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Research Article

Paternal Programming of Liver Function and Lipid Profile Induced by a Paternal Pre-Conceptional Unhealthy Diet: Potential Association with Altered Gut Microbiome Composition

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Keywords
Gut microbiota · Liver function · High-fat-sucrose-salt diet · Paternal programing

Abstract
Background/Aims: Paternal exposure to adverse environmental conditions can act on offspring's phenotype and influence offspring's later life disease risk. Our study was designed to examine the effect of feeding male rats before mating a high-fat, high-sucrose and high-salt diet (HFSSD) over two generations (F0 and F1) on their offspring's (F2) liver function and gut microbiome composition. ***Methods:*** Male F0 rats and male F1 rats were fed either control diet or HFSSD before mating. Liver function of F2 offspring was investigated, and their gut microbiome composition was analyzed by 16S rRNA gene sequencing in the F2 offspring of rats whose fathers and grandfathers were fed with control diet (CD) (F0CD+F1CD-F2 group) or HFSSD prior to mating (F0HD+F1HD-F2 group). ***Results:*** F2 offspring had higher serum as-

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partate aminotransferase activity (female, $p < 0.05$ and male, $p < 0.01$ respectively) compared with control. Shannon indexes of gut microbiota indicated a significantly higher diversity in the female F0HD+F1HD-F2 as compared to F0CD+F1CD-F2 female offspring ($p < 0.01$). The dominant phyla of all the groups were *Bacteroidetes*, *Firmicutes* and *Proteobacteria*. There were significant differences in gut bacterial community composition at phyla and genus level between the F0CD+F1CD-F2 and F0HD+F1HD-F2. Furthermore, the variation in the relative abundance (percentage) of bacterial genus in the F2 offspring was associated with liver function alterations induced by a paternal pre-conceptional unhealthy diet. Male F0HD+F1HD-F2 offspring had higher serum cholesterol, high density lipoproteins as well as low density lipoproteins concentrations compared to the corresponding male control rats. ***Conclusion:*** Taken together, our findings suggested that a paternal pre-conceptional unhealthy diet predisposes the offspring to mild liver function alterations and alterations of gut microbiota in later life. Effects on lipids were sex-specific and only seen in male offspring.

Introduction

Disadvantageous nutrition of the parents can impact on later life disease susceptibility of the offspring. Suboptimal early life conditions that affect germ cells, the intrauterine environment or early postnatal conditions may be responsible for this. The concept connecting the impact of early life conditions on later life disease susceptibility is known as fetal programming or developmental origins of adult disease [1–3].

Recently, gut microbiota, which plays important roles in maintaining host health and in disease development, has become a major and promising research area in biomedicine [4–7]. Previous studies have suggested that gut microbiota is important in regulating metabolic pathways in healthy people and in patients with obesity, diabetes and cardiovascular diseases [8, 9]. The developmental origins of health and disease (DOHaD) hypothesis, firstly formulated in the early 1990s, proposed that adverse in utero conditions can influence developmental pathways in early life that result in long-term changes to offspring disease susceptibility [1, 2, 10, 11]. Recent work on the human microbiome indicates that gut microbiota may additionally explain the observations put forth by the DOHaD hypothesis [12–14].

Alterations in maternal microbiota composition, associated with both diet and obesity status, have been widely reported [15–18]. Disruption of the mother's gut microbiota can influence early pioneering bacteria in the newborn and may be a prime candidate for intergenerational transmission of metabolic disease risk given the considerable influence of these microbes on infant growth [19, 20], immune system development [21–23], and even neural development [24, 25]. Interestingly, emerging evidence has additionally indicated that transmission of microbiota from mother to offspring may occur before delivery, which has since reemphasized the importance of the pregnancy period to the development of the neonatal microbiome [12, 13]. Gut microbiota is an important factor that affects energy harvest from the diet and energy storage in the host. Indeed, gut microbiota exerts a strong influence on host lipid and cholesterol metabolism. It was reported that the severity of nonalcoholic fatty liver disease (NAFLD) postnatally is strongly associated with gut dysbiosis and a shift in gut metabolic function [26], including production and utilization of short-chain fatty acids and bile acids.

The above-described mechanisms are of maternal origin and are caused by either maternal environmental factors or maternal genes that act on the epigenome of the growing fetus. Several studies showed that also paternal nutritional factors, in particular during maturation of the sperm, may alter the epigenome and the phenotype of the offspring [27–30].

Table 1. Composition of CD, and HFSSD

	CD	HFSSD
Components		
Casein	20.0	23.3
Corn starch	39.8	8.7
Maltodextrin	13.2	10.5
Sucrose	10.0	20.1
Fiber	5.0	5.0
Soybean oil	7.0	2.9
Lard	0.0	20.7
Salt	0.3	3.7
Minerals	3.5	3.5
Vitamins	1.0 (0.01 folate)	1.0 (0.01 folate)
Energy		
Energy value, kJ/kg	16,736	19,539
Lipids	15.8	45.5
Sucrose	10.00	17.25
Proteins	20.30	20.23

All values are given as percentages unless indicated otherwise. All components are wt/wt. CD, control diet; HFSSD, high-fat, high-sucrose and high-salt diet.

It was shown that feeding of male rat founders with high-fat diet before mating induces impaired glucose tolerance in female offspring [31], most likely due to epigenetic adaptations in liver. However, investigations into the later-life effects of paternal pre-conceptional unhealthy diet on gut microbiota in the offspring are limited. Given the multifaceted nature of this topic, in the current study, we analyzed the effect of feeding male rats high-fat, high-sucrose and high-salt diet (HFSSD) over two generations (F0 and F1) on their offspring's (F2) liver function and gut microbiome composition.

Materials and Methods

Animals

The present study utilized Sprague-Dawley rats of both sexes, including F0 generation (30 males and 32 females), F1 generation (80 males and 80 females) and F2 generation (49 males and 38 females) animals. The F0 rats were purchased from Hunan SJA Laboratory Animal Co., Ltd (Changsha, China) and were delivered at the age of 4 weeks. The animals were housed in temperature-controlled chambers under control lighting with 12:12-h light-dark cycles. All the animals were allowed ad libitum access to water and food. The experimental protocols were approved by the Experimental Animal Center of the Hunan Normal University (Changsha, China) in accordance with the ethical standards of Hunan Province, China.

Study Design

F0 male rats were randomly divided into two study groups: (1) control diet (CD, n = 15) and (2) HFSSD (n = 15). All animals were allowed to consume tap water and food ad libitum. A detailed description of the diet compositions is indicated in Table 1.

The 14-week-old F0 founder males fed either a CD or HFSSD were mated with F0 12-week-old normal weight naturally cycling CD-fed dams to produce F1 offspring. The presence of a vaginal plug was designated as gestational day 1. The rats were mated for 4 days. After mating and throughout the gestational period, all the F1 dams were fed CD. The F1 male offspring was randomly allocated into two study groups. (1) Paternal control diet group (PatCD group): male F1 offspring of CD-fed F0 founders received CD from birth until the 24th week of age and were mated with CD-fed dams and their F2 offspring represented the F0CD+F1CD

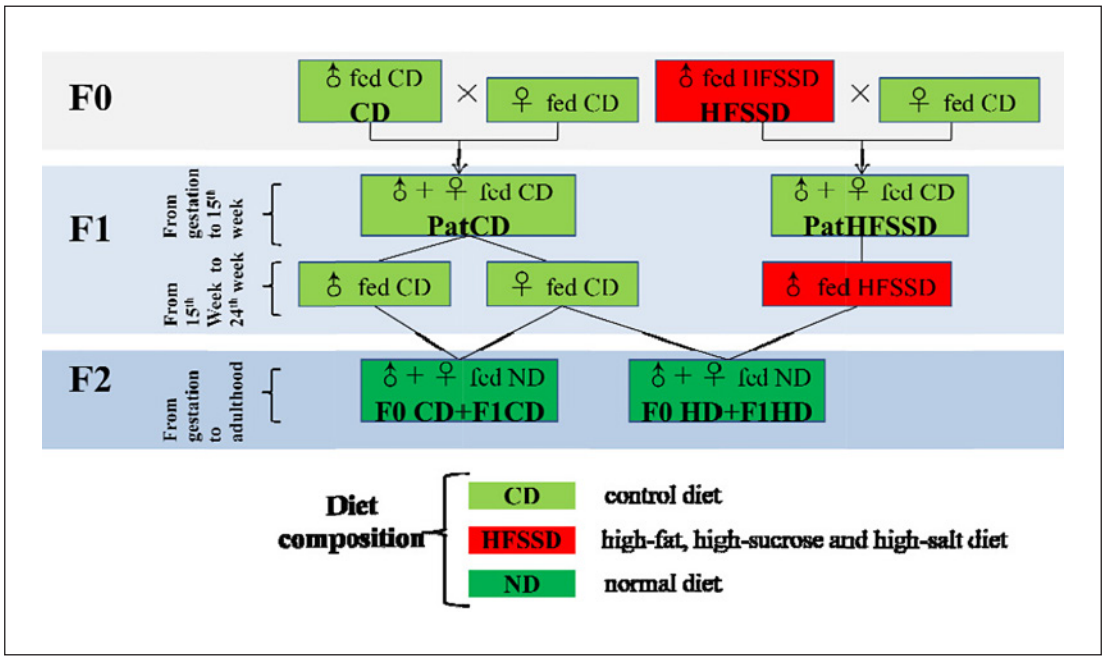

Fig. 1. F0 male rats were randomly divided into two study groups: (1) control diet (CD, *n* = 15); (2) high-fat, high-sucrose and high-salt diet (HFSSD, *n* = 15). The F1 male offspring was randomly allocated to two study groups: (1) paternal control diet group (PatCD group): male F1 offspring of CD-fed F0 founders received CD from birth until the 24th week of age and were mated with CD-fed dams and their F2 offspring represented the F0CD+F1CD group (F0CD+F1CD-F2 group); (2) paternal high-fat, high-sucrose and high-salt diet group (PatHFSSD group): male F1 offspring of HFSSD-fed F0 founders received CD from birth until the 15th week of age followed by HFSSD until the 24th week of age and were mated with CD-fed dams and their offspring represented the F0HD+F1HD group (F0HD+F1HD-F2 group). Based on F0 and F1 paternal diet before mating, the F2 offspring can be divided into two study groups: (1) F0CD+F1CD group: F2 offspring of F0 and F1 male founders fed a CD; (2) F0HD+F1HD group (F0CD+F1CD-F2 group): F2 offspring of F0 and F1 male founders fed a HFSSD (F0HD+F1HD-F2 group).

group (F0CD+F1CD-F2 group); (2) paternal high-fat, high-sucrose and high-salt diet group (PatHFSSD group): male F1 offspring of HFSSD-fed F0 founders received CD from birth until the 15th week of age followed by HFSSD until the 24th week of age and were mated with CD-fed dams and their offspring represented F0HD+F1HD group (F0HD+F1HD-F2 group). In summary, based on F0 paternal diet and F1 paternal diet before mating, the F2 offspring can be divided into two study groups (Fig. 1): (1) F0CD+F1CD group: F2 offspring of F0 and F1 male founders fed a CD; (2) F0HD+F1HD group: F2 offspring of F0 and F1 male founders fed a HFSSD. All F2 offspring (F0CD+F1CD and F0HD+F1HD) were fed normal diet (ND) from birth until the 25th week of age. Then, they were sacrificed under deep anesthesia induced by intraperitoneal injection of 3% (wt/vol.) sodium pentobarbital solution. Afterwards, blood samples were collected, the organs were harvested, and feces were aseptically taken from the cecum of the rats.

Metabolic Tests

Blood samples were taken via aortic puncture at study end. Serum glucose (GLU), cholesterol (CHOL), triacylglycerols (TG), high-density lipoproteins (HDL), low-density lipoproteins (LDL), alanine aminotransferase (ALT), and aspartate transaminase (AST) were measured using Hitachi 7020 automatic biochemistry analyzer (Hitachi High-Technologies, Tokyo, Japan).

Fecal DNA Extraction and 16S rRNA Gene Sequencing

Fresh stool samples were collected after sacrifice and immediately stored at –80 °C for subsequent analysis. The sample sizes were 25 (16 male samples and 9 female samples) in F0CD+F1CD (F2 offspring of F0 and F1 male founders fed a CD) group and 29 (16 male samples and 13 female samples) in F0HD+F1HD

Table 2. Body weight and serum metabolite levels in F0 founders males

Parameter	CD (n = 15)	HFSSD (n = 15)
Body weight, g		
On arrival in the lab	175.5±2.9	177.2±5.2
At 10 weeks	415.5±7.3	396.1±4.9
At 12 weeks	472.4±7.5	447.1±4.1**
At 14 weeks	504.4±8.1	475.8±4.8**
At 18 weeks	562.6±8.4	539.6±4.1*
Serum metabolites		
GLU, mmol/L	7.6±1.5	7.6±1.7
TG, mmol/L	1.1±0.1	1.0±0.1
CHOL, mmol/L	1.4±0.1	1.7±0.1*
BUN, mmol/L	7.0±0.3	8.3±0.3**
Cr, μmol/L	35.3±2.2	35.4±2.1
AST, U/L	144.3±14.2	142.5±9.6
ALT, U/L	26.6±6.3	59.8±16.2*

GLU, glucose; TG, triacylglycerols; CHOL, cholesterol; BUN, blood urea nitrogen; Cr, creatinine; AST, aspartate aminotransferase; ALT, alanine aminotransferase; CD, control diet; HFSSD, high-fat, high-sucrose and high-salt diet. Values are given as mean ± SEM. * $p < 0.05$ versus CD, ** $p < 0.01$ versus CD.

(F2 offspring of F0 and F1 male founders fed a HFSSD) group. Genomic DNA of microbiota was extracted from fecal samples by QIAamp DNA Stool Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. DNA was quantified by the Nanodrop 2000. The extracted DNA from each sample was used as the template to amplify the V3 and V4 hypervariable regions of ribosomal 16S rRNA genes. Briefly, the purified 1 μg of genomic DNA was fragmented to an average size of 300–400 bp and ligated with adapters. The PCR was performed using a primer cocktail that anneals to the ends of the adapters to enrich DNA fragments that have adapter molecules on both ends and followed by clean up and quantification. Sequencing was performed using a 300-bp paired-end sequencing protocol on the Illumina MiSeqplatform (Illumina, San Diego, CA, USA) at Oebiotech Company, Shanghai, China. Raw paired-end reads were subjected to quality filtering using Trimmomatic software before paired-end read assembling with FLASH software. All chimeras of assembling sequences were eliminated to reach high-quality sequences.

Bioinformatics and Statistical Analysis

The optimized sequences were clustered into Operational Taxonomic Units (OTUs) using Vsearch software with 97% threshold of pairwise identity, and the OTUs were used to estimate community diversity and richness. The Shannon index, PD_whole_tree, and Chao were calculated using QIIME. The representative sequences of OTUs were used to generate a phylogenetic tree using FasTree. The phylogenetic tree was then used for unweighted UniFrac principal coordinate analysis (PCoA) [32]. The relative abundances of gut microbiota in each sample and other measurement data were expressed as mean ± SEM and evaluated with unpaired *t*-test. Correlation analyses between relative abundance (percentage) of bacterial genus and liver function index were performed by using Spearman's correlation analyses. Statistical significance was accepted as $p < 0.05$ (two-sided significance testing). All statistical analyses were performed using SPSS 22.0 statistical software (Chicago, IL, USA).

Results

F0 Founders

Male F0 founders were fed either CD or HFSSD for 9 weeks before mating. The body weight gain and serum metabolite levels of the F0 founders are presented in Table 2. No effect of HFSSD on the number of pubs was observed.

Table 3. Body weight, liver weight and serum metabolites of female F2 offspring

Parameter	F0CD+ F1CD (n = 11)	F0HD+F1HD (n = 15)
Body weight, g		
At 9 weeks	214.35±4.79	232.36±3.56**
At 12 weeks	241.20±5.51	254.17±3.32
At 15 weeks	262.96±5.39	279.87±3.77*
At 18 weeks	278.07±4.95	301.32±3.75**
At 22 weeks	298.15±5.34	316.65±3.88*
At 25 weeks	308.1±5.55	322.64±3.71*
Liver weight		
Liver weight, g	9.78±0.22	10.22±0.18
Relative liver weight, % to body weight	3.08±0.01	3.13±0.01
Serum metabolites		
AST, U/L	88.75±6.55	112.10±7.05*
ALT, U/L	33.03±4.37	40.72±2.62
GLU, mmol/L	7.99±0.24	8.03±0.45
TG, mmol/L	2.00±0.31	1.77±0.18
CHOL, mmol/L	2.17±0.12	2.33±0.10
HDL, mmol/L	0.82±0.04	0.91±0.05
LDL, mmol/L	0.13±0.01	0.12±0.01

AST, aspartate aminotransferase; ALT, alanine aminotransferase; GLU, glucose; TG, triacylglycerols; CHOL, cholesterol; HDL, high density lipoproteins; LDL, low density lipoproteins; F0CD+F1CD, F2 offspring of F0 and F1 male founders fed a CD; F0HD+F1HD, F2 offspring of F0 and F1 male founders fed a HFSSD. Values are given as mean ± SEM. * p < 0.05 versus F0CD+F1CD, ** p < 0.01*versus* F0CD+F1CD.

F2 Offspring

Body Weight and Food Intake

Compared with the F0CD+F1CD group, the body weight of the female F0HD+F1HD group increased significantly at the 9th, 15th, 18th, 22nd and 25th week (Table 3). However, no significant differences in body weight, except at the age of 12 weeks, were observed between the F0CD+F1CD and F0HD+F1HD male offspring (Table 4). No significant differences in food and water consumption were observed among the groups throughout the experiment (data not shown).

Liver function and Serum Metabolites

There were no significant differences in liver weights and serum ALT activity between the F0CD+F1CD and F0HD+F1HD of both sexes. However, AST activity was significantly higher in female and male F0HD+F1HD groups than comparable F0CD+F1CD groups (Tables 3, 4).

No significant differences in other serum metabolites were observed between the female F0CD+F1CD group and the female F0HD+F1HD group (Table 3). However, serum CHOL, HDL and LDL were significantly higher in the male F0HD+F1HD group than in the male F0CD+F1CD group (Table 4). The above results indicate that the grandfather's HFSSD and paternal HFSSD resulted in liver function and lipid metabolism abnormalities of F2 offspring.

Analysis of the Diversity and Richness of the Microbiome using 16S rRNA Sequencing

High-throughput sequencing of the bacterial 16S rRNA gene was performed in fecal samples of F0CD+F1CD and F0HD+F1HD groups at the age of 25 weeks to characterize their

Table 4. Body weight, liver weight and serum metabolites of male F2 offspring

Parameter	F0CD+F1CD (n = 20)	F0HD+F1HD (n = 19)
Body weight, g		
At 9 weeks	353.39±4.26	356.22±4.10
At 12 weeks	402.52±7.97	427.02±5.84*
At 15 weeks	465.66±5.91	461.43±10.86
At 18 weeks	520.89±6.97	527.28±8.97
At 22 weeks	565.74±7.91	584.77±7.44
At 25 weeks	585.78±7.98	604.77±8.50
Liver weight		
Liver weight, g	19.55±0.57	19.98±0.33
Relative liver weight, % to body weigh	3.34±0.01	3.27±0.01
Serum metabolites		
AST, U/L	84.66±3.39	102.15±4.80**
ALT, U/L	32.07±1.62	35.49±2.01
GLU, mmol/L	11.89±0.66	12.04±0.60
TG, mmol/L	1.92±0.25	1.96±0.12
CHOL, mmol/L	1.80±0.07	2.01±0.06*
HDL, mmol/L	0.69±0.03	0.79±0.03**
LDL, mmol/L	0.10±0.01	0.11±0.01*

AST, aspartate aminotransferase; ALT, alanine aminotransferase; GLU, glucose; TG, triacylglycerols; CHOL, cholesterol; HDL, high density lipoproteins; LDL, low density lipoproteins; F0CD+F1CD, F2 offspring of F0 and F1 male founders fed a CD; F0HD+F1HD, F2 offspring of F0 and F1 male founders fed a HFSSD. Values are given as mean ± SEM. * $p < 0.05$ versus F0CD+F1CD, ** $p < 0.01$ versus F0CD+F1CD.

Table 5. Bacterial diversity analyzed by high-throughput sequencing

Estimator	Female		Male	
	F0CD+F1CD (n = 9)	F0HD+F1HD (n = 13)	F0CD+F1CD (n = 16)	F0HD+F1HD (n = 16)
OTUs	649.00±30.19	543.08±28.91	652.50±39.51	728.38±21.81
PD_whole_tree	24.86±1.15	20.15±0.99	23.84±1.28	26.68±0.79
Chao	840.85±48.15	683.68±43.82	837.24±48.87	909.19±22.58**
Shannon	5.65±0.38	6.43±0.10**	6.44±0.18	6.59±0.11

OTUs, operational taxonomic units. The number of OTUs, diversity estimator PD_whole_tree, Chao and Shannon were calculated at 3% distance. F0CD+F1CD, F2 offspring of F0 and F1 male founders fed a CD; F0HD+F1HD, F2 offspring of F0 and F1 male founders fed a HFSSD. Values are given as mean ± SEM. * $p < 0.05$ versus F0CD+F1CD, ** $p < 0.01$ versus F0CD+F1CD.

gut microbiome. A total of 2,095,124 high-quality sequences were produced in the present study, with an average of 38,798 sequences per sample. The Good's coverage of each group was over 97%, indicating that the 16S rDNA sequences identified in these groups represent the majority of bacteria present in the samples of the present study. Community diversity was estimated using the PD_whole_tree, Chao, and Shannon index, and richness was evaluated based on the number of OTUs. The Shannon index was higher in the female F0HD+F1HD group than in the female F0CD+F1CD group (Table 5). The Chao index was higher in the male

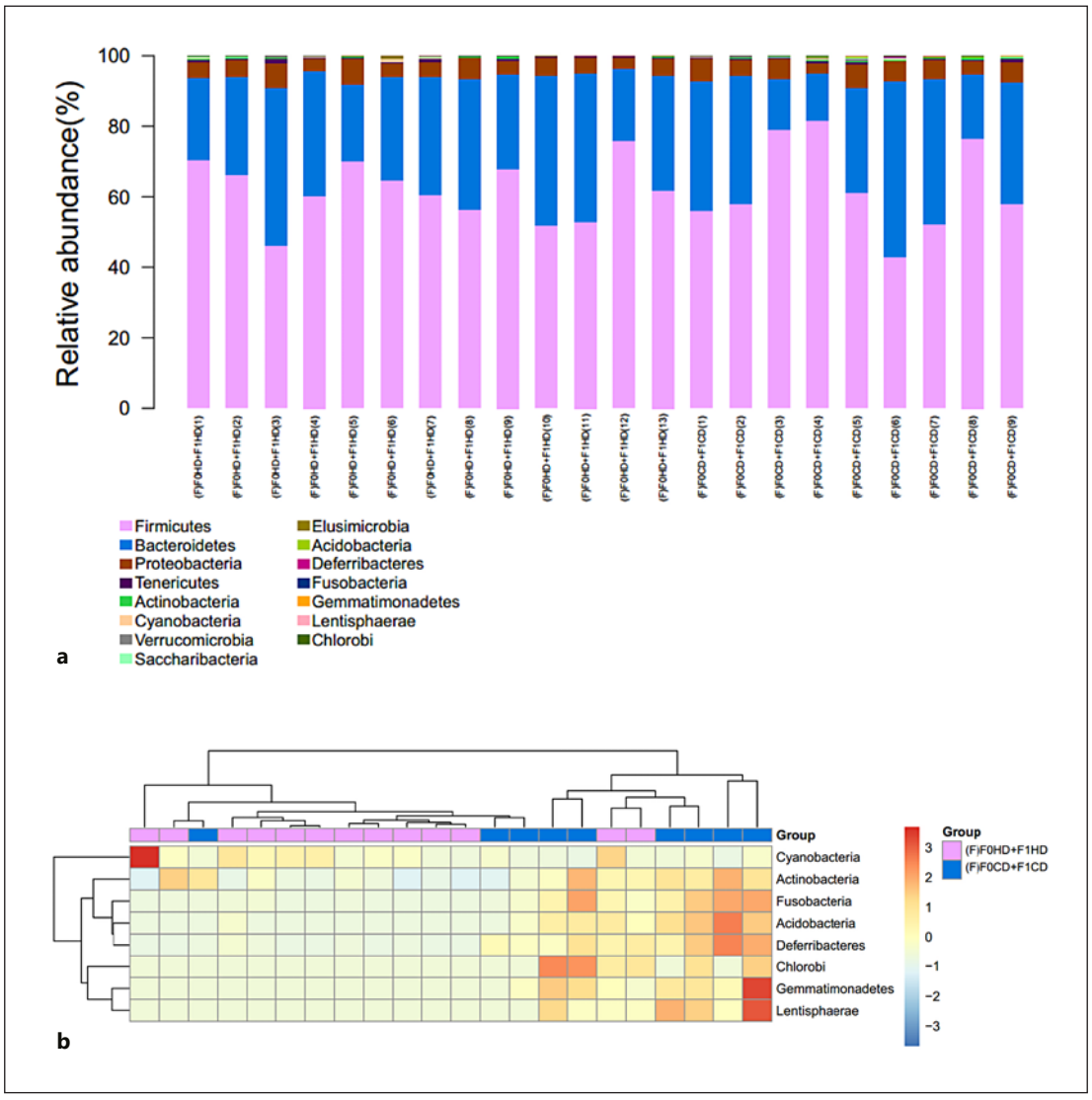

Fig. 2. The gut microbiota composition in F2 generation female offspring. **a** Relative abundance of the main phyla in the intestinal microbiota. **b** Heat map showing different phylotypes at the phyla level. **c** The heat map reflects the bacterial abundances at the genus level. **d** Principal coordinate analysis (PCoA) plots of gut microbial communities in F2 generation female offspring.

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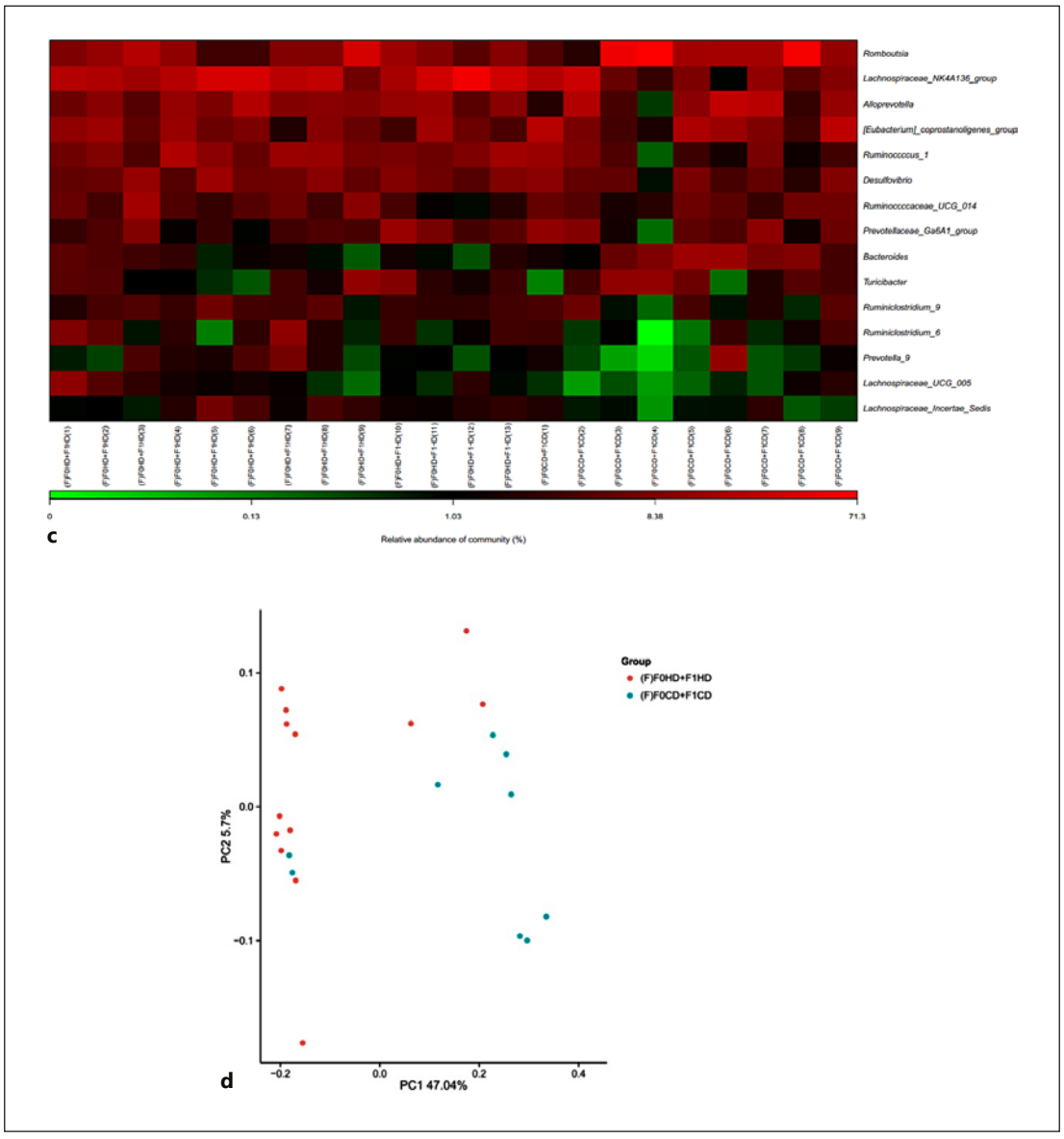
F0HD+F1HD offspring as compared to female offspring (Table 5). However, no significant differences in the OTUs or PD_whole_tree were observed between the F0CD+F1CD and F0HD+F1HD groups of both sexes (Table 5).

Comparison of the Gut Bacterial Community Composition among Different Groups

We analyzed the abundance of various bacteria in fecal samples. At the phyla level, *Firmicutes, Bacteroidetes* and *Proteobacteria* were dominant in the F2 offspring of both sexes (Fig. 2a, 3a). There was no difference in the relative abundances of the three main phyla between the F0CD+F1CD and F0HD+F1HD group of both sexes (Table 6). However, *Fusobacteria, Deferribacteres, Gemmatimonadetes, Acidobacteria, Lentisphaerae, Actinobacteria* and *Chlorobi* were significantly decreased whereas *Cyanobacteria* was significantly increased in



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the female F0HD+F1HD group ($p < 0.05$) compared to the female F0CD+F1CD group (Fig. 2b). A significant increase in the abundance of *Gemmatimonadetes* and *Lentisphaerae* was observed in the male F0HD+F1HD group ($p < 0.05$) compared to the male F0CD+F1CD group (Fig. 3b).

At the genus level, we analyzed the abundance of the genera in the studied groups and presented the results as a heat map. The resulted heatmap showed that paternal HFSSD led to a lower proportion of *Romboutsia (Firmicutes)*, *[Eubacterium]_coprostanoligenes_group (Firmicutes)*, *Bacteroides (Bacteroidetes)*, *Alloprevotella (Bacteroidetes)* in F2 female offspring ($p < 0.05$), and a higher proportion of *Alloprevotella (Bacteroidetes)*, *Ruminococcaceae_UCG_014 (Firmicutes)* and *Ruminococcus_1 (Firmicutes)* ($p < 0.05$) and a lower proportion of *Desulfovibrio (Proteobacteria)* in F2 male offspring ($p < 0.05$) (Fig. 2c, 3c).

Unweighted UniFrac PCoA of Illumina MiSeq amplicon data demonstrated separate clustering of the gut communities between the F0CD+F1CD group and the F0HD+F1HD group with PC1 percent variation explained = 47.04% and PC2 percent variation explained = 5.7% in the F2 female offspring (Fig. 2d), and PC1 percent variation explained = 27.73% and PC2

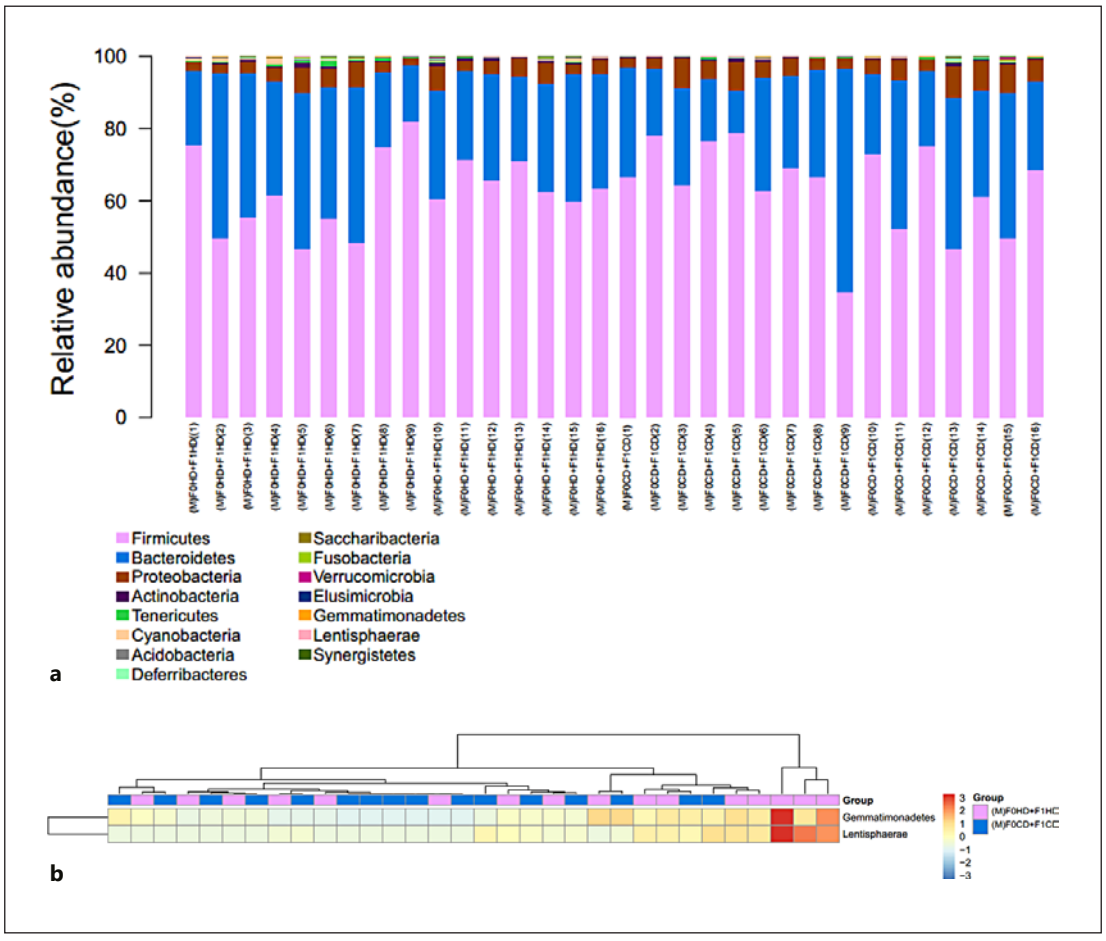

Fig. 3. The gut microbiota composition in F2 generation male offspring. **a** Relative abundance of the main phyla in the intestinal microbiota. **b** Heat map showing different phylotypes at the phyla level. **c** The heat map reflects the bacterial abundances at the genus level. **d** Principal coordinate analysis (PCoA) plots of gut microbial communities in F2 generation male offspring.

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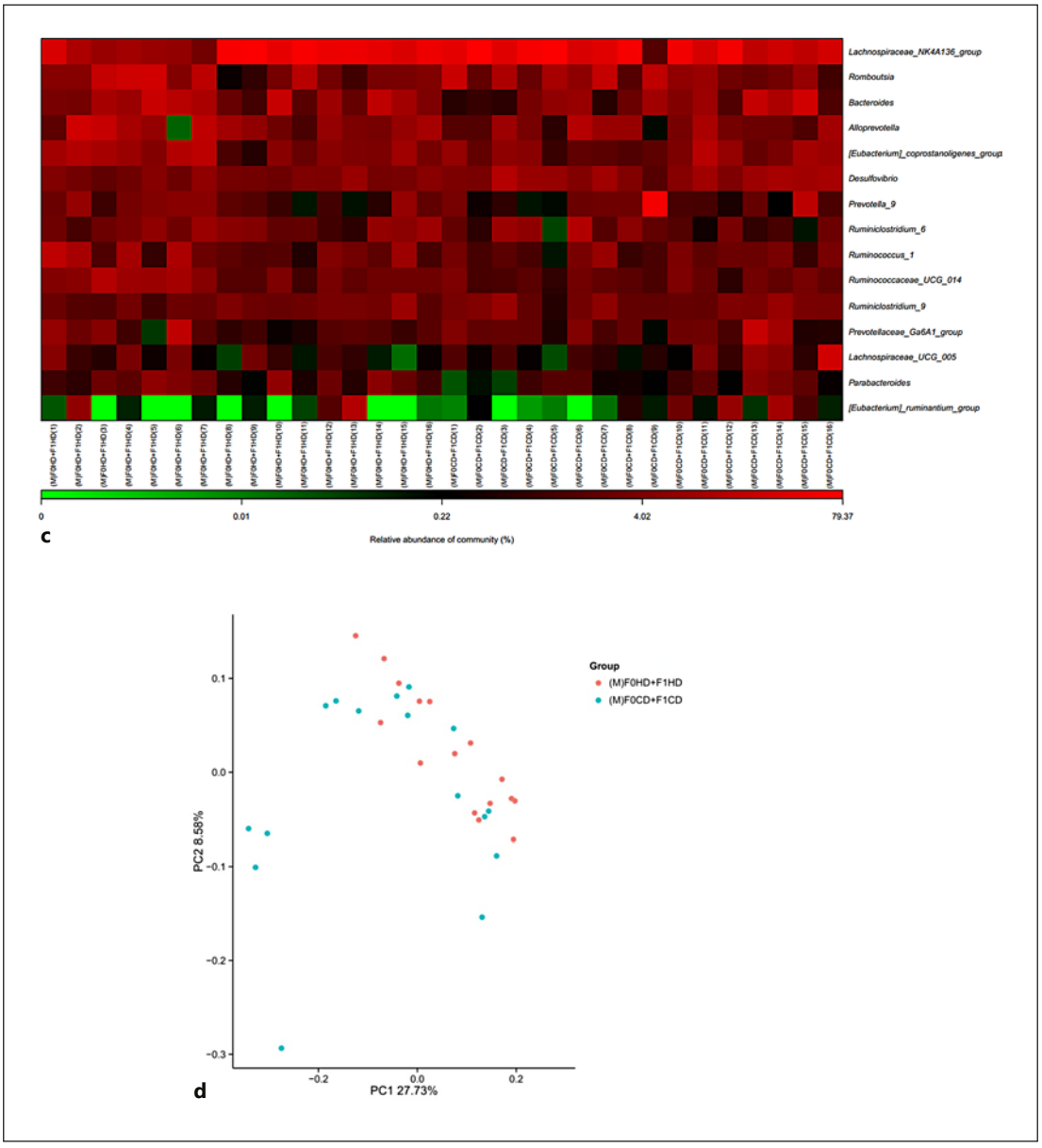
percent variation explained = 8.58% in the F2 male offspring (Fig. 3d). PCoA revealed striking shifts in microbiota between the F0CD+F1CD group and the F0HD+F1HD group in the F2 offspring, which was mainly reflected on the PC1 axis.

Correlation Analysis

Spearman's correlation analyses between relative abundance (percentage) of sequences belonging to main bacterial genus and liver function were performed. In the F2 female offspring, the correlation analyses indicated that *Lachnospiraceae_NK4A136_group (Firmicutes)* ($r^2 = 0.446$, $p < 0.05$) and *Ruminiclostridium_9 (Firmicutes)* ($r^2 = 0.525$, $p < 0.05$) percentage were positively associated with AST activity whereas *Romboutsia (Firmicutes)* ($r^2 = -0.510$, $p < 0.05$) and *Bacteroides (Bacteroidetes)* ($r^2 = -0.423$, $p < 0.05$) percentage were negatively associated with AST activity. The abundance of *Lachnospiraceae_NK4A136_group (Firmicutes)* ($r^2 = 0.553$, $p < 0.01$), *Desulfovibrio* (*Proteobacteria*) ($r^2 = 0.441$, $p < 0.05$) and *Ruminiclostridium_9 (Firmicutes)* ($r^2 = 0.616$, $p < 0.01$) were positively associated with ALT activity whereas *Romboutsia (Firmicutes)* ($r^2 = -0.649$, $p < 0.01$) and *Bacteroides (Bacteroidetes)* ($r^2 = -0.584$, $p < 0.01$) were negatively associated with ALT activity. The abundance



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of *Alloprevotella (Bacteroidetes)* (r^2 = –0.642, p < 0.01) was negatively associated with GLU and *Ruminococcus_1 (Firmicutes)* (r^2 = 0.601, p < 0.01) was positively associated with TG (Table 7).

In the F2 male offspring, the correlation analyses indicated that the abundance of *Alloprevotella (Bacteroidetes)* (r^2 = –0.478, p < 0.01) and *Ruminiclostridium_6 (Firmicutes)* (r^2 = –0.529, p < 0.01) were negatively associated with ALT activity. *Ruminiclostridium_6 (Firmicutes)* (r^2 = 0.429, p < 0.05) percentage were positively associated with GLU. The abundance of *Alloprevotella (Bacteroidetes)* (r^2 = 0.416, p < 0.05) and *Ruminococcaceae _UCG _014 (Firmicutes)* (r^2 = 0.449, p < 0.01) were positively associated with CHOL and *Desulfovibrio (Proteobacteria)* (r^2 = –0.393, p < 0.05) was negatively associated with CHOL. The abundance of *Ruminococcaceae_UCG_014 (Firmicutes)* (r^2 = 0.405, p < 0.05) was positively associated with HDL and *Desulfovibrio (Proteobacteria)* (r^2 = –0.419, p < 0.05) was negatively associated with HDL (Table 8).

Table 6. Relative abundance (%) of *Firmicutes*, *Bacteroidetes* and *Proteobacteria* in the F2 generation offspring of both sexes

Taxon	Female		Male	
	F0CD+F1CD (*n* = 9)	F0HD+F1HD (*n* = 13)	F0CD+F1CD (*n* = 16)	F0HD+F1HD (*n* = 16)
Firmicutes	62.90±0.04	61.89±0.02	63.95±0.13	62.71±0.10
Bacteroidetes	30.37±0.04	32.13±0.02	29.62±0.12	31.28±0.09
Proteobacteria	5.29±0.04	4.89±0.03	5.40±0.02	4.21±0.02

F0CD+F1CD, F2 offspring of F0 and F1 male founders fed a CD; F0HD+F1HD, F2 offspring of F0 and F1 male founders fed a HFSSD. Values are given as mean ± SEM. * $p < 0.05$ versus F0CD+F1CD, ** $p < 0.01$ versus F0CD+F1CD.

Table 7. Correlation analysis between relative abundance (%) of main bacterial genus and liver function/serum lipids in F2 generation female offspring

	Lachnospiraceae_NK4A136_group	Romboutsia	Bacteroides	Alloprevotella	[Eubacterium]_coprostanoligenes_group	Desulfovibrio	Prevotella_9	Ruminiclostridium_6	Ruminococcus_1	Ruminococcaceae_UCG_014	Ruminiclostridium_9	Prevotellaceae_Ga6A1_group	Lachnospiraceae_UCG_005
AST	0.446*	−0.510*	−0.423*	−0.090	−0.104	0.334	0.273	0.007	0.161	−0.076	0.525*	−0.190	0.274
ALT	0.553**	−0.649**	−0.584**	−0.057	−0.096	0.441*	0.343	0.153	0.277	−0.107	0.616**	0.032	0.237
GLU	0.141	−0.299	−0.191	−0.642**	−0.362	0.231	0.202	−0.034	0.011	−0.145	0.089	−0.310	0.211
TG	0.010	0.003	0.007	−0.399	0.001	0.055	0.028	0.306	0.601**	0.007	0.058	−0.332	0.284
CHOL	0.331	−0.268	−0.384	−0.087	−0.138	0.238	−0.129	−0.051	0.028	−0.240	0.023	0.024	−0.016
HDL	0.423	−0.356	−0.420	0.063	−0.110	0.189	−0.052	0.031	0.079	−0.202	0.118	0.080	−0.018
LDL	0.316	−0.412	−0.190	−0.331	0.174	0.122	−0.020	−0.064	0.353	−0.022	0.334	−0.240	0.027

AST, aspartate aminotransferase; ALT, alanine aminotransferase; GLU, glucose; TG, triacylglycerols; CHOL, cholesterol; HDL, high density lipoproteins; LDL, low density lipoproteins. * $p < 0.05$, ** $p < 0.01$.

Table 8. Correlation analysis between relative abundance (%) of main bacterial genus and liver function/serum lipids in F2 generation male offspring

	Lachnospiraceae_NK4A136_group	Romboutsia	Bacteroides	Alloprevotella	[Eubacterium]_coprostanoligenes_group	Desulfovibrio	Prevotella_9	Ruminiclostridium_6	Ruminococcus_1	Ruminococcaceae_UCG_014	Ruminiclostridium_9	Prevotellaceae_Ga6A1_group	Lachnospiraceae_UCG_005
AST	−0.126	0.049	0.319	−0.171	0.031	−0.274	−0.184	−0.129	0.008	0.173	−0.058	−0.086	−0.116
ALT	−0.032	0.041	0.294	−0.478**	−0.021	−0.096	−0.110	−0.529**	0.026	0.130	−0.029	0.012	0.093
GLU	−0.032	−0.131	0.069	0.120	−0.214	−0.112	−0.061	0.429*	−0.184	−0.116	0.073	−0.085	−0.354*
TG	−0.041	0.034	0.033	0.104	−0.021	−0.087	0.046	0.219	−0.072	0.107	0.117	−0.063	−0.046
CHOL	−0.143	0.308	0.124	0.416*	0.235	−0.393*	0.183	0.193	0.205	0.449**	−0.176	−0.225	−0.091
HDL	−0.085	0.229	0.089	0.344	0.166	−0.419*	0.173	0.096	0.234	0.405*	−0.211	−0.263	−0.176
LDL	0.082	−0.058	0.164	0.043	−0.047	−0.029	−0.345	−0.112	−0.014	−0.001	0.218	−0.236	−0.056

AST, aspartate aminotransferase; ALT, alanine aminotransferase; GLU, glucose; TG, triacylglycerols; CHOL, cholesterol; HDL, high density lipoproteins; LDL, low density lipoproteins. * $p < 0.05$, ** $p < 0.01$.

Discussion

Most studies investigating fetal programming focused on maternal programming. So far, many different maternal programming factors have been discovered (for example over- or undernutrition [33–35], lack of micronutrients [36, 37], and stress during pregnancy [38–40]). However, more recent studies showed that also paternal exposure to adverse environmental conditions can act on the offspring's phenotype in terms of fetal programming and influence later life disease risk [11, 28]. Paternal programming has been likewise described in clinical observation studies [41, 42]. So far most of the animal studies dealing with paternal programming used paternal high-fat diet models prior to mating to investigate potential underlying mechanisms. We exposed male rats to a high-salt-fat-sugar diet mimicking an unhealthy fast food diet often eaten by young men as recently described [30]. Our study was designed to examine the effect of feeding male rats HFSSD over two generations (F0 and F1) on their offspring's (F2) liver function, lipid profile and gut microbiome composition. Similar to other studies [43–45], we have shown that the F0HD+F1HD rats (two generations of paternal unhealthy diet) of both sexes had higher body weight, and higher serum aspartate aminotransferase activity at the age of 25 weeks. In addition, serum cholesterol, high density lipoproteins, low density lipoproteins were significantly higher in the male F0HD+F1HD group than in the male F0CD+F1CD group. This indicates that paternal high-salt-fat-sugar diet could lead to abnormal lipid metabolism in the later life of the offspring in a sex-specific manner. The clinical implications of these findings are unclear, since higher HDL levels are associated with a decreased risk for cardiovascular events in humans, whereas higher LDL concentrations have the opposite association with cardiovascular events.

It is important to mention the gut-liver axis, which refers to the close anatomical and functional relationship between the gastrointestinal tract and the liver [46, 47]. Such an association includes transfer of molecules associated with the gut microbiome to the liver and vice versa [48]. A growing body of evidence indicated that gut-liver axis malfunction (small intestinal bacterial overgrowth, intestinal dysbiosis, and increased intestinal permeability ["leaky gut"]) is a leading factor in the development and progression of NAFLD and obesity [49–53].

Our present study indicated that the dominant phyla of both F0CD+F1CD group and F0HD+F1HD group in the F2 offspring were *Bacteroidetes*, *Firmicutes* and *Proteobacteria*. These data were consistent with previous findings [54, 55]. Further analysis showed that there were significant differences in gut bacterial community composition at phyla and genus level between the F0CD+F1CD group and F0HD+F1HD group in the F2 offspring of both sexes. Of interest, our present study showed that the variations of the relative abundance (percentage) of bacterial genus of the F2 offspring were associated with liver function disorder induced by a paternal pre-conceptional unhealthy diet. Although we demonstrated correlations between liver functional parameters/lipids and the variations of the relative abundance (percentage) of bacterial genus, the causality remains to be established in further studies. Stool transplantation studies transferring stool from F2 offspring to rats with parents and grandparents on a completely normal rat diet might be a useful tool to analyze whether the observed alterations of liver function and/or lipid metabolism in the F2 offspring are causally related to alterations in the gut microbiome.

Conclusion

Our data suggest that paternal HFSSD over two generations resulted in mild liver functional alterations and intestinal microbial disorders in the F2 offspring as well as lipid profile alterations in male offspring only in later life. Our work is novel in showing the interplay

between paternal pre-conceptional unhealthy diet, liver function alterations, lipid profile and gut microbiota. Therefore, as both gut microbiota and paternal programming become more evident and prevalent players in obesity and T2DM, a better understanding of the role of the gut microbiota in paternal programming might provide novel perspectives regarding its pathophysiological relevance and pave the way for new therapeutic principles of obesity and T2DM. Further studies should be performed to confirm these results.

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Statements of Ethics

The study was approved by the animal welfare committee of the Hunan Normal University, Changsha, China

Disclosure Statement

The authors declare that there are no competing financial interests.

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