and identify specific biomarkers for each sex in both fecal and liver samples in obese mice.

From fecal metabolomic analysis, several biomarkers, such as BCAAs, short-chain fatty acids (SCFAs), and glutamate, were shown significant changes impacted by HFD. First, a s one of the important BCAAs, isoleucine, and its first degradation product 3-methyl-2-oxovalerate were observed to be significantly elevated across all time points (5th, 9th, and 12th months) in the female HFD group compared to its control group. Isoleucine plays an important role in the growth, protein and fatty acid metabolism, immunity, and regulation of key signaling pathways like mTOR (Zhang et

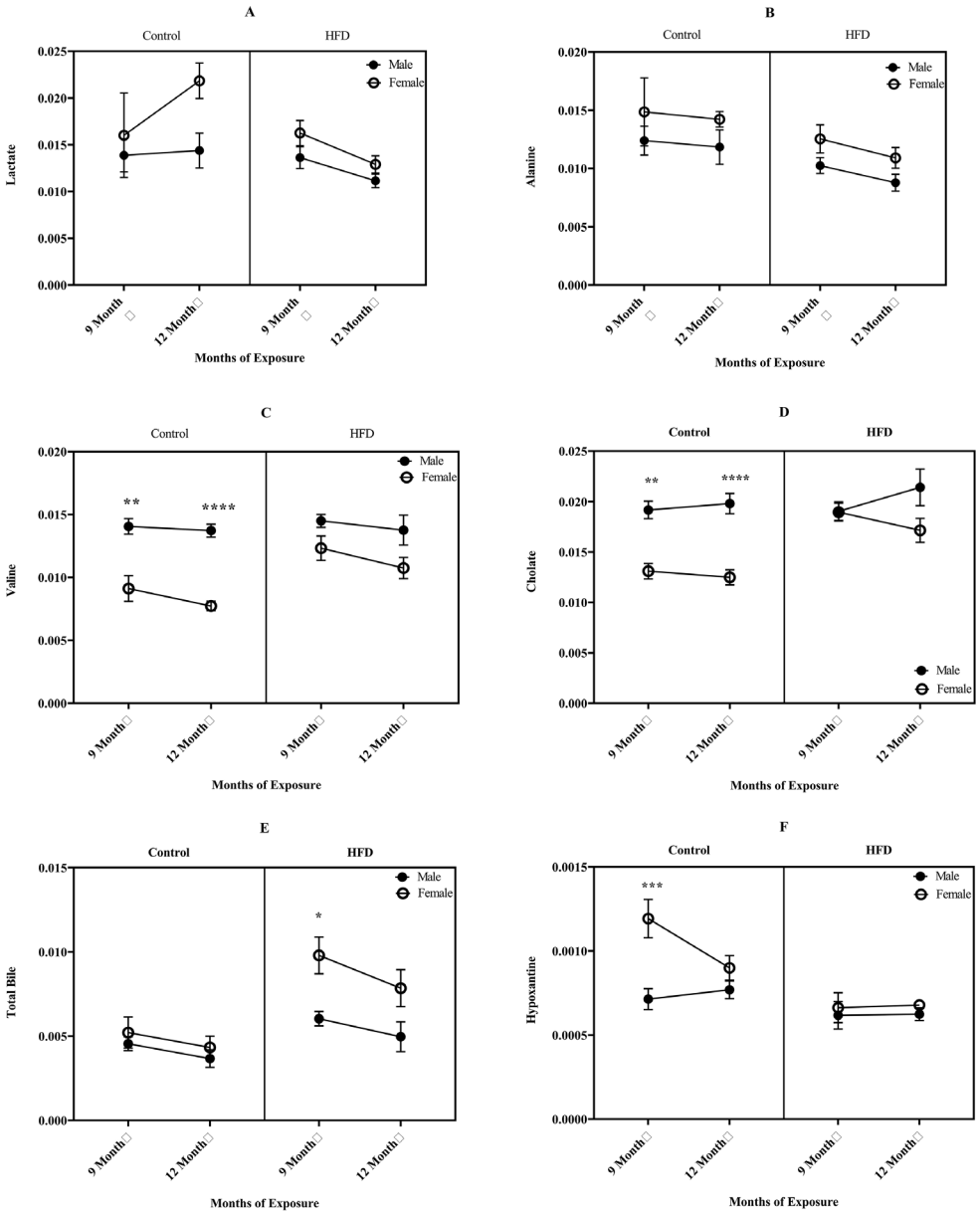


Fig. 4 Sex-specific differences on key metabolites while comparing trends in male and female fecal samples. Data expressed are mean ± SEM. ***p < 0.001, **p < 0.01, *p < 0.05

al., 2017). Amino acid levels in the body are tightly regulated, and very little is being excreted through the urine or feces. Therefore, elevated fecal isoleucine excretion in our study may indicate increased peripheral isoleucine in the host due to decreased amino acid catabolism or increased gut bacterial metabolism in the female HFD group. Recent studies have shown a strong correlation of BCAA levels with insulin resistance and blood glucose levels in obese human individuals in comparison to non-obese individuals(Allam-Ndoul et al., 2015; Lynch & Adams, 2014; White et al., 2021). Thus, increased fecal BCAA levels can be used as an effective biomarker for identifying early metabolic alteration associated with obesity in females.

Fecal SCFAs, such as acetate and propionate, were reduced significantly in 9th month female HFD fecal samples compared to its control. The gut bacteria produce SCFAs via fermentation of undigested dietary starch and fibers. SCFAs supplementation is considered a strategy to reduce weight, as it plays an important role in appetite regulation, lipid and glucose metabolism, and integrity of the gastrointestinal barrier. Moreover, propionate regulates lipolysis and lipogenesis rate in fat cells (Kimura et al., 2014). Decreased acetate and propionate indicate a decline in SCFA production by gut bacteria in female mice, which exacerbate the pathophysiology of obesity (Kimura et al., 2013).

Bile acid plays an important role in fat digestion, absorption. It serves as a signaling molecule keeping up a homeostatic state (Agellon, 2002; Zeng et al., 2019). Bile acid cholate is synthesized in the liver and is conjugated before

Table 4 The liver metabolites analyzed 12th month on male and female samples. The fold change (FC) was calculated using HFD vs. Control. False discovery rate (FDR) is expressed as a q value

	Male			Female		
Metabolites	p value	FDR (q)	FC	p value	FDR (q)	FC
3-Hydroxybutyrate	3.63E-01	5.21E-01	1.15	2.04E-01	3.54E-01	1.16
Acetate	2.15E-01	3.94E-01	1.22	5.01E-02	2.07E-01	1.33
Alanine	4.53E-01	5.54E-01	1.08	1.95E-01	3.79E-01	1.15
Aspartate	3.69E-01	5.07E-01	2.05	6.15E-02	2.03E-01	3.11
Azelate	8.43E-02	3.48E-01	1.18	1.45E-02	9.57E-02	1.21
Choline	7.51E-02	3.54E-01	1.63	4.35E-04	1.44E-02	1.30
Creatine	1.63E-01	3.59E-01	1.34	6.52E-02	1.96E-01	1.21
Creatinine	2.41E-01	4.19E-01	1.47	2.37E-01	3.56E-01	1.10
Fumarate	3.00E-01	4.95E-01	1.76	6.69E-01	7.61E-01	1.29
Glucose	8.80E-01	9.37E-01	0.99	9.77E-01	9.77E-01	1.00
Glutamate	5.68E-03	4.69E-02	1.78	1.20E-02	9.90E-02	1.21
Glutamine	3.89E-04	1.28E-02	1.64	6.97E-02	1.92E-01	1.17
Glutathione	1.02E-01	3.37E-01	1.18	2.34E-02	1.29E-01	1.24
Glycine	9.37E-02	3.44E-01	1.12	1.45E-01	3.19E-01	1.07
Histidine	5.10E-01	6.01E-01	0.81	1.30E-01	3.30E-01	0.60
IMP	1.95E-01	3.79E-01	0.49	2.02E-01	3.70E-01	0.08
Inosine	3.58E-01	5.37E-01	1.15	2.18E-01	3.43E-01	1.09
Isoleucine	9.92E-01	9.92E-01	1.00	5.34E-01	6.29E-01	1.08
Lactate	9.57E-01	9.87E-01	1.00	9.06E-03	9.97E-02	1.18
Leucine	1.50E-01	3.54E-01	1.23	1.46E-01	3.01E-01	1.13
Lysine	1.72E-01	3.55E-01	1.25	3.06E-01	4.21E-01	1.09
Malonate	1.18E-01	3.25E-01	1.68	1.38E-01	3.25E-01	1.25
Maltose	1.16E-01	3.48E-01	0.89	2.17E-01	3.58E-01	0.92
Methionine	1.05E-03	1.16E-02	1.38	3.39E-02	1.60E-01	1.19
Nicotinurate	3.47E-01	5.45E-01	0.72	4.22E-01	5.36E-01	0.89
Phenylalanine	8.16E-01	8.98E-01	0.92	2.84E-01	4.07E-01	0.71
Propylene glycol	3.69E-01	4.87E-01	0.81	6.81E-01	7.49E-01	1.09
Pyruvate	7.41E-03	4.89E-02	1.53	4.03E-03	6.65E-02	1.21
Sarcosine	4.06E-01	5.15E-01	1.66	8.21E-01	8.47E-01	1.04
Succinate	7.07E-02	3.89E-01	1.48	4.07E-01	5.37E-01	1.19
Taurine	3.96E-04	6.53E-03	1.33	5.17E-01	6.32E-01	1.03
Tyrosine	7.30E-01	8.31E-01	1.09	7.92E-01	8.43E-01	1.04
Valine	1.45E-01	3.68E-01	1.23	5.57E-02	2.04E-01	1.14

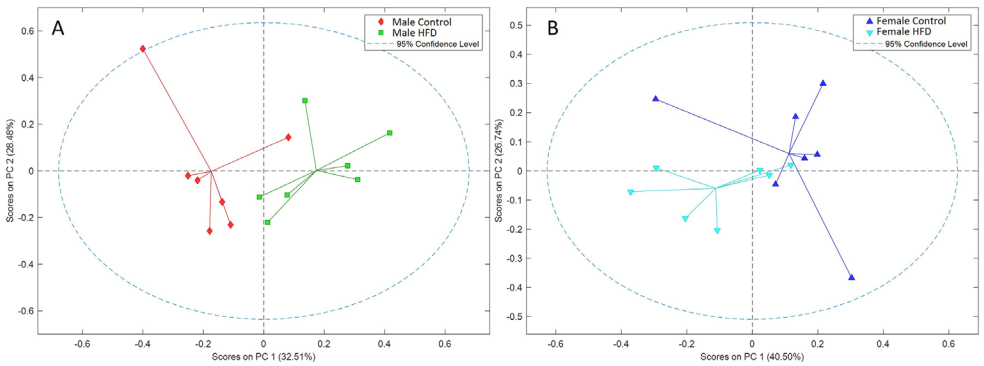
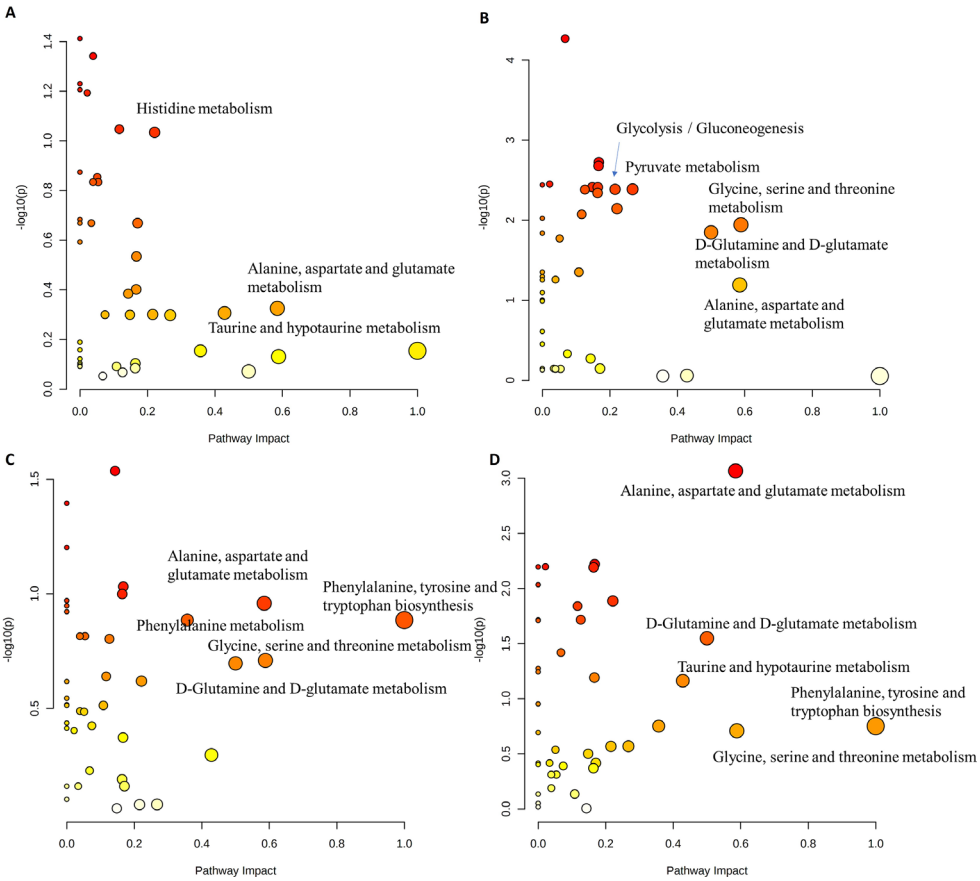


Fig. 5 The PCA score plots on the liver samples. **A**, the male samples (Control vs. the HFD). **B**, female samples (Control vs. the HFD)

secreting into the small intestine. About 95% of bile acid secreted into the small intestine is reabsorbed and recirculated through enterohepatic circulation. The leftover portion is passed on to the distal intestine, which is further transformed into secondary metabolites by gut microbes. Thus, little bile acids are lost in the feces (Chiang, 2013; Lin et al., 2019a). However, long-term consumption of HFD increased the secretion of bile acid and its secondary products. A study done only in male rats showed increased bile acids secretion and fats absorption in the development of hepatic steatosis



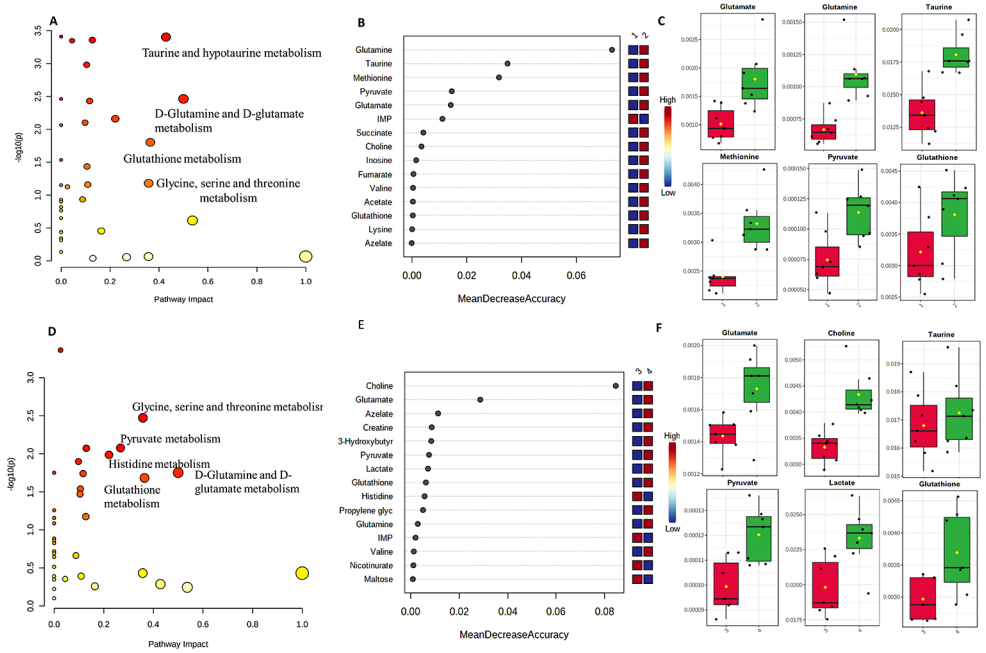


Fig. 6 The fecal metabolic pathway analysis using the plot of the global t-test vis the pathway impact. The pathways with higher impacts and lower p values are considered as imported in the plot. **A**, Male samples at 9th month. **B**, Female samples at 9th month. **C**, Male samples at 12th month. **D**. Female samples at 12th month. The -log(p) is 1.3 when p-value is 0.05

Fig. 7 The liver extracts metabolites analysis for control vs. HFD. **A**, The metabolic pathway analysis using the plot of the global t-test vis the pathway impact for the male samples. The pathways with higher impacts and lower p values are considered as imported in the plot. The -log(p) is 1.3 when p-value is 0.05. **B**, The random forest analysis of the important metabolites for the male samples, and the high mean decrease accuracy mean high importance in the classification. **C**, The box plot of selected metabolites in the male group where red represent control and green HFD group. **D**, the same as figure A for the females. **E**, The same plot as B for females. **F**, The box plot of selected metabolites in the female group. The group numbers are as follows, 1, Male control, 2, Male HFD, 3, Female control and 4, female HFD

(Hori et al., 2020). Non-alcoholic steatohepatitis (NASH) patients consuming high fat diet showed increased serum and urine bile metabolites in NASH patients compared to healthy individuals (Ferslew et al., 2015). Our study showed significantly increased levels of total bile acid, cholate, and glycocholate in both the 9th and 12th month of female HFD fecal samples compare to its control, but not in male HFD mice. Furthermore, the impact of HFD on bile acid catabolism is apparently sex-specific demonstrated by our study. All these findings imply that chronic HFD consumption is evident to hamper the bile acid metabolism, and the elevated bile acids secretion may further lead to intestinal hyperpermeability (Murakami et al., 2016; Yoshitsugu et al., 2019, 2020). The HFD induced altered bile acid level maybe also due to increased gut microbial communities, which are essential to convert conjugated bile acids into secondary metabolites (Lin et al., 2019b).

Glutamate is an important marker in both fecal and liver metabolites. It plays an important role in maintaining intestinal integrity, synthesis of glutathione, enteric bacterial nitrogen metabolism, and brain health (Mazzoli & Pessione, 2016; Newsholme et al., 2003). A cross-sectional study showed lower fecal glutamate levels in obese individuals (sex unspecified) compared to lean subjects (Palomo-Buitrago et al., 2019). Intestinal glutamate is interchangeable with glutamine, which fuels enterocytes (Burrin & Reeds, 1997). In our study, the female HFD group showed significantly lower levels of glutamate in the 9th and 12th month's fecal samples. The reduced fecal glutamate level in HFD female group may be due to the increased intestinal absorption and liver utilization of glutamate (Blachier et al., 2009; Yang et al., 2016; Windmueller & Spaeth, 1975). Moreover, glutamate is also used as substrate by enteric anaerobic bacteria to produce acetate and butyrate (Lombardi & Dicks, 2022; Mardinoglu et al., 2015). Our study showed a trend of decreased acetate and butyrate in both male and female HFD mice compared to their controls ($p > 0.05$). Our finding in general is supported by the fact that obese individuals have reduced glutamate fermenting enteric bacterial abundance (Liu et al., 2017). Therefore, reduced fecal excretion especially in female HFD group is due to increased intestinal glutamate catabolism and altered bacterial fermentation of glutamate.

In contrast with decreased fecal glutamate level, both the male and female HFD liver extracts showed significant increase of glutamine and glutamate levels compare to their controls. Liver is central in nitrogen metabolism where glutamine is catabolized to produce glutamate and ammonia. Glutamate further enters TCA cycle and act as a substrate for gluconeogenesis (Newsholme et al., 2003), while ammonia generated detoxified through urea cycle. The long-term HFD exposure leads to increased level of glutamine synthetase in the liver of male C57BL/6 mice (Soontornniyomkij et al., 2016). It also leads to fatty liver, which further reduces liver ammonia detoxification capacity (Green & Hodson, 2014). In such circumstances, hepatotoxicity is avoided through alternative metabolomics pathway. This pathway synthesize glutamine through glutamate while utilizing the ammonia (Soontornniyomkij et al., 2016; Varani et al., 2022). Increased liver glutamine and glutamate levels in both male and female HFD groups resonates the effect of chronic HFD feeding on liver detoxification capacity and adaptation to the alternative glutamine synthesis(Ruiz et al., 1991). Furthermore, glutamate is a precursor for glutathione which is an important antioxidant. Elevated levels of glutathione found in female HFD liver samples only indicates more active defensive mechanism was used to overcome liver dysfunction in female HFD group (Ghosh et al., 2011; Yu et al., 1999).

In conclusion, this study implemented a systematic comparison of metabolites between sexes using HFD exposed C57BL/6J mice. In our study, female HFD mice exhibited less HFD induced weight gain compared to male mice over time but also a more discernable perturbation and more robust changes in metabolites. Results from our study of obesity-related changes over time may be overall significant in establishing early fecal and liver biomarkers as they may be first discerned and for their sex-specific characteristics. Future studies will investigate the sex-specific gut microbial composition and gut-liver axis of key metabolites with larger sample size, preferably $n \geq 15$, as well as include basic chemistry data such as plasma cholesterol or triglyceride levels to help link the phenotypes with the key metabolites. Such information will likely provide additional insight on molecular mechanisms of HFD-induced specific responses.

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Author contributions BW and VJ performed metabolomics experiments and data analysis. BW, VJ, and JH wrote the manuscripts. AO, EC, and AC carried out mice husbandry and physiological experiments. SHH and AMD revised the manuscript. JH, BW, and AMD designed the experiments and provided general project guidance for students. JLG revised the manuscript and supported the graduate student AO to conduct the research.

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Data availability The raw data supporting the conclusions of this article will be made available by the authors without undue reservation.

Declarations

Competing interests The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest. All authors have read the version and approved it to be published.

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