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Chronic exposure to parabens promotes non-alcoholic fatty liver disease in association with the changes of the gut microbiota and lipid metabolism†

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Non-alcoholic fatty liver disease (NAFLD) has become a serious public health issue due to changing dietary patterns and composition. However, the relationship between NAFLD occurrence and food additives, such as preservatives, remains unknown. This study aimed to evaluate the toxicity of parabens, namely methylparaben (MeP) and ethylparaben (EtP), in relation to NAFLD occurrence in mice under different dietary conditions. Exposure to MeP and EtP exacerbated high-fat diet (HFD)-induced obesity, glucose intolerance, higher serum lipid concentrations, and fat accumulation by upregulating genes involved in lipid metabolism. Untargeted metabolomics revealed that arachidonic acid (AA) metabolism was the top enriched pathway upon MeP and EtP exposure in the presence of HFD. 11,12-Epoxyeicosatrienoic acid (11,12-EET) was the most abundant AA metabolite and was significantly reduced upon exposure to MeP or EtP. Moreover, an integrative analysis of differential fecal taxa at the genus level and serum AA metabolites revealed significant associations. In addition, MeP and EtP enhanced lipid accumulation in AML12 cells and HepG2 cells cultured with oleic acid. 11,12-EET supplementation could significantly alleviate lipid accumulation by suppressing the expression of lipid metabolism-related genes and proteins. The present study suggests that chronic exposure to MeP and EtP promoted NAFLD *via* gut microbiota-dependent AA metabolism. These results highlight the need for reducing oral exposure to synthetic preservatives to improve metabolic disturbance under HFD conditions.

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1. Introduction

Non-alcoholic fatty liver disease (NAFLD) is a major public health concern affecting approximately 25% of the global population.[1] It is one of the most common hepatic diseases worldwide and is increasing owing to its close association with obesity and insulin resistance.[2] Excess caloric consumption, which leads to obesity and related comorbidities, is a primary risk factor for NAFLD. Intensive studies showed that the gut microbiota plays a vital role in the pathophysiology of metabolic diseases, including NAFLD, through the gut–liver axis.[3,4] In addition, dietary composition, such as fructose and polyunsaturated or monounsaturated fats, could promote NAFLD by altering the gut microbiota, increasing intestinal permeability and elevating hepatic inflammation and lipid peroxidation.[5] Interestingly, several environmental pollutants, such as organochlorine pesticides,[6] polyfluoroalkyl substances,[7] and microplastics,[8] have been shown to alter lipid influx–efflux balance in the liver and promote hepatic inflammation, steatosis and NAFLD. However, the potential toxicity of many food additives known to be associated with the development of NAFLD, such as preservatives, remains unknown.

Food additives are extensively used in the food industry to improve food quality and safety during processing, storage and packing.[9] Parabens, including methyl- (MeP), ethyl- (EtP), propyl- (PrP), butyl- (BuP), and benzyl-parabens (BeP), are among the most widely used preservatives in foodstuffs, cosmetics and pharmaceuticals due to their antimicrobial properties.[10] MeP and EtP were found in all of the milk-based infant formulas, while MeP, EtP, and BeP were detected in 85.6–100% of cereal-based complementary food.[11] The median concentrations of MeP, EtP, PrP, BuP and BeP in sewage sludge

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from China were 66, 12, 9, 8 and 2 ng g^{-1}, respectively.[12] The predominant paraben in adult and pregnant women's urine was also MeP, followed by EtP, PrP, BuP and BeP; their median concentrations were 5.78, 0.39, 0.35, 0.01 and 0.02 μg L^{-1}, respectively.[13,14] Additionally, positive correlations were found between concentrations of MeP and EtP in paired dust and urine samples.[15] This highlights the need to investigate the potential health risks of parabens.

Increasing evidence from epidemiological and toxicological studies showed that paraben exposure might be associated with metabolic disorders, including obesity and diabetes mellitus (DM). Dietary exposure to parabens was positively associated with overweight/obesity in adolescent girls.[16] MeP and EtP exposure increased the risk of DM; EtP showed positive associations with a higher risk of obesity.[17] Urinary EtP was associated with gestational DM, with risk ratios of 1.12, 1.11 and 1.70 for the second, third and highest quartiles, respectively.[18] A positive association was found between maternal urinary concentrations of BuP and childhood overweight within the first eight years of life, and maternal exposure to BuP increased weight in the offspring of female mice.[19] However, the effects of paraben exposure on the onset of NAFLD and the underlying molecular mechanism have not been clearly elucidated.

In this study, the effects of paraben exposure on the development of NAFLD were investigated in female mice under normal diet (ND) and HFD conditions by screening typical NAFLD symptoms, including glucose intolerance, lipid accumulation, liver inflammation and the expression of gluconeogenesis, glycolysis, adipogenic differentiation, fatty acid intake, beta oxidation, lipogenesis, and inflammation related genes. In addition, serum metabolic profiling and the gut microbiota upon MeP and EtP exposure were investigated to delineate the molecular mechanism and seek an effective intervention. 11,12-EET was found to be the most abundant metabolite involved in the AA metabolism pathway, identified by serum metabolomics and fecal metagenomics. Furthermore, the potential protective role of the AA metabolite 11,12-EET against lipid metabolism disorder was verified *in vitro*.

2. Materials and methods

2.1. Animal experiments

Sixty female SPF C57BL/6J mice, 3 weeks old, were purchased from the Medical Laboratory Animal Center of Nanjing Medical University, and housed with a 12-hour light/dark cycle, with no restrictions on their food or water, under controlled conditions of humidity (50% ± 5%) and temperature (22 ± 2 °C). All the experimental programs for mice have been approved by the Nanjing Medical University Institutional Animal Care and Use Committee (IACUC-2207017).

A total of 60 mice were randomly divided into 6 groups, each containing 10 mice (5 mice per cage). A schematic diagram of the experimental study design is shown in Fig. 1A. After one week of adaptation, the animals were gavaged with 100 mg per kg per day MeP (purity > 99.0%, Tokyo Chemical Industry Co.), 100 mg per kg per day EtP (purity > 99.0%, Tokyo Chemical Industry Co.), or vehicle (olive oil) for 12 weeks. This dose is 10-fold that of the current recommended acceptable daily intake for the sum of MeP, EtP, and their sodium salts by the European Food Safety Authority.[20] Parabens were dissolved in ethanol at a concentration of 1.0 g mL^{-1} and then diluted with olive oil to achieve a final concentration of 10 mg mL^{-1}. The mice received a daily gavage of 10 μL per g bw, equivalent to 100 mg per kg bw paraben. After exposure to Mep or EtP for 4 weeks, mice were fed with normal diet (ND, XTCON50J, 10% fat) or high-fat diet (HFD, XTHF60, 60% fat) until sacrifice (lasting for 8 weeks). ND and HFD were updated daily without restricting mice to arbitrary consumption. The composition of ND and HFD (Jiangsu Xietong Pharmaceutical Bioengineering Co., Ltd, Nanjing, China) is listed in ESI Table 1.† Mice were weighed daily and the stool samples were collected at 12 weeks for subsequent metagenomics analysis. Blood was collected after 12 h fasting at 12 weeks. After exsanguination, the heart, liver, spleen, kidneys, colon, small intestine, large abdominal omental fat pad and periuterine fat pad were precisely dissected, weighted and immediately immersed in liquid nitrogen and they were stored at −80 °C for further analysis.

2.2. Oral glucose tolerance test (OGTT)

OGTT was performed according to a previous study with minor modification.[21] At 14 weeks of age, mice underwent oral gavage with a dextrose solution at a dose of 1.5 g kg^{-1} after overnight fasting. Blood glucose was measured using a glucometer (Bayer Healthcare Co. Ltd, Germany) before (0 min) and after (15, 30, 60, 90, and 120 min) the glucose test.

2.3. Biochemical assays

Serum biochemical indices including aspartate aminotransferase (AST), alanine aminotransferase (ALT), triglyceride (TG), total cholesterol (TC) and glucose contents were detected using an automatic biochemical analyzer (Chemray 800, Rayto Life and Analytical Sciences Co., Shenzhen, China). Serum insulin levels were measured using an ELISA kit from Uscn Life Science Inc. (Wuhan, China). Thawed liver tissue was cut, weighted, and homogenized for hepatic TG and TC analysis using assay kits from Jiancheng Bioengineering Institute (Nanjing, China). Hepatic 11,12-EET content was analyzed using an ELISA kit from Coibo Bio Biotech Technology Co., Ltd (Shanghai, China).

2.4. Histopathology

Liver, colon and adipose tissues were collected and immediately fixed with 4% paraformaldehyde for 24 h. Then, these fixed tissues were paraffin-embedded and sectioned at a thickness of 4 μm. Deparaffinized sections were stained with hematoxylin and eosin (H&E). Subsequently, all sections were observed and analyzed using a Pannoramic digital slide

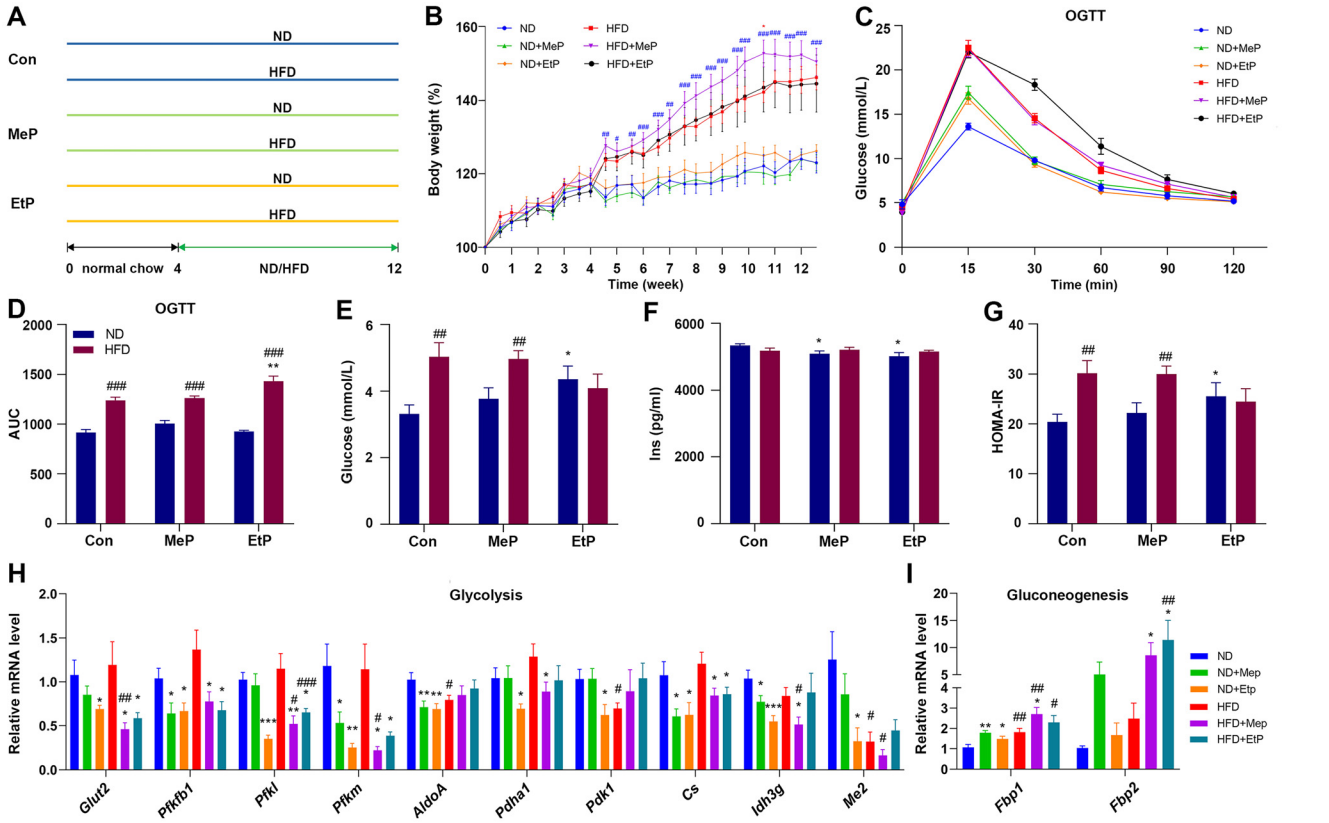

Fig. 1 Paraben exposure disrupted aggravated glucose metabolism in mice fed with ND or HFD. (A) Schematic diagram of the experimental study design. (B) Weight change data is expressed as the percentage change from the starting body weight. Mice were fasted overnight for the oral glucose tolerance test (OGTT). (C) OGTT curve and (D) area under curve. (E) Serum glucose, (F) insulin, and (G) homeostasis model assessment-insulin resistance (HOMA-IR). The relative expression of genes involved in (H) glycolysis (*Glut2, Pfkfb1, Pfkl, Pfkm, AldoA, Pdha1, Pdk1, Cs, Idh3g* and *Me2*) and (I) gluconeogenesis (*Fbp1* and *Fbp2*). Data are presented as the mean ± SEM and analyzed by ordinary one-way ANOVA with Tukey's multiple comparisons. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, compared with the respective control; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$, compared with the ND group.

scanner (Pannoramic SCAN, 3DHISTECH Kft, Budapest, Hungary).

2.5. 16S rRNA sequencing and analysis

Stool samples were collected to extract DNA. The V3–V4 hypervariable region of the bacterial 16S rRNA gene was selected for amplification using 338F-806R and sequenced by I-Sanger Inc. (Shanghai, China). The data were analyzed according to our previous study.[22] Briefly, partial least squares discriminant analysis (PLS-DA) was performed using the Bray–Curtis algorithm based on the operational taxonomic unit (OTU) level. Phylogenetic Investigation of Communities by Reconstruction of Unobserved States 2 (PICRUSt 2) was used to predict the metagenome function. Statistical Analysis of Metagenomic Profiles (STAMP) was used to identify the significantly disrupted pathways.

2.6. Serum metabolomes and data processing

Serum metabolic profiling was analyzed using a UHPLC Ultimate 3000 system coupled with a Q Exactive hybrid quadrupole-orbitrap mass spectrometer (UPLC-MS) according to our previous study.[22] Briefly, thawed serum samples (50 μL) were spiked with ice-cold methanol (150 μL). The quality control (QC) sample was prepared by pooling an aliquot of the same volume from each sample. Data acquisition and analysis were performed using SIEVE and SIMCA-P 14.0 software. PLS-DA was performed for detecting the distributions of different groups, their classifications, and comparisons among different groups. Variables with VIP > 1.0 and $P < 0.05$ were considered statistically differential metabolites. Pathway analysis was performed by using MetaboAnalyst 5.0 (**https://www.metaboanalyst.ca**) and the Kyoto encyclopedia of genes and genomes (KEGG) database ($P < 0.05$).

2.7. Cell culture and treatment

AML12 cells and HepG2 cells were obtained from YuChi Biology Co., Ltd (Shanghai, China) and Shanghai Institute of Cell Biology, Chinese Academy of Sciences (Shanghai, China), respectively. AML12 cells were cultured in the AML12 complete medium (YuChi Biology Co., Ltd, Shanghai, China) containing Dulbecco's Modified Eagle's medium/nutrient mixture F-12 (DMEM/F12) basal medium, 10% fetal bovine serum (FBS), 1%

tryptic iron–selenium transfer protein, and 40 ng mL^{-1} dexamethasone at 37 °C in a 5% CO_2 incubator. HepG2 cells were cultured in DMEM (TransGen Biotech Co., Ltd, Beijing, China) containing 20% FBS. Oleic acid (OA) is commonly employed to induce steatosis *in vitro* for its ability to produce morphological similarities to steatotic hepatocytes.[23] Cells were seeded at a density of 5×10^5 cells per mL in a 6-well plate, incubated for 24 h, and treated with 0.5 mmol L^{-1} OA (Sangon Biotech. Co., Ltd, Shanghai, China) alone or combined with 10 nmol L^{-1} 11,12-EET (MedBio Pharmaceutical Technology Inc., Shanghai, China) or 1 μmol L^{-1} parabens (MeP or EtP) for 24 h.

2.8. Quantification of 11,12-EET, TG and TC content

The culture medium was collected for 11,12-EET analysis after centrifugation at 1000 rpm for 10 min with an ELISA kit from Coibo Bio Biotech Technology Co., Ltd (Shanghai, China). The cell precipitate was homogenized in 0.2 mL of ice-cold PBS; the protein concentration was determined using a BCA protein assay kit (Beyotime Biotech. Co., Ltd, Shanghai, China). Then, intracellular TG and TC levels were determined with assay kits from Jiancheng Bioengineering Institute (Nanjing, China). In addition, lipid accumulation in these cells was assessed microscopically using Oil Red O staining (Beyotime Biotech. Co., Ltd, Shanghai, China) and quantified by lipid droplets of whole cell from each well with Image-Pro Plus 6.0.

2.9. RNA extraction and qPCR for gene expression

Total RNA from liver tissue, AML12 cells, or HepG2 cells was extracted using the TRIzol reagent (Tiangen Biotech, Beijing). RNA quality was determined using a NanoDrop 2000 spectrophotometer, and then 1 μg and RNA from each sample was used for reverse transcription into cDNA using HiScript II Q RT SuperMix (Vazyme Biotech, Nanjing, China). The primers, listed in ESI Table 2,† were synthesized by GenScript (Nanjing, China). *GAPDH* was used as the internal reference gene to calculate the results.

2.10. Western blot

Protein extraction from cell samples was performed using a protein extraction kit (KeyGEN Biotech, KGB5303-50), and concentrations were determined with the BCA protein assay reagent. Protein samples were boiled at 95 °C for 5 min with an upwelling buffer, resolved by 10% SDS-PAGE, and transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA). The membranes were incubated overnight at 4 °C with rabbit anti-Cpt1a (1 : 1000, Proteintech, 15184-1-AP), rabbit anti-PPARγ (1 : 1000, Proteintech, 16643-1-AP) or rabbit anti-GAPDH (1 : 1000, Proteintech, 10494-1-AP). Subsequently, the membranes were then incubated with the respective secondary antibodies; the immune complex was detected using an enhanced chemiluminescence immunoblotting assay kit. ImageJ was used for semi-quantitative determination of protein expression.

2.11. Statistical analysis

The data of body weight, organ weight, biochemical parameters, and gene expression are expressed as mean ± SEM and they were analyzed using ordinary one-way ANOVA with Tukey's multiple comparisons. The Wilcoxon rank-sum test was performed to compare taxon abundance after false discovery rate (FDR) adjustment. The predicted functions of the metagenome were determined using Welsh's *t*-test after FDR adjustment. The correlation between metagenomic and metabolomic or serum chemistry data was analyzed using the Mantel test and Spearman rank test. A significance threshold of $P < 0.05$ was considered statistically significant.

3. Results

3.1. Paraben exposure aggravated glucose intolerance in HFD-fed mice

Expectedly, mice fed with HFD exhibited higher body weight; MeP enhanced this elevation (Fig. 1B). Neither MeP nor EtP affected the body weight of mice fed with ND. HFD impaired glucose tolerance, and EtP exposure aggravated this effect (Fig. 1C and D). EtP significantly increased fasting blood glucose and homeostasis model assessment-insulin resistance (HOMA-IR) in mice fed with ND (Fig. 1E and G). MeP and EtP also reduced serum insulin levels in ND-fed mice (Fig. 1F). MeP and EtP exposure downregulated hepatic mRNA expression of several gluconeogenesis genes, such as *Pfkfb1*, *Pfkm*, *Cs* and *Idh3g*, in ND-fed mice; HFD exacerbated this effect (Fig. 1H). MeP and EtP upregulated the expression of *Fbp1* and *Fbp2* in mice fed with ND or HFD, respectively (Fig. 1I).

3.2. Paraben exposure promoted HFD-induced NAFLD

No significant effects of MeP or EtP exposure on heart weight, colon length, small intestine length or histopathological changes of the colon were observed (Fig. S1†). EtP exposure enhanced HFD-induced weight gain of the spleen and kidneys. HFD-fed mice showed higher weight of the livers; animals coexposed to HFD and MeP showed the significantly heavier livers (Fig. 2A). Histopathological analysis showed slight microvesicular steatosis in the livers of HFD-fed mice; paraben exposure, especially EtP, exacerbated this phenotype (Fig. 2B). HFD increased TC but not TG contents in the liver and serum (Fig. 2C–F). MeP exposure consistently increased hepatic levels of TG (Fig. 2C) and TC (Fig. 2D) in mice fed with ND or HFD. In parallel, MeP exposure increased serum TG and TC in HFD-fed mice (Fig. 2E and F). EtP exposure could aggravate HFD-induced elevation of hepatic TG (Fig. 2C) and serum TC (Fig. 2F). In addition, MeP enhanced the elevation of abdominal fat (Fig. 2G) and periuterine fat (Fig. 2H) of HFD-fed mice. HFD enlarged the adipocyte diameter in the periuterine fat; this effect was more pronounced in the MeP and EtP groups (Fig. 2I and J).

To confirm these effects, the expression of hepatic genes involved in lipid metabolism and inflammation was investi-

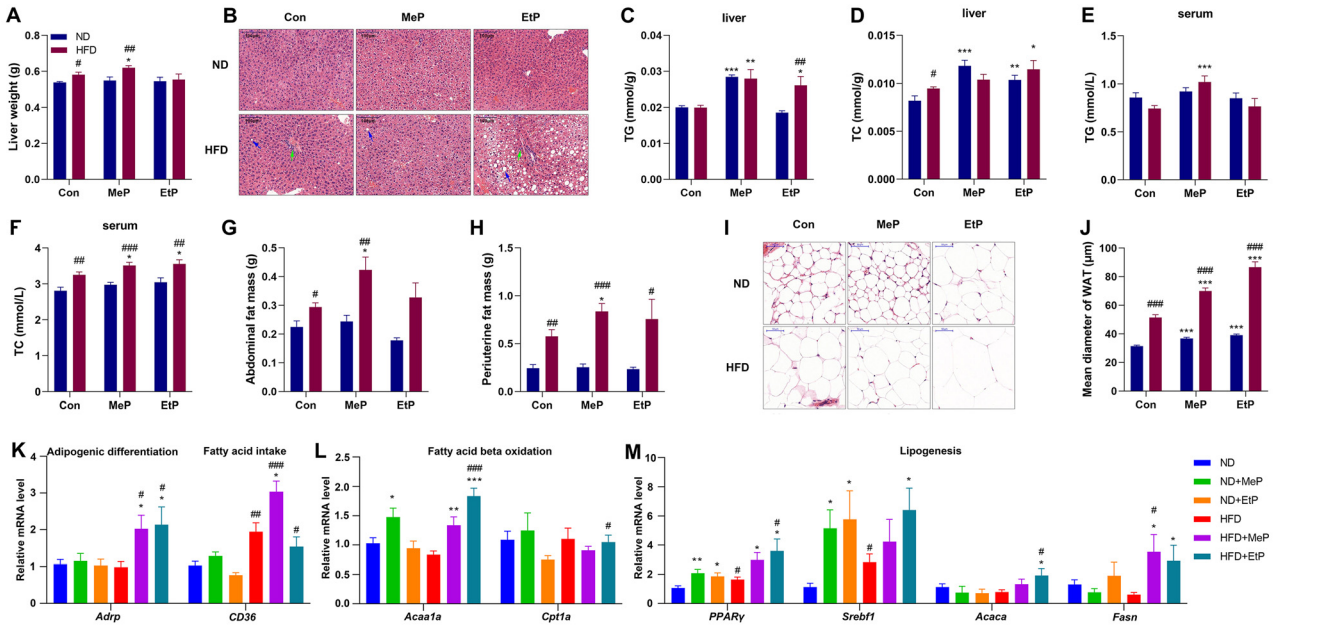

Fig. 2 Paraben exposure triggered hepatic steatosis in mice fed with ND or HFD. (A) Liver weight. (B) Representative histopathological observation of liver sections. The tissue was stained with hematoxylin and eosin (H&E) and observed by optical microscopy. Blue arrow, lipid droplets; green arrow, inflammatory cells; scale bar: 100 μm. (C) Hepatic triglyceride (TG) and (D) total cholesterol (TC) contents. (E) Serum TG and (F) TC contents. (G) Abdominal fat mass and (H) periuterine fat mass. (I) Representative histopathological observation of periuterine fat, scale bar: 50 μm. (J) Quantification of adipocyte diameter. The relative expression of genes involved in (K) adipogenic differentiation (*Adrp*) and fatty acid intake (*CD36*), (L) fatty acid beta oxidation (*Acaa1a* and *Cpt1a*) and (M) lipogenesis (*PPARγ*, *srebf1*, *Acaca* and *Fasn*). Data are presented as the mean ± SEM and analyzed by ordinary one-way ANOVA with Tukey's multiple comparisons. $^{*}P < 0.05$, $^{**}P < 0.01$, compared with the respective control; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$, compared with the ND group.

gated. In ND-fed mice, MeP increased the expression of *Acaa1a* (Fig. 2L), and both MeP and EtP significantly upregulated the expression of lipogenesis genes, including *PPARγ* and *srebf1* (Fig. 2M). Additionally, MeP and EtP induced the expression of genes involved in adipogenic differentiation (*Adrp*), fatty acid beta oxidation (*Acaa1a*), and lipogenesis (*PPARγ* and *Fasn*) in HFD-fed mice (Fig. 2K–M). Compared with ND-fed mice, the increased expression of *Adrp*, *CD36*, *Acaa1a*, *Cpt1a*, *PPARγ*, *Acaca* and *Fasn* was observed in HFD-fed mice tested with Mep or EtP (Fig. 2K–M). EtP exposure significantly enhanced the increase in serum ALT but not AST in HFD-fed mice (Fig. S2A and B†). MeP exposure upregulated the expression of *Adgre1* and *MCP-1*; EtP exposure induced the expression of *Adgre1* in the liver of ND-fed mice (Fig. S2F and G†). EtP exposure aggravated the upregulation of *IL6*, *TNFα*, *Adgre1* and *MCP-1* in the liver of mice under HFD test (Fig. S2D–G†).

3.3. Paraben exposure disrupted lipid metabolism *in vitro*

Compared to the control, AML12 cells and HepG2 cells exposed to OA exhibited more and larger cellular lipid droplets. MeP and EtP exacerbated OA-induced cellular lipid accumulation (Fig. 3A, B and E). In parallel, markedly increased TG and TC contents were observed in both AML12 cells (Fig. 3C and D) and HepG2 cells (Fig. 3F and G) exposed to OA. EtP and MeP increased the TG content in AML12 cells and HepG2 cells tested with OA, respectively (Fig. 3C and F). These data confirmed that paraben exposure promoted intracellular lipid accumulation.

3.4. Paraben exposure altered AA metabolism

The serum metabolome was analyzed to the molecular mechanisms underlying lipid metabolism disorder induced by paraben exposure. PLS-DA analysis showed that the serum metabolic profile in mice exposed to Mep or EtP was significantly different from that in the control group, especially that in mice fed with HFD (Fig. S3A†). Five and twenty seven serum metabolites were altered in ND-fed mice after MeP and EtP tests, respectively. MeP and EtP exposure in combination with HFD significantly altered 338 and 99 metabolites, respectively (Fig. S3B†). No significant pathways were enriched in ND-fed mice upon exposure to MeP or EtP. However, 11 and 5 enriched pathways were observed in HFD-fed mice following MeP and EtP exposure, respectively (Fig. S3C and D†). AA metabolism was the top enriched pathway in HFD-fed mice exposed to MeP (Fig. 4A) and EtP (Fig. 4B). Among 11 differential metabolites involved in the AA metabolism pathway, 11,12-EET was the most abundant metabolite and it was significantly reduced by both MeP and EtP exposure compared to that in HFD-fed mice (Fig. 4C). EtP significantly decreased the hepatic content of 11,12-EET in HFD-fed mice (Fig. 4D). The expression of *CYP4A14*, *CYP2C50*, and *CYP2J9* was significantly downregulated after MeP or EtP exposure, while the expression

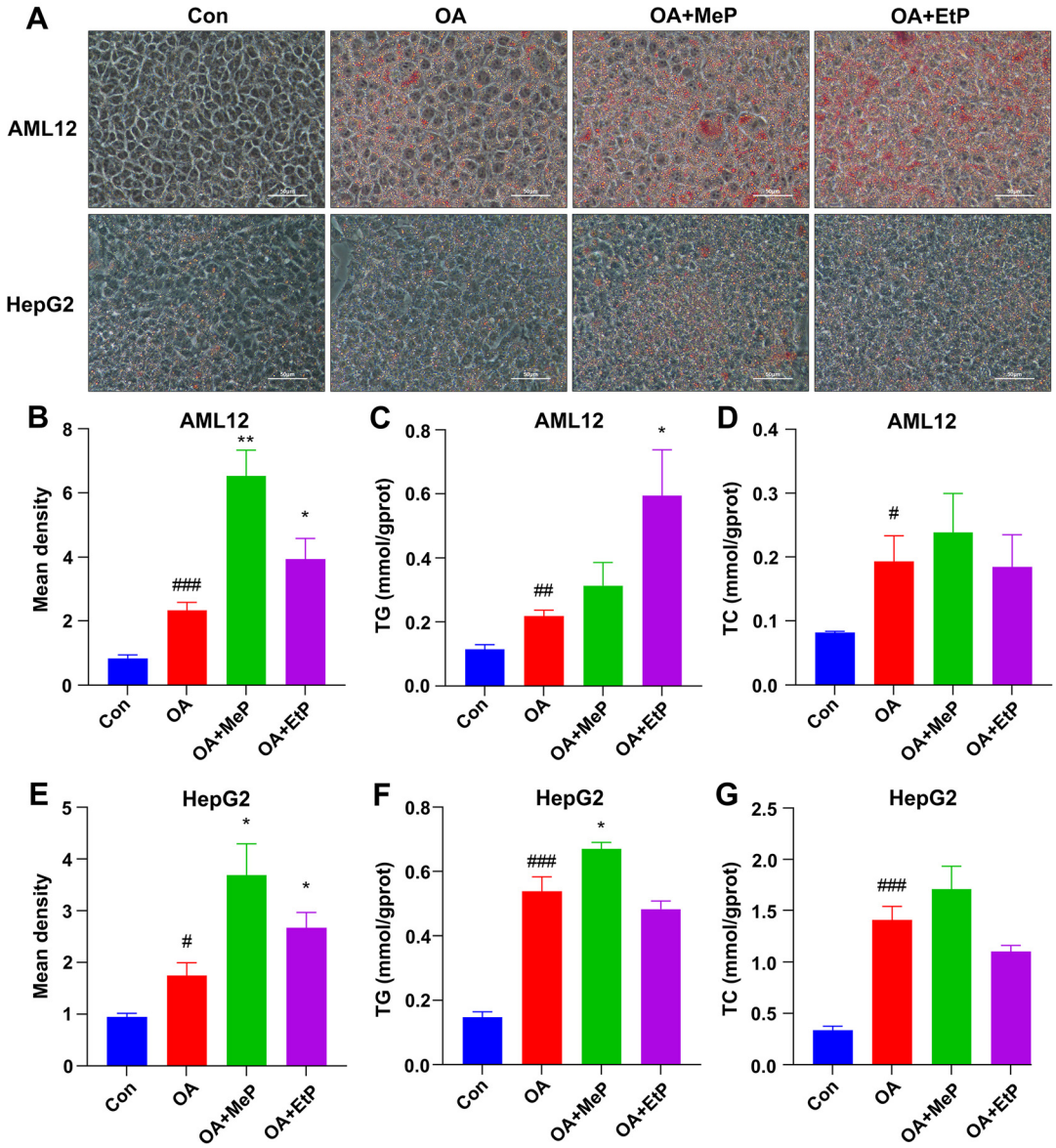

Fig. 3 Paraben exposure disrupted lipid metabolism in AML12 cells and HepG2 cells. (A) Representative images of lipid droplet staining using Oil Red O. (B) Quantification of integrated optical density of AML12 cells and HepG2 cells (E). (C) TG and (D) TC contents of AML12 cells. (F) TG and (G) TC contents of HepG2 cells. Data are presented as the mean ± SEM and analyzed by ordinary one-way ANOVA with Tukey's multiple comparisons. $^{*}P < 0.05$, $^{**}P < 0.01$, compared with the OA group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$, compared with the control group.

of *Ephx2* was significantly increased after MeP exposure compared to that in HFD-fed mice (Fig. 4E).

MeP exposure reduced 11,12-EET content in HepG2 cells, and EtP exposure reduced 11,12-EET content in AML12 cells and HepG2 cells compared to that in OA-treated cells (Fig. 4F and H). The expression of *CYP4A30B*, *CYP2C67*, and *CYP2J9* was downregulated by OA in AML12 cells. In AML12 cells treated with OA, MeP reduced the expression of *CYP2J9*, while both MeP and EtP upregulated the expression of *Ephx2* (Fig. 4G). MeP or EtP exposure reduced *CYP4A11*, *CYP2C8* and *CYP2J2* gene expression and upregulated *Ephx2* gene expression in HepG2 cells (Fig. 4I).

3.5. Gut microbiota contributed to lipid metabolism disorders induced by parabens

The Shannon and Simpson indices showed that MeP could significantly reduce the diversity of fecal microbiota of ND-fed mice. HFD increased the microbial diversity of mice after MeP or EtP exposure compared with that in ND-fed mice (Fig. 5A and B). Despite significant inter-individual variation, PLS-DA analysis revealed significant clustering of fecal microbiota among the control, MeP and EtP groups, especially in HFD-fed mice (Fig. 5C and D). Several differential bacterial taxa at the genus level were identified in mice treated with parabens

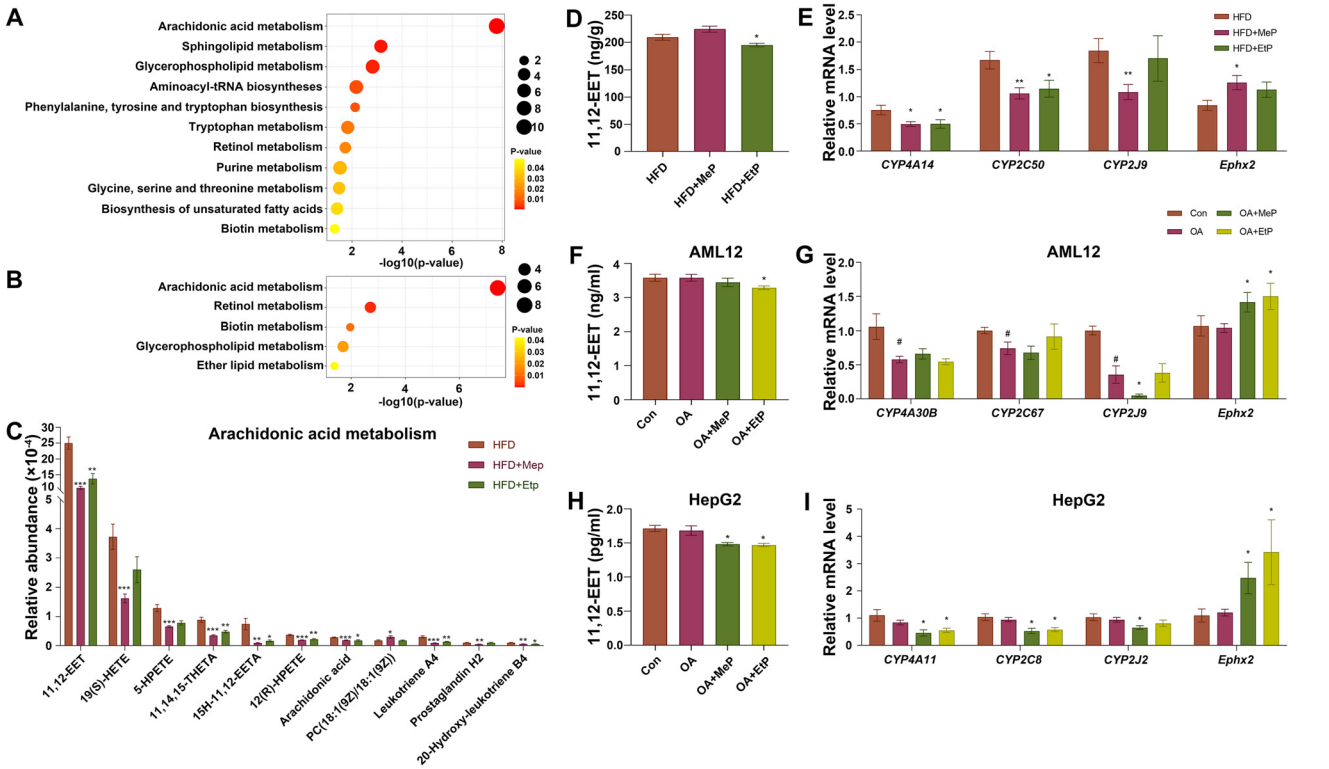

Fig. 4 Paraben exposure altered arachidonic acid (AA) metabolism. (A) Pathway analysis of differential metabolites between the HFD and HFD + MeP groups or (B) HFD + EtP groups. (C) The relative intensity of metabolites involved in the AA metabolism pathway. (D) Hepatic 11,12-EET content. (E) Relative expression of genes involved in AA metabolism in HFD-fed mice. (F) 11,12-EET content and (G) expression of AA metabolism-related genes in AML12 cells. (H) 11,12-EET content and (I) expression of AA metabolism-related genes in HepG2 cells. Data are represented as the mean ± SEM. $*P < 0.05$, $*P < 0.01$, $***P < 0.001$, compared with the HFD or OA group. 11,12-EET, 11,12-epoxyeicosatrienoic acid; 15*H*-11,12-EETA, 15-hydroxy-11,12-epoxyeicosatrienoic acid; 11,14,15-THETA, 11,14,15-trihydroxyicosatrienoic acid; 12(*R*)-HPETE, 12*R*-hydroperoxyeicosatetraenoate; 19(*S*)-HETE, 19(*S*)-hydroxy-5,8,11,14-eicosatetraenoic acid; 5-HPETE, 5-hydroperoxy-(6*E*,8*Z*,11*Z*,14*Z*)-eicosatetraenoic acid.

alone or combined with HFD (Fig. S4†). 112 and 52 metabolic pathways were identified after MeP exposure in ND and HFD groups, respectively (Fig. S5A†). Ten common pathways were enriched (Fig. S5B†), among which 7 showed the same trend, such as AA metabolism (Fig. 5E and S5B, D, E†). In HFD-fed mice, EtP exposure enriched 103 pathways, 31 of which were common to those with MeP exposure (Fig. S5C†), characterized by AA metabolism and glycerolipid metabolism (Fig. 5E, F and S5F, G†). Altogether, these data demonstrated that paraben exposure promoted NAFLD through the disruption of AA metabolism, a process intricately mediated by alteration in the gut microbiota.

The Mantel test was performed to assess the relationship between paraben-associated genera and key metabolites in the AA metabolism. The relative abundance of *unclassified_o_Oscillospirales* was correlated with 11,12-EET between the control group and both the MeP and EtP groups (Fig. 5G and H). Further Spearman correlation test revealed that the relative abundance of *Parvibacter* was negatively correlated with serum TG contents between the control and MeP or EtP groups (Fig. S6A and B†). The relative abundance of *Akkermanisa* was negatively correlated with serum TG and AST contents between the control and EtP groups (Fig. S6A and B†). 11,12-EET was positively correlated with *Escherichia–Shigella* and *Christensenella* between control and MeP or EtP groups. The relative abundance of *unclassified_c_Clostridia* was negatively correlated with 11,12-EET between the control and Mep or EtP groups (Fig. S6A and B†).

3.6. 11,12-EET alleviated lipid metabolism disorder induced by paraben exposure

Supplementation with 11,12-EET effectively curtailed cellular lipid accumulation induced by the combination of OA and MeP or EtP in both AML12 cells and HepG2 cells (Fig. 6A, B and D). Compared to the control, higher expression of *Cpt1a*, *Srebf1*, *Acaca* and *Fasn* was observed in AML12 cells treated with OA, this effect was more pronounced in the MeP and EtP groups. 11,12-EET supplementation inhibited the upregulation of *Adrp*, *Cpt1a*, *Acaca*, and *Fasn* induced by OA combined with MeP or EtP (Fig. 6C). Furthermore, 11,12-EET supplementation suppressed the upregulation of *Adrp*, *Cpt1a*, *PPARγ*, *Srebf1*, *Acaca* and *Fasn* in HepG2 cells coexposed to OA and MeP or EtP (Fig. 6E). MeP and EtP enhanced OA-induced upregulation of PPARγ in AML12 cells (Fig. 6F and G) but not in HepG2 cells (Fig. 6H and I). MeP and EtP enhanced the upregulation

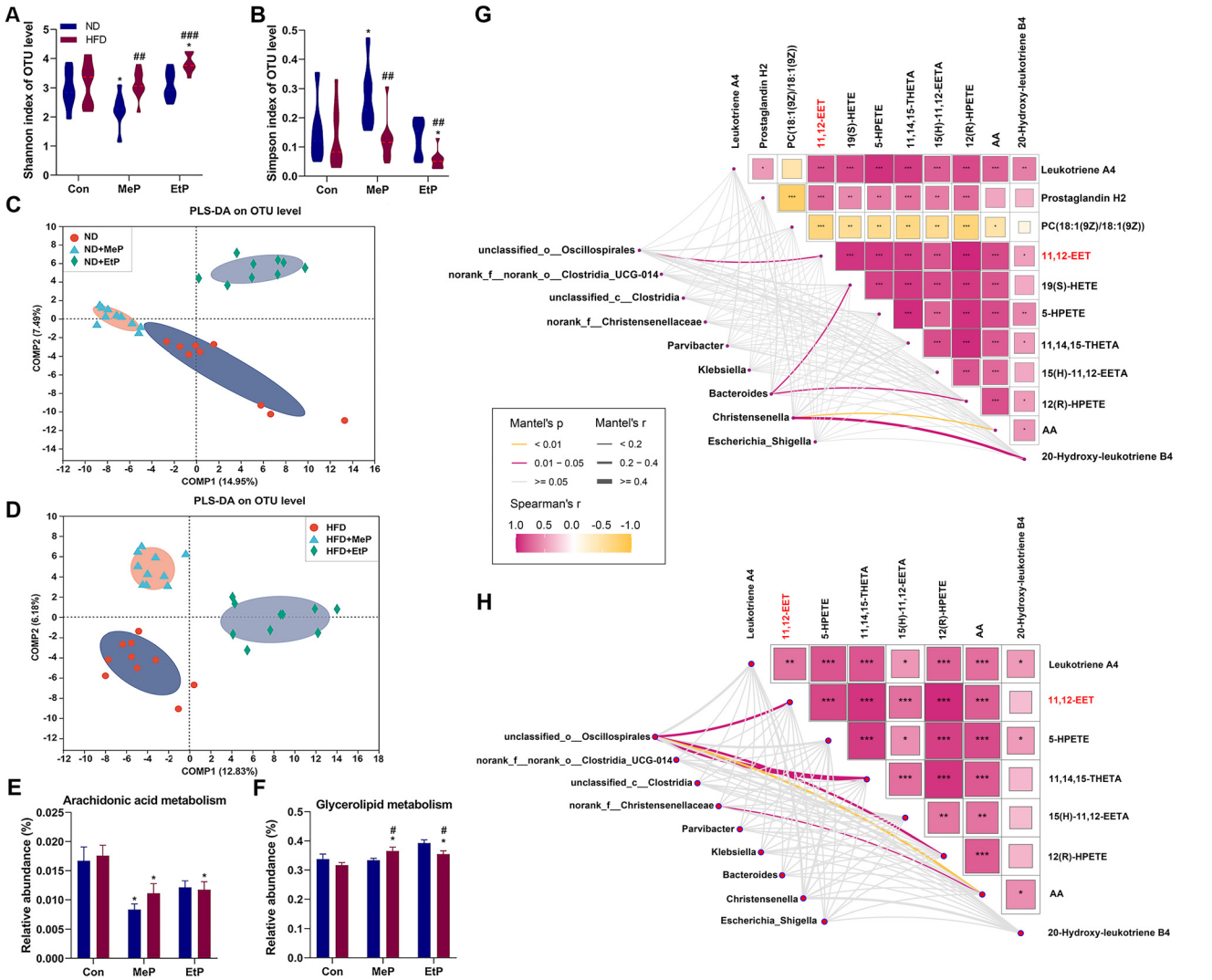

Fig. 5 Alterations in the composition and function of the gut microbiota induced by paraben exposure. (A) Alpha diversity analysis of Shannon and (B) Simpson indices of the three treatments at the OTU level. Beta diversity analysis using the projection to latent structures discriminant analysis (PLS-DA) based on binary-Lennon analysis (beta diversity) on the OTU among control, Mep and EtP groups fed with (C) ND or (D) HFD. Predicted functions of the metagenome from 16S rRNA sequencing were conducted by PICRUSt 2.0 to identify pathways. Enrichment of arachidonic acid metabolism pathway (E) and glycerolipid metabolism (F) in the gut microbiota. Data are presented as the mean ± SEM and analyzed by Wilcoxon rank-sum test. $^{*}P < 0.05$, compared with the respective control; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$, compared with the ND group. Mantel test of differential genera with metabolites in the AA metabolism pathway between HFD and HFD + MeP (G) or (H) HFD + EtP groups. Significant correlations are denoted by stars ($^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$).

of *Cpt1a* induced by OA, 11,12-EET significantly suppressed these effects (Fig. 6F–I).

4. Discussion

Parabens are among the most widely used preservatives in food, pharmaceuticals and consumer products because of their advantageous properties. Parabens are extensively introduced into our diet as food additives and they frequently coexist.[15] Following oral administration, parabens are rapidly absorbed from the gastrointestinal tract and blood.[24] MeP showed the highest detection rate in food products (99%), followed by EtP (84%) and BeP (37%).[25] Therefore, MeP and EtP were chosen to trigger NAFLD *via* gavage in this study. Although emerging evidence revealed adverse effects of parabens on human health, including endocrine, reproductive and metabolic disorders,[10] the potential effects of parabens on NAFLD remain unclear. The global prevalence of NAFLD has dramatically increased in parallel with the epidemic of obesity. NAFLD induced by HFD or a western diet exhibits greater severity in males than females, manifested by histological analysis and biochemical measurements.[26] In addition, women experienced higher exposure to paraben levels than men

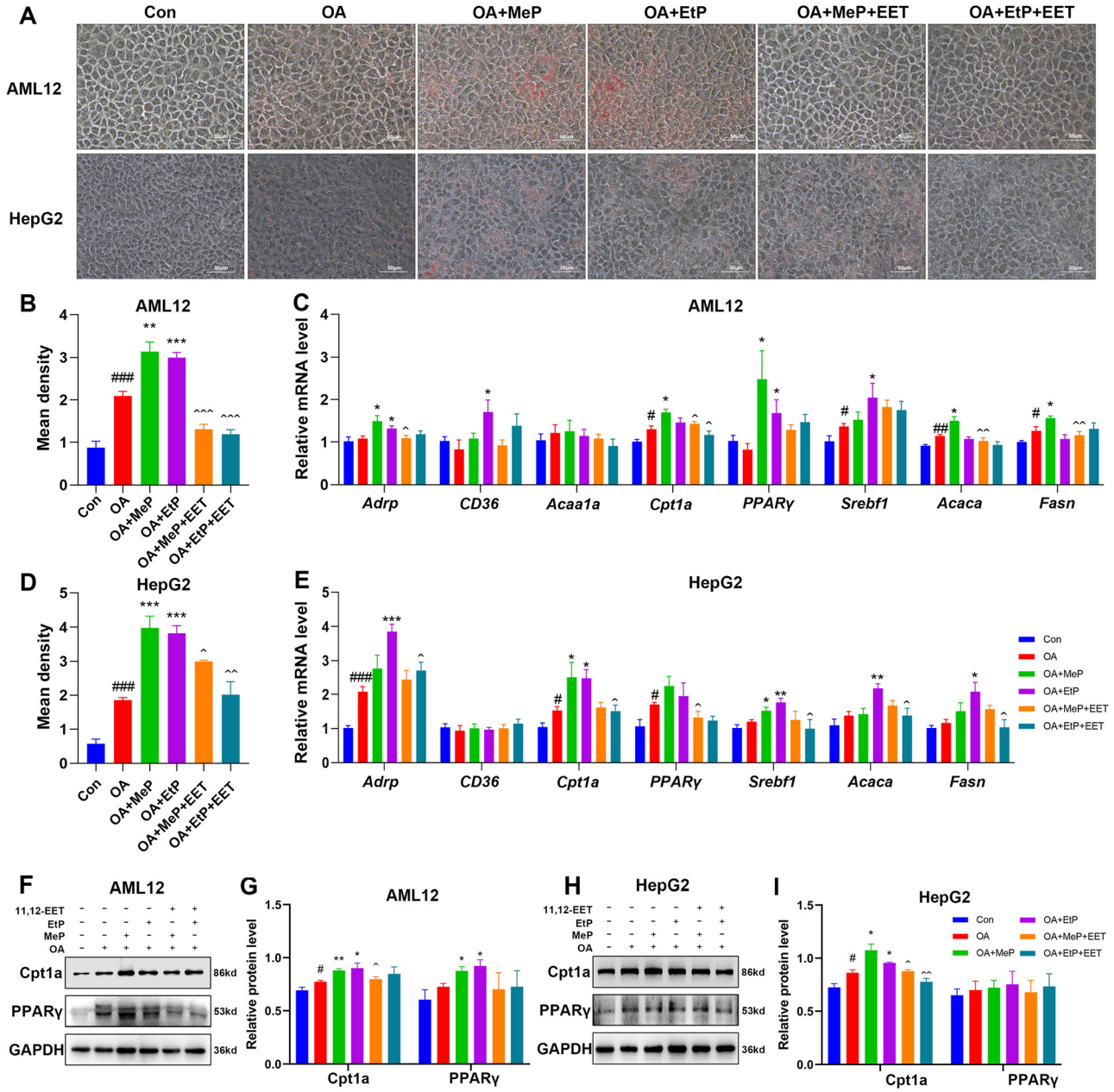

Fig. 6 11,12-EET alleviated paraben-induced lipid metabolism disorder. (A) Representative images of lipid droplet staining using Oil Red O. Quantification of integrated optical density of AML12 cells (B) and HepG2 cells (D). The relative expression of genes involved in lipid metabolism in AML12 cells (C) and HepG2 cells (E). Protein expression of *Cpt1a* and *PPARγ* in AML12 cells (F and G) and HepG2 cells (H and I) treated with OA in combination with MeP, EtP or 11,12-EET detected by western blot assay. *GAPDH* was used as an internal reference. Data are presented as the mean ± SEM and analyzed by ordinary one-way ANOVA with Tukey's multiple comparisons. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$, compared with control group; $^{*}P < 0.05$, $^{*}P < 0.01$, $^{***}P < 0.001$, compared with OA; $^{\wedge}P < 0.05$, $^{\wedge\wedge}P < 0.01$, $^{\wedge\wedge\wedge}P < 0.001$, compared with the OA + MeP or OA + EtP group.

through use of consumer products.[27] High total parabens and MeP daily intake were associated with a high BMI in girls, but this association was not observed in boys.[16] Moreover, women were found to have a greater mortality risk associated with exposure to MeP and EtP.[28] Thus, this study aimed to determine whether MeP and EtP exposure in combination with HFD promote the development of NAFLD in female mice.

Emerging evidence indicated that paraben exposure was in association with metabolic disorders. Although MeP exposure did not impact body weight in ND-fed mice, MeP significantly increased weight gain induced by HFD, accompanied by greater liver weight and fat mass. MeP increased adipogenesis and inhibited glucose uptake and basal lipolysis of 3T3-L1 white adipocytes, indicating disruption in differentiation and metabolic process.[29] In ND-fed mice, MeP significantly

increased the hepatic contents of TG and TC. While EtP exposure did not affect body weight or liver lipid accumulation in mice fed with ND or HFD, it significantly increased fasting blood glucose and the AUC of ND-fed mice, indicating that EtP may exhibit the hyperglycemic effect. EtP exposure also exacerbated HFD-induced glucose intolerance, hepatic steatosis and enlarged adipocytes. In addition, MeP and EtP exposure also increased lipid accumulation in AML12 cells and HepG2 cells. These data indicated that paraben exposure could promote the development of NAFLD in mice, especially in response to HFD.

NAFLD is associated with the dysregulation of lipid metabolism and hepatic inflammation.[30] Serum levels of ALT and AST in female mice fed with HFD were significantly increased. EtP exposure significantly worsened the elevation of ALT and hepatic infiltration of inflammatory cells, suggesting that paraben exposure could trigger hepatic inflammation, which contributed to the development of NAFLD. Genes related to hepatic inflammation and energy metabolism were further analyzed to illustrate the underlying mechanism. EtP significantly enhanced the upregulation of *IL6* and *MCP-1* induced by HFD. In addition, MeP also induced the expression of *Adgre1* and *MCP-1* in ND-fed mice. Both paraben exposure significantly upregulated hepatic expression of genes involved in gluconeogenesis, adipogenic differentiation, fatty acid intake, beta oxidation and *de novo* lipogenesis, while they inhibited glycolysis-related genes. These results suggest that parabens may contribute to NAFLD by inducing inflammatory response and disrupting the energy metabolism balance.

A metabolomics approach provided insightful evidence of the altered metabolic profile and enriched metabolic pathways in NAFLD.[31] Metabolomics data from pregnant women revealed that paraben exposure altered several metabolites enriched in purine metabolism and fatty acid β-oxidation pathways.[32] Here, paraben exposure had a modest yet detectable impact on the metabolome, only 5 and 27 metabolites were identified in ND-fed mice after exposure to MeP and EtP, respectively. In contrast, they altered 338 and 99 metabolites involved in four common pathways, including AA metabolism, glycerophospholipid metabolism, retinol metabolism, and biotin metabolism in HFD-fed mice. AA, one of the most abundant polyunsaturated fatty acid (PUFA) in the human body, had been linked to NAFLD. The activation of the AA metabolism pathway was a conserved molecular hallmark during non-alcoholic steatohepatitis (NASH) development across species.[33] AA and its metabolites, including leukotriene A4 and leukotriene B4, play an important role in proinflammatory cytokine production and insulin resistance, and dramatically increase with obesity and NAFLD.[34]

AA can be metabolized by *CYP450*, such *CYP2J* and *CYP2C*, to generate EETs (5,6-EET, 8,9-EET, 11,12-EET, and 14,15-EET), which are predominantly metabolized by soluble epoxide hydrolases (Ephx) to diols or dihydroxyeicosatrienoic acids (DHET).[35] MeP and EtP exposure could significantly decrease the serum level of 11,12-EET, which was the most abundant among 11 differential metabolites involved in the AA metabolism pathway enriched by paraben exposure. They also reduced the intracellular content of 11,12-EET in AML12 cells and HepG2 cells cultured with OA. The expression of *CYP4A*, *CYP2C* and *CYP2J* was markedly inhibited by HFD-treated liver and OA-treated cells, while the expression of *Ephx2* was upregulated. Compared with healthy controls, total EET, 11,12-EET, 14,15-EET, total DHET, 11,12-DHET and 14,15-DHET were significantly lower in individuals with steatosis and NASH.[36] An EET agonist alleviated typical NAFLD symptoms, including lipid accumulation, liver inflammation and fibrosis in HFD-fed db/db mice.[37] Thus, the potential protective role of 11,12-EET in NAFLD was verified *in vitro*. 11,12-EET supplementation significantly alleviated lipid accumulation in AML12 cells and HepG2 cells.

It is well established that the gut microbiota is an important contributor to health and plays a causal role in development of NAFLD.[38] As antimicrobial agents, parabens inhibited Gram-positive bacteria and promoted oxidative stress-tolerant bacteria in the river ecosystem.[39] Paraben exposure was able to disrupt the composition and diversity of the gut microbial community in zebrafish and rodents.[40–42] Thus, the role of the gut microbiota in NAFLD induced by HFD was investigated in this study. The present study revealed that HFD significantly decreased the relative abundance of beneficial bacteria, specifically *Lactobacillus* and *Bifidobacterium*, while increased inflammation-related *Erysipelatoclostridium*.[43] As expected, MeP significantly shifted the microbial community of mice fed with ND or HFD, as evidenced by decreased diversity and a clear separation of PLS-DA plots. EtP increased the diversity of the gut microbiota in mice fed with HFD, but this effect was not observed in mice fed with ND. *Akkermansia muciniphila* was proved to be a therapeutic target to prevent human obesity and associated disorders.[44] EtP may decrease the abundance of *Akkermansia*, potentially exacerbating obesity and NAFLD induced by HFD. The abundance of the *Klebsiella*, *Escherichia* and *Bacteroides* genera was increased in NASH patients.[45] However, both MeP and EtP decreased the relative abundance of *Klebsiella*, *Bacteroides*, and *Escherichia–Shigella*. Therefore, the results from this study should be interpreted with caution due to the ongoing controversy regarding the microbiome profile in NAFLD.

Evidence is accumulating that metabolites derived from the gut microbiota, such as short-chain fatty acids, secondary bile acids, and aromatic amino acids, play a role in the etiology of obesity and NAFLD.[46] The gut microbiota could metabolize dietary omega-6 PUFA, which is abundant in the Western diet contributed to obesity and related diseases.[47] Although AA metabolism takes place in several organs, including the brain, heart, kidneys, liver, lungs, and vasculature, it was also found to be associated with intestinal flora disturbance.[48] Interestingly, MeP and EtP significantly disrupted AA metabolism of the gut microbiota in two dietary patterns. After MeP and EtP exposure, *unclassified_c_Clostridia* showed a negative correlation with 11,12-EET, 11,14,15-THETA and 15*H*-11,12-EETA. *Clostridium bifermentans* JCM 1386 was found to saturate AA during anaerobic cultivation.[49] Mantel test revealed a significant association of *unclassified_o_Oscillospirales* with 11,12-

EET. These data suggest that MeP and EtP exposure can disrupt AA metabolism mediated by the gut microbiota, potentially contributing to the development of NAFLD.

This study, for the first time, reveals that chronic exposure to parabens promotes the development of NAFLD under HFD conditions, evidenced by a notable increase in weight gain, fat accumulation, serum lipid, and liver injury, by the regulation of hepatic genes involved in inflammation and energy metabolism. The disruption of the AA metabolism in the serum metabolic profile and the gut microbiota induced by MeP and EtP exposure might contribute to promoting NAFLD. Supplementation with the AA metabolite 11,12-EET diminished lipid accumulation *in vitro*. This study underscores the importance of reducing oral exposure of parabens through the reasonable use of food additives to alleviate metabolic disturbances under HFD conditions. However, several shortcomings remain in this study. Well-designed sex-based comparative studies are needed to characterize the effects of co-exposure of parabens *via* various routes on NAFLD development. Furthermore, large-scale population studies will help confirm the association of paraben exposure with NAFLD. Finally, studies using germ-free mice are needed to verify the crucial role of the gut microbiota in paraben-triggered NAFLD and elucidate the underlying molecular mechanism.

Data availability

The 16S rRNA sequencing data from mice feces have been submitted to NCBI (Sequence Read Archive, SRA). The BioProject accession number is PRJNA924972 (**https://dataview.ncbi.nlm.nih.gov/object/PRJNA924972?reviewer=td1oai9jgmaj9lmce8l55bdsb3**).

Author contributions

Z. Z. designed the experiments. Y. L. R., S. Y. L. and X. Q carried out animal experiments. Y. L. R., X. Y. S. and J. M. performed the microbiome analysis. W. L. P., S. H. N. and Z. D. Z. performed the metabolomics analysis. Z. Z. and Y. L. R. drafted the manuscript. Z. Z., L. L. directed the research.

Conflicts of interest

The authors declare that there are no conflicts of interest.

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