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Basic nutritional investigation

Effect of a high-fat high-fructose diet on the composition of the intestinal microbiota and its association with metabolic and anthropometric parameters in a letrozole-induced mouse model of polycystic ovary syndrome

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ABSTRACT

Objective: It has been suggested that dysbiosis of the gut microbiota is associated with the pathogenesis of Polycystic Ovary Syndrome (PCOS), and that improper diet can aggravate these changes. This study thus aimed to investigate the effects of a high-fat/high-fructose (HF/HFr) diet on the gut microbial community and their metabolites in prepubertal female mice with letrozole (LET)-induced PCOS. We also tested the correlations between the relative abundance of microbial taxa and selected PCOS parameters.
Research methods & procedures: Thirty-two C57BL/6 mice were randomly divided into four groups (n = 8) and implanted with LET or a placebo, with simultaneous administration of a HF/HFr diet or standard diet (StD) for 5 wk. The blood and intestinal contents were collected after the sacrifice.
Results: Placebo + HF/HFr and LET + HF/HFr had significantly higher microbial alpha diversity than either group fed StD. The LET-implanted mice fed StD had a significantly higher abundance of *Prevotellaceae_UCG-001* than the placebo mice fed StD. Both groups fed the HF/HFr diet had significantly lower fecal levels of short-chain fatty acids than the placebo mice fed StD, while the LET + HF/HFr animals had significantly higher concentrations of lipopolysaccharides in blood serum than either the placebo or LET mice fed StD. Opposite correlations were observed between *Turicibacter* and *Lactobacillus* and the lipid profile,
Conclusion: HF/HFr diet had a much stronger effect on the composition of the intestinal microbiota of prepubertal mice than LET itself.

Introduction

Polycystic ovary syndrome (PCOS) is a common endocrine disease that affects 6% to 20% of women of reproductive age and is associated with a high risk of infertility, obesity, and insulin resistance [1]. Although genetic, neuroendocrine, metabolic, environmental, and lifestyle-related factors are known to cause PCOS, its etiology remains unclear.

There is growing evidence that dysbiosis of the gut microbiota is associated with the pathogenesis of PCOS. A recent review confirmed that PCOS women with altered testosterone/estrogen profiles had different gut microbiota compositions, including in beta diversity and a lower alpha diversity than healthy women [2]. Moreover, changes in the relative abundances of specific taxa of gut bacteria have been correlated with clinical manifestations of PCOS, such as obesity and insulin resistance [3]. Kelley et al. [4] were the first to confirm changes in gut microbiota after induction of PCOS in mice with letrozole (LET), including a significant decrease in the total gut microbial species count and phylogenetic richness. Moreover, Torres et al. observed that LET-induced PCOS in adult mice was associated with a distinct shift in gut microbial

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diversity, unlike in LET-induced PCOS in pubertal mice [5]. This finding shows that the timing of androgen exposure in animal models may significantly affect metabolism dysregulation and the gut microbiome in PCOS.

Furthermore, improper diet, such as a high-fat diet (HFD), can aggravate the intestinal dysbiosis in LET-induced PCOS in mice [6]. The study of Zheng et al. [6] also observed that the influence of LET on gut bacteria was not as significant as that of HFD; they showed that the abundance of the *Vibrio* genus significantly increased in the LET treatment group, that the *Bacteroides* and *Phascolarctobacterium* genera were enriched in the HFD group, and that the *Bacteroides, Phascolarctobacterium, Blautia, Parabacteroides, Akkermansia, [Ruminococcus]_torques_group*, and *Anaerotruncus* genera were enriched in the LET group fed with HFD [6]. High levels of consumption of highly processed food that is rich in simple sugars—particularly fructose and saturated fat—have been associated with obesity and metabolic disorders [7,8]. The group that is most vulnerable to these effects is young people around adolescence, who are overexposed to diets high in fats and sugar, and especially in fructose [9], from soft drinks, energy drinks, and fruit juices [10]. It is also known that this type of diet can affect the composition of the gut microbiota [11]. However, the exact direction of these microbial changes and their effects on the severity of PCOS symptoms have not been unequivocally assessed so far.

It is worth noting that the use of an animal model of PCOS allows the observation and validation of new biomarkers related to this disease [12]. It also enables identification of the molecular mechanisms that underlie the metabolic features of PCOS, which may result in the development of innovative treatment methods [13]. The application of letrozole in the induction of PCOS allows one-time subcutaneous implantation, while the use of androgens often involves daily injections, which may translate into higher levels of stress in animals [14]. Moreover, the use of letrozole enables induction in both the "lean" PCOS phenotype when used individually, and in the "classic" phenotype, when its effect is enhanced by a factor causing metabolic disorders, such as improper diet [14,15].

This study thus aims to investigate the effects of high-fat/high-fructose (HF/HFr) diet on gut microbial community and their metabolites in prepubertal female mice with letrozole-induced PCOS. We moreover intended to determine whether there is a correlation between the relative abundance of microbial taxa and selected parameters associated with PCOS, such as body weight gain, adipose tissue, blood testosterone concentration, Homeostasis Model Assessment of Insulin Resistance (HOMA-IR), triglycerides (TG), low-density lipoprotein cholesterol (LDL-C), and Castelli's Risk Index. The findings of this study may help achieve a better understanding of the effects of the HF/HFr diet and LET-induced PCOS on the composition of the gut microbiota, and this would be valuable for further study of new PCOS therapies.

Materials and methods

Experimental animals and treatment

In PCOS animal models, it is advised to induce the disease during the prepubertal period, as this leads to more stable PCOS outcomes than when it is induced after puberty [16]. Thirty-two prepubertal female C57BL/6 mice (average body weight 13.5 g) with an age of 3 wk were therefore exposed to LET for a period of 5 wk [16]. The animals were purchased from Mossakowski Institute of Experimental and Clinical Medicine, Polish Academy of Sciences, Warsaw, Poland and housed in the vivarium at the Department of Physiology, Biochemistry and Animal Biostructure, part of the Faculty of Veterinary Medicine and Animal Sciences at Poznań University of Life Sciences. The animals were allowed to adapt to the laboratory environment for 10 d. All animals were housed in standard polycarbonate cages and maintained in a controlled environment with a temperature of 21 ± 1°C, humidity of 55%–65%, and a 12-h light–dark cycle. After acclimatization, at 4 wk of age, the mice were randomly assigned to four groups: (1) Placebo, consisting of mice injected with a placebo pellet and fed a standard diet (StD) (n = 8); (2) Placebo + HF/HFr, consisting of mice injected with a placebo pellet and fed the HF/HFr diet (n = 8); (3) LET, consisting of mice injected with a LET pellet and fed a standard diet (n = 8); and (4) LET + HF/HFr, consisting of mice injected with a LET pellet and fed the HF/HFr diet (n = 8). Subcutaneous implantation of continuous release letrozole (3 mg, 50 μg/d) or a placebo pellet was performed to induce PCOS or to create a control group. Both the active product and the placebo control pellets contained a matrix of carrier–binder consisting of cholesterol, lactose, celluloses, phosphates, and stearates, with the only difference being that the pellets inducing PCOS had an additional active substance, letrozole. The letrozole was purchased from Innovative Research of America. Induction of PCOS was confirmed through histopathological examination of the ovaries. The methodology and results of this are presented in our previous article [15].

Two groups of mice were fed a standard laboratory diet (3.8 kcal/g, energy supply ratio: protein 18%, carbohydrate 66%, fat 16%). The other two groups were fed the HF/HFr diet (4.7 kcal/g, energy supply ratio: protein 17%, carbohydrate 37.5% (mainly fructose), fat 45.5%). The experimental diets were bought from Morawski Animal Feed (Kcynia, Poland). The animals had unlimited access to water and food throughout the experimental period. Once a week, the animals were weighed using a Sartorius MSE2202S-100-D0 precision balance (Germany). During the last week of the experiment, the body composition of the mice was analyzed by in vivo time-domain nuclear magnetic resonance using a Bruker Minispec LF90 body analyzer (USA). The study was approved by the Local Ethical Commission under permission No. 51/2021 and was carried out in line with the ARRIVE 2.0 guidelines for animal research [17].

Sample collection

Fresh fecal samples were collected from all groups of mice during the last week of the experiment and were immediately frozen at −80°C until the analysis was performed. After the end of the experiment (5 wk), two individuals from each group were randomly selected until the number of individuals was depleted. These individuals were sacrificed by decapitation between the two time points ZT 3 (9 am) and ZT5 (11 am). Blood was collected in nonheparinized tubes. The blood was centrifuged (3500 × g, 15 min, 4°C) to obtain serum samples, which were then frozen at −80°C for future biochemical analysis. The intestinal contents of the cecum were collected and the bacterial DNA was immediately isolated using the commercially available QIAamp fecal DNA minikit (QIAGEN, Hilden, Germany), following the manufacturer's protocol.

Biochemical analysis

The concentration of lipopolysaccharides in blood serum was measured using an immunoassay (ELISA) kit obtained from Sunlong Biotech (Hangzhou, Zhejiang, China). Serum glucose (GLU), triglycerides (TG), total cholesterol (TC), HDL-C, and LDL-C were measured using commercially available colorimetric and enzymatic assays from Pointe Scientific (Lincoln Park, MI, USA). The concentrations of insulin and lipopolysaccharide (LPS) in blood serum were measured using an immunoassay (ELISA) kit from Sunlong Biotech (Hangzhou, Zhejiang, China). Testosterone level was determined using an immunoassay (ELISA) kit from LDN (Nordhorn, Germany). The optical density of these samples was measured using a Synergy 2 microplate reader (Biotek, Winooski, VT, USA).

Calculation of the HOMA-IR and Castelli indices

Insulin resistance and β-cell function were evaluated using the Homeostasis Model Assessment Method with the following formula:

HOMA − IR = fasting glucose [mmol/L] × fasting insulin [μIU/mL]/22.5 [18]

Castelli's Risk Index I (CRI-I) was calculated as follows:

CRI-I = total cholesterol [mg/dL] / high-density lipoprotein [mg/dL] [19]

Fecal SCFA analysis

Determination and quantification of short-chain fatty acids (SCFAs) in the mice feces were performed by gas chromatography coupled with flame ionization detection (GC-FID) [20]. Thawed stool samples weighing 100 mg were homogenized with a spatula and then acidified with 50% sulfuric acid. After centrifugation, 50 μL of internal standard solution (IC6, concentration 330 μM) was added. This mixture was extracted using 1 mL of ethyl ether and centrifuged (5 min, 2800 × g). The extraction was repeated three times and 3 mL of the organic phase was collected each time. Finally, 0.5 μL of the harvested organic phase was injected into the gas chromatograph (GC) for analysis. The individual acids were quantified using gas chromatography equipped with a flame ionization detector (Agilent 7890 series II Agilent Technologies, Santa Clara, CA, USA) and a BPX 70 column (BPX70, 25 m × 0.22 mm ID × 0.25 μm, SGE Analytical Science, Ringwood, Australia). The acids were identified by mass spectrometry (Agilent 5975C, Agilent Technologies, Santa Clara, CA, USA). Peak integration was performed using MSD ChemStation (Agilent Technologies, Santa Clara, CA, USA). Acid concentrations are expressed in [μmol/g] [20].

Gut microbiota analysis

Bacterial DNA isolated from the cecal contents of the mice was sent to Genomed (Warsaw, Poland) for 16S rRNA gene, V3–V4 region sequencing using a MiSeq platform with paired-end (PE) technology, 2 × 300 nt (Illumina, San Diego, CA, USA). Specific sequences of the 341F and 785R primers (metagenomic 16S rRNA analysis) were used to amplify the selected region and to prepare the library. PCR was performed using a Q5 Hot Start High-Fidelity 2X Master Mix under the reaction conditions recommended by the manufacturer. Bioinformatic analysis of the raw sequences was performed using QIIME 2 software. OTUs were classified to taxonomic levels based on the Silva 138 reference sequence database.

Statistical analysis

The sample size was calculated using G*Power software (RRID:SCR_013726), following the previous study of Zheng et al. [6]. The sample size was calculated to be eight mice per group on the basis of the differences in HOMA-IR between the HFD (high-fat diet) group (0.8 ± 0.26) and the PCOS+HFD group (1.21 ± 0.11), with an alpha value of 0.05 and a power of 0.95. The normality of the data distribution was tested using the Shapiro–Wilk test. The Kruskal–Wallis test was then used for nonnormally distributed data, such as SCFA concentrations, and the Tukey HSD test was used for normally distributed data [21], such as LPS concentration. Both tests were carried out using Statistica 13.3.0 (TIBCO Software, Palo Alto, CA, USA; 2017). A *P* value of less than 0.05 was considered statistically significant.

The microbiota composition was analyzed using RStudio (R version 4.0.3 (2020-10-10)) using a set of packages that included *phyloseq, microbiome*, and *vegan*. The taxa were filtered by removing all those not assigned to any phylum. Only taxa with abundances over 0.25% in at least one sample were left in the dataset [22]. In total, 302 OTUs were identified. All analyses of gut microbiota composition were performed on the basis of the relative abundances (RA) of the OTUs. As the data was not normally distributed, the Kruskal–Wallis test and the Dunn test (with *P* adjusted using the method of Benjamini and Hochberg) were used to assess differences in the RA of the individual taxa, grouped at different taxonomic levels between the study groups. To assess the association between study groups and microbial β-diversity, a bacterial distance matrix was constructed using the Bray–Curtis distance, and PCoA and PERMANOVA analysis was performed. The α-diversity was compared between groups using the Shannon and Simpson indices. These indices was calculated for the samples using QIIME (v1.7.0) based on the rarefied OTU counts. Correlations between the relative abundance of microbiome genera and the metabolic and anthropometric markers were calculated using Spearman's correlation test. Only strong correlations ($q < 0.01$, $R > 0.6$) are presented in the body of this report, while other correlations ($q < 0.05$, $R < 0.06$) are shown in Supplementary Figure S3. The microbiota features that differentiate intestinal microbiota were characterized using the LEfSe method (with the strategy of multi-class analysis all-against-all) for biomarker discovery [23], which uses the Kruskal–Wallis rank–sum test to detect features with significantly different abundance levels between assigned taxa, and which performs an LDA to determine the effect size of each feature. A q value of less than 0.05 was considered statistically significant. All the results are presented in the tables and figures as arithmetic means ± standard deviations (SD).

Results

Anthropometric, hormonal, and metabolic parameters

The anthropometric, hormonal and metabolic results have been thoroughly described previously [15]. In brief, the LET+HF/HFr group saw significantly greater weight gain than did the LET group, by approximately 11.9%. Additionally, the LET+HF/HFr group exhibited significantly increased testosterone levels and deteriorated lipid profile and HOMA-IR. Only in the Placebo+HF/HFr group were similar changes observed, other than for changes in insulin sensitivity. Both the LET+HF/HFr and Placebo+HF/HFr groups developed polycystic ovaries. Although the LET-treated group did not display endocrine or metabolic abnormalities, polycystic ovaries were nonetheless observed.

Intestinal microbiota diversity

The alpha diversity metric determined at the OTU level with the Shannon index showed that the Placebo group had significantly lower diversity and richness than the Placebo + HF/HFr group (3.48 ± 0.25 vs. 4.06 ± 0.14, $q < 0.001$) or the LET + HF/HFr group (3.48 ± 0.25 vs. 3.82 ± 0.21, $q < 0.05$). Significantly lower alpha-diversity was also observed in the LET group than in the Placebo + HF/HFr group (3.62 ± 0.27 vs. 4.06 ± 0.14, $q < 0.001$) (Fig. 1). Similar differences were observed using Simpson's index (Placebo vs. Placebo + HF/HFr 0.92 ± 0.03 vs. 0.96 ± 0.01, $q < 0.001$; Placebo vs. LET + HF/HFr 0.92 ± 0.03 vs. 0.95 ± 0.01, $q < 0.01$; Placebo + HF/

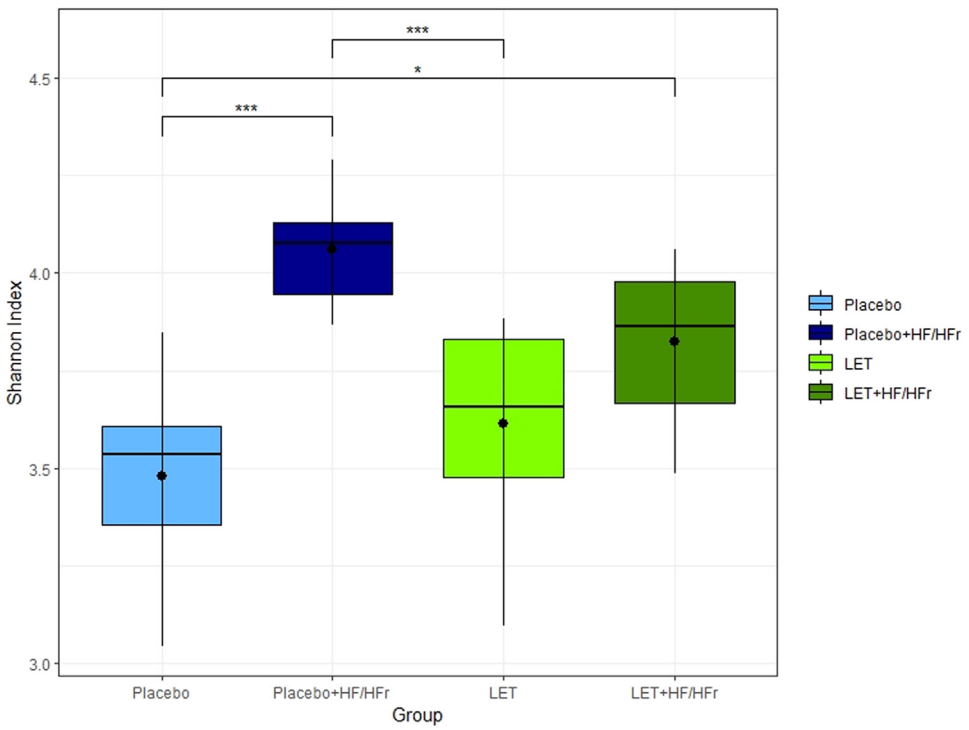

Fig. 1. Boxplots comparing cecal microbial α-diversity (measured by the Shannon Index) of experimental groups. *: $q < 0.05$; **: $q < 0.01$; ***: $q < 0.001$.

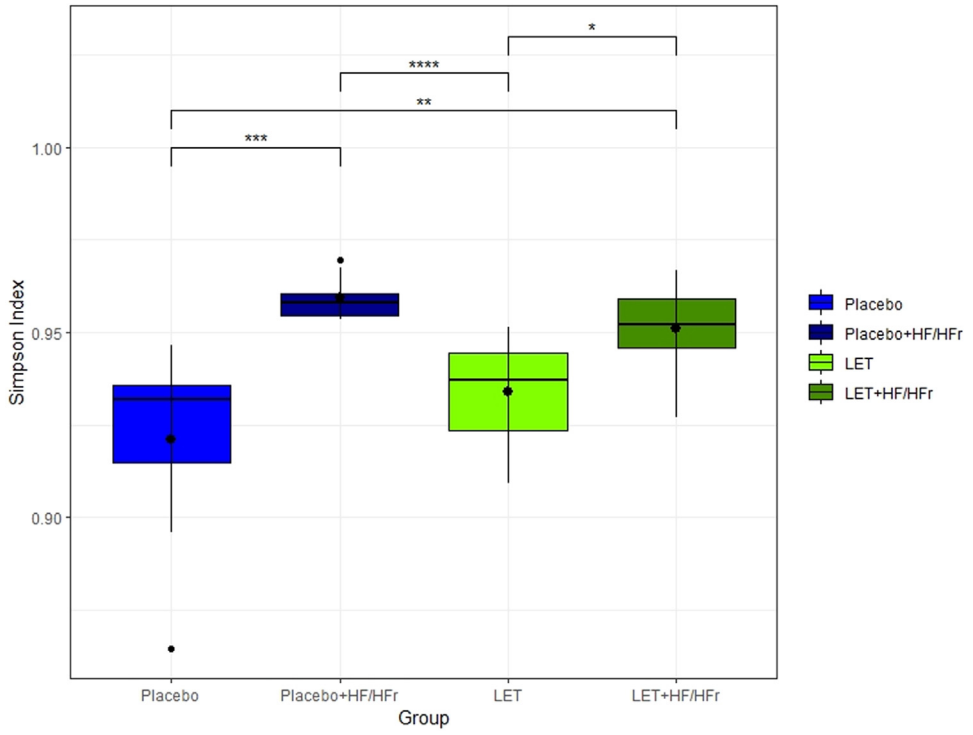

Fig. 2. Boxplots of the cecal microbial α-diversity (measured by Simpson's index) of the experimental groups. *: $q < 0.05$; **: $q < 0.01$; ***: $q < 0.001$.

HFr vs. LET 0.96 ± 0.01 vs. 0.93 ± 0.01, $q < 0.001$). However, the use of Simpson's index allowed us to note significant differences between the LET and LET + HF/HFr groups (0.93 ± 0.01 vs. 0.95 ± 0.01, $q < 0.05$) (Fig. 2).

A PCoA plot using the Bray–Curtis dissimilarity metric demonstrated a distinct clustering of the Placebo + HF/HFr group than the other three groups (Fig. 3). The significance of these differences was confirmed by the Kruskal–Wallis and Dunn tests, which compared coordinate values for points located within each group on the *x* axis (Placebo HF/HFr and LET: $q < 0.001$; Placebo HF/HFr and LET + HF/HFr: $q < 0.01$; Placebo HF/HFr and Placebo: $q < 0.001$).

Composition of the intestinal microbiota

Bacterial abundances and prevalence at the phylum and genus levels are compared and presented in Table 1, while comparisons of other taxonomic levels (class, order, and family) are presented in Supplementary Table S1.

The five major phyla in our study groups are shown in Figure 4. *Firmicutes* and *Bacteroidota* were the most abundant phyla in all groups. Moreover, the *Firmicutes:Bacteroidota* ratio did not significantly differ across groups (data not shown).

On the phylum level, *Actinobacteriota* was present only in the Placebo + HF/HFr group (0.13% ± 0.13%). Differences between groups in the majority of remaining phyla were not statistically significant.

To determine the effects of the HF/HFr diet, mice in the Placebo +HF/HFr group were compared with those in the Placebo group. On the genus level, the Placebo + HF/HFr group had a significantly higher abundance than the Placebo group for *Alloprevotella* (5.54% ± 1.69% vs. 1.06% ± 0.90%, $q < 0.01$), *Muribaculum* (2.16% ± 0.32% vs. 0.81% ± 0.31%, $q < 0.01$), *Rikenella* (1.17% ± 0.51% vs. 0.33% ± 0.18%, $q < 0.01$), and *Parasuterella* (0.22% ± 0.15% vs. 0.001% ± 0.004%, $q < 0.01$). *Lactobacillus* abundance and prevalence were also significantly lower in the Placebo + HF/HFr group than in the Placebo group (0.001% ± 0.003% vs. 0.11% ± 0.12%, $q < 0.001$; prevalence 12,5% vs. 100%).

Furthermore, to determine the effects of LET, animals in the LET group were compared with those in the Placebo group. The LET group had a significantly higher abundance of *Prevotellaceae_UCG-001* than did the Placebo group (0.66% ± 0.29% vs. 0.29% ± 0.55%, $q < 0.01$).

The Placebo + HF/HFr and LET groups were then analyzed in order to compare the effects of both LET and HF/HFr. The Placebo + HF/HFr group showed significantly higher abundances than the LET group for *Alloprevotella* (5.54% ± 1.69% vs. 1.50% ± 1.10%, $q < 0.01$), *Muribaculum* (2.16% ± 0.32% vs. 0.74% ± 0.33%, $q < 0.001$), *Rikenella* (1.17% ± 0.51% vs. 0.42% ± 0.24%, $q < 0.01$), and *Clostridia_vadinBB60_group* (11.14% ± 2.59% vs. 4.40% ± 1.99%, $q < 0.01$). However, the Placebo + HF/HFr group had significantly lower abundances of *Lactobacillus* (0.001% ± 0.003% vs. 0.06% ± 0.09%, $q < 0.05$), *Butyricicoccus* (0.04% ± 0.04% vs. 0.38% ± 0.46%, $q < 0.05$), and *Prevotellaceae_NK3B31_group* (0.00% ± 0.00% vs. 0.21% ± 0.25%, $q < 0.001$). The prevalence of *Prevotellaceae_NK3B31_group* was 0% in the Placebo + HF/HFr group.

Finally, the LET+ HF/HFr group was compared with the Placebo+ HF/HFr group to determine whether LET and HF/HFr had an additive harmful effect on the composition of the intestinal microbiota. The Placebo + HF/HFr group also had significantly higher abundances of *Muribaculum* (2.16% ± 0.32% vs. 1.02% ± 0.42%, $q < 0.01$), *Romboutsia* (0.69% ± 0.54% vs. 0.01% ± 0.03%, $q < 0.01$), *Turicibacter* (0.63% ± 0.32% vs. 0.07% ± 0.16% $q < 0.01$), *Clostridium_sensu_stricto _1* (0.23% ± 0.24% vs. 0.01% ± 0.04% $q < 0.01$), and *Clostridia_vadinBB60_group* (11.14% ± 2.59% vs. 3.47% ± 1.79%, $q < 0.001$) than did the LET + HF/HFr group.

Furthermore, linear discriminant analysis (LDA) effect size (LEfSe) was determined in order to identify significant differentially abundant microbiota. The results of this analysis are presented in Supplementary Figures S1 and S2. The results for genus taxonomic levels are comparable with those from the previous

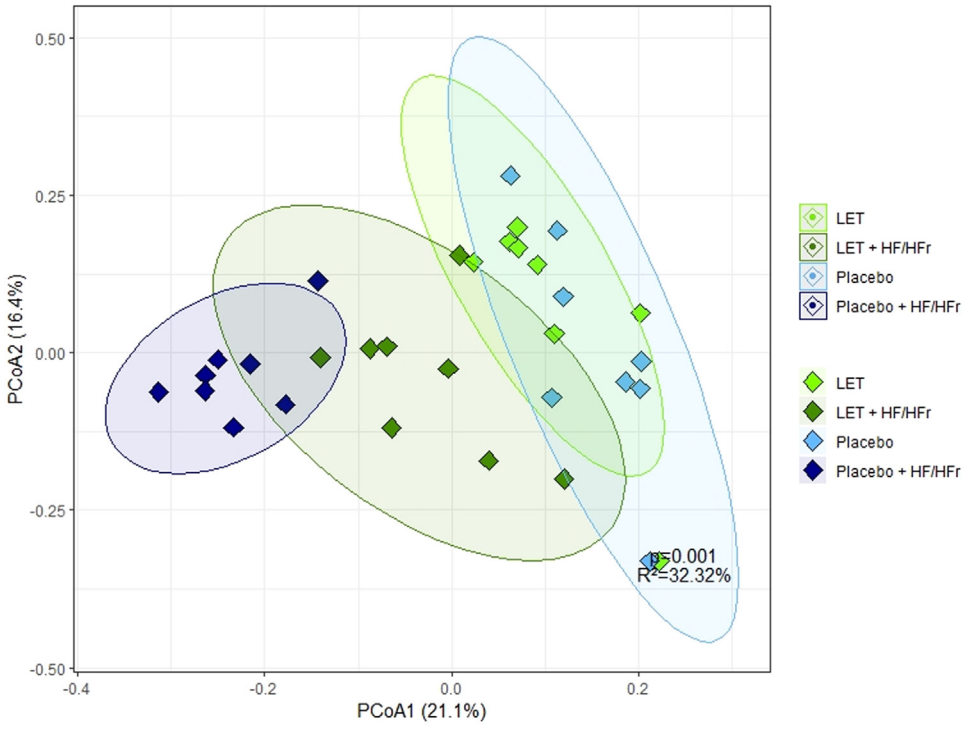

Fig. 3. PCoA plot of microbial β-diversity (based on the Bray–Curtis distance) of cecal samples showing PERMANOVA results on associations with study groups.

Table 1
Relative abundances and prevalence of selected taxa at the phylum and genus levels (values are presented as percentages)

Taxonomic level	Placebo			Placebo + HF/HFr			LET			LET + HF/HFr		
	Mean	SD	Prevalence	Mean	SD	Prevalence	Mean	SD	Prevalence	Mean	SD	Prevalence
PHYLUM												
Bacteroidota	37.67	6.60	100	48.22	7.21	100	46.08	7.62	100	50.54	14.05	100
Firmicutes	38.97	10.23	100	33.94	6.55	100	34.40	5.43	100	30.52	13.06	100
Campylobacterota	18.04	5.30	100	12.75	3.45	100	14.34	5.28	100	13.73	3.96	100
Deferribacterota	3.96	1.50	100	1.99	0.78	100	3.73	1.98	100	2.92	1.40	100
Cyanobacteria	0.52	0.32	100	1.15	1.60	100	0.41	0.34	100	0.59	0.89	87.5
Proteobacteria	0.32	0.27	100	0.97	0.64	100	0.33	0.51	100	0.49	0.57	100
Desulfobacterota	0.24	0.22	100	0.44	0.37	100	0.28	0.21	100	0.73	0.63	100
Patescibacteria	0.18	0.25	50	0.22	0.14	100	0.17	0.19	66,7	0.15	0.16	87.5
Verrucomicrobiota	0.09	0.14	100	0.20	0.15	100	0.26	0.40	100	0.34	0.34	100
Actinobacteriota	0.00 ^{a}	0.00	0	0.13 ^{b}	0.13	100	0.00 ^{a}	0.00	0	0.00 ^{a}	0.00	0
GENUS												
Clostridia_vadinBB60_group	8.69 ^{bc}	4.19	100	11.14 ^{c}	2.59	100	4.40 ^{ab}	1.99	100	3.47 ^{a}	1.79	100
Alloprevotella	1.06 ^{a}	0.90	87.5	5.54 ^{b}	1.69	100	1.50 ^{a}	1.10	77.8	3.04 ^{ab}	2.51	75
Muribaculum	0.81 ^{a}	0.31	100	2.16 ^{b}	0.32	100	0.74 ^{a}	0.33	100	1.02 ^{a}	0.42	100
Rikenella	0.33 ^{a}	0.18	100	1.17 ^{b}	0.51	100	0.42 ^{a}	0.24	100	1.05 ^{b}	0.76	100
Prevotellaceae_UCG-001	0.29 ^{a}	0.55	75	0.36 ^{ab}	0.17	100	0.66 ^{b}	0.29	100	0.32 ^{ab}	0.15	100
Butyricicoccus	0.19 ^{ab}	0.18	87.5	0.04 ^{a}	0.04	100	0.38 ^{b}	0.46	88.9	0.05 ^{a}	0.05	87.5
Prevotellaceae_NK3B31_group	0.18 ^{ab}	0.32	50	0.00 ^{a}	0.00	0	0.21 ^{b}	0.25	100	0.01 ^{a}	0.02	12.5
ASF356	0.15 ^{b}	0.25	75	0.35 ^{b}	0.25	87.5	0.04 ^{a}	0.11	11.1	0.00 ^{a}	0.00	0
Lactobacillus	0.11 ^{b}	0.12	100	0.001 ^{a}	0.003	12.5	0.06 ^{b}	0.09	77.8	0.00 ^{a}	0.00	0
Parasutterella	0.001 ^{a}	0.004	12.5	0.22 ^{b}	0.15	75	0.09 ^{ab}	0.11	77.8	0.14 ^{b}	0.15	87.5
Romboutsia	0.00 ^{a}	0.00	0	0.69 ^{b}	0.54	87.5	0.00 ^{a}	0.00	0	0.01 ^{a}	0.03	25
Turicibacter	0.00 ^{a}	0.00	0	0.63 ^{b}	0.32	100	0.00 ^{a}	0.00	0	0.07 ^{a}	0.16	50
Clostridium_sensu_stricto_1	0.00 ^{a}	0.00	0	0.23 ^{b}	0.24	87.5	0.00 ^{a}	0.00	0	0.01 ^{a}	0.04	12.5
GCA-900066575	0.00 ^{a}	0.00	0	0.16 ^{b}	0.13	87.5	0.11 ^{ab}	0.14	55.6	0.13 ^{ab}	0.21	62.5
Bifidobacterium	0.00 ^{a}	0.00	0	0.13 ^{b}	0.13	100	0.00 ^{a}	0.00	0	0.00 ^{a}	0.00	0
[Eubacterium]_siraeum_group	0.00 ^{a}	0.00	0	0.13 ^{b}	0.16	87.5	0.00 ^{a}	0.00	0	0.00 ^{a}	0.00	0
[Eubacterium]_coprostanoligenes_group	0.00 ^{a}	0.00	0	0.06 ^{b}	0.11	75	0.00 ^{a}	0.00	0	0.07 ^{b}	0.13	75

Results are expressed as means ± SDs (n = 8 per group). Values with different letters (a, b, c) show statistically significant differences ($q < 0.05$).

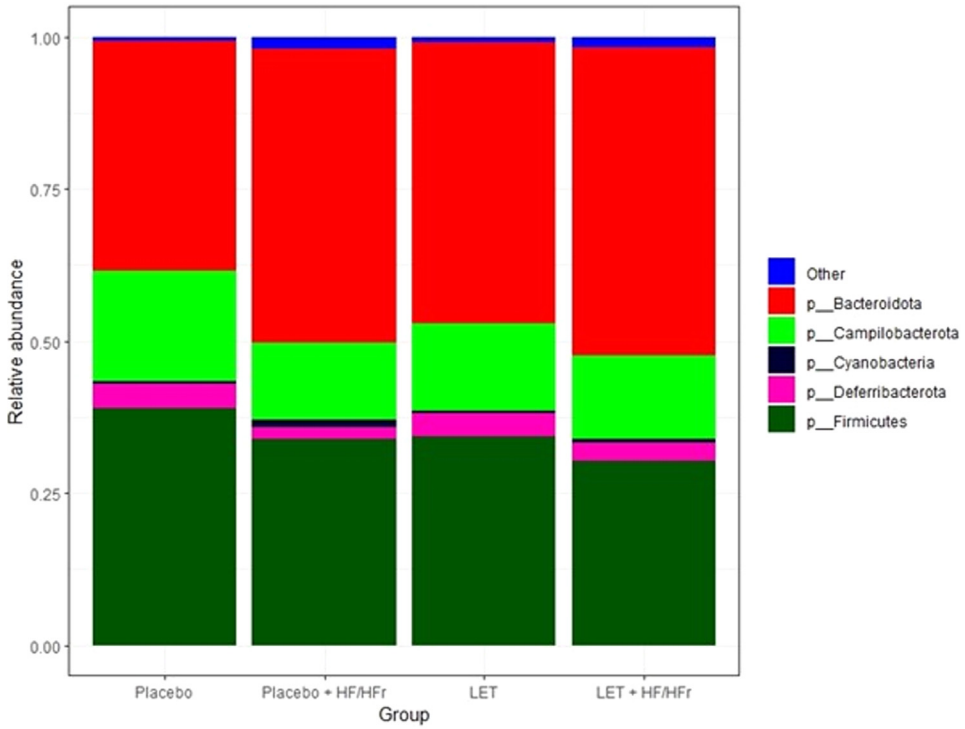

Fig. 4. Relative abundances of gut microbiota on the phylum level.

analysis, but there are notable discrepancies on the phylum level. The LET + HF/HFr group was characterized by the presence of *Bacteroidota*, Placebo + HF/HFr by the presence of *Proteobacteria*, and the Placebo group had an increased abundance of bacteria from *Deferribacterota*.

Bacterial metabolites

Both groups fed the HF/HFr diet (the Placebo + HF/HFr and the LET + HF/HFr groups) showed significantly lower fecal levels of three short-chain fatty acids—acetic, propionic, and butyric acid—than did the Placebo group ($P < 0.05$). The LET + HF/HFr group had a significantly higher plasma lipopolysaccharide (LPS) concentration than the Placebo and LET groups ($P < 0.05$). Detailed values are presented in Table 2.

Correlation of microbial relative abundance with selected parameters associated with PCOS

Potential correlations of relative abundance in bacterial phyla and genera were investigated for all mice using measurements of seven metabolic parameters associated with PCOS. The parameters tested were body weight gain, adipose tissue, concentration of testosterone, HOMA-IR, TG, LDL-C, and Castelli's Risk index.

No significant correlations were observed on the phylum level. On the genus level, however, significant correlations were noted between the abundance of selected genera and lipid profile parameters. Figure 5 shows only the strongest Spearman correlations ($q < 0.01$, $R > 0.6$).

We note that there are opposing correlations of *Turicibacter* and *Lactobacillus* abundances with total cholesterol concentrations (R = 0.65, R = -0.63, respectively, $q < 0.01$). A positive correlation was observed between the concentration of HDL-C and the abundance of the *[Eubacterium]_coprostanoligenes_group* (R = 0.64, $q < 0.01$) and *Turicibacter* (R = 0.66, $q < 0.01$). In turn, abundances of *Lactobacillus* and *Butyricicoccus* were inversely correlated with the concentration of the HDL-L cholesterol fraction (R = -0.7, R = -0.64, respectively, $q < 0.01$). Furthermore, higher abundances of *[Eubacterium]_coprostanoligenes_group, Romboutsia, Turicibacter*, and *Rikenella* were also associated with higher concentrations of LDL-C (R = 0.61, R = 0.61, R = 0.60, R = 0.61, respectively, $q < 0.01$), unlike in the case of *Lactobacillus* and *Prevotellaceae_NK3B31_group* (R = -0.72, R = -0.65, respectively, $q < 0.01$).

There was also a positive correlation between the abundance of *[Eubacterium]_siraeum_group* (R = 0.52, $q < 0.05$), *Muribaculum*

Table 2
Levels of selected short-chain fatty acids in the feces of mice and the concentrations of LPS in blood

Parameters/study groups	Placebo	Placebo + HF/HFr	LET	LET + HF/HFr
Acetic acid (μmol/L)	30.41 ± 7.04 ^{b}	4.16 ± 0.47 ^{a}	10.09 ± 3.45 ^{ab}	3.85 ± 0.74 ^{a}
Propionic acid (μmol/L)	1.49 ± 0.45 ^{b}	0.30 ± 0.09 ^{a}	0.52 ± 0.14 ^{ab}	0.30 ± 0.10 ^{a}
Butyric acid (μmol/L)	2.63 ± 0.96 ^{b}	0.29 ± 0.09 ^{a}	0.61 ± 0.12 ^{ab}	0.32 ± 0.08 ^{a}
LPS (μg/mL)	23.44 ± 4.43 ^{a}	26.47 ± 5.00 ^{ab}	23.52 ± 2.21 ^{a}	28.78 ± 7.86 ^{b}

Data are presented as means ± standard deviations and should be read horizontally. LPS concentration was analyzed by one-way analysis of variance followed by Tukey's HSD test, while SCFA content was analyzed using the nonparametric Kruskal–Wallis test.
Values with different letters show statistically significant differences ($P < 0.05$).
LPS, lipopolysaccharide; HF/HFr, high-fat/high-fructose diet; LET, letrozole.

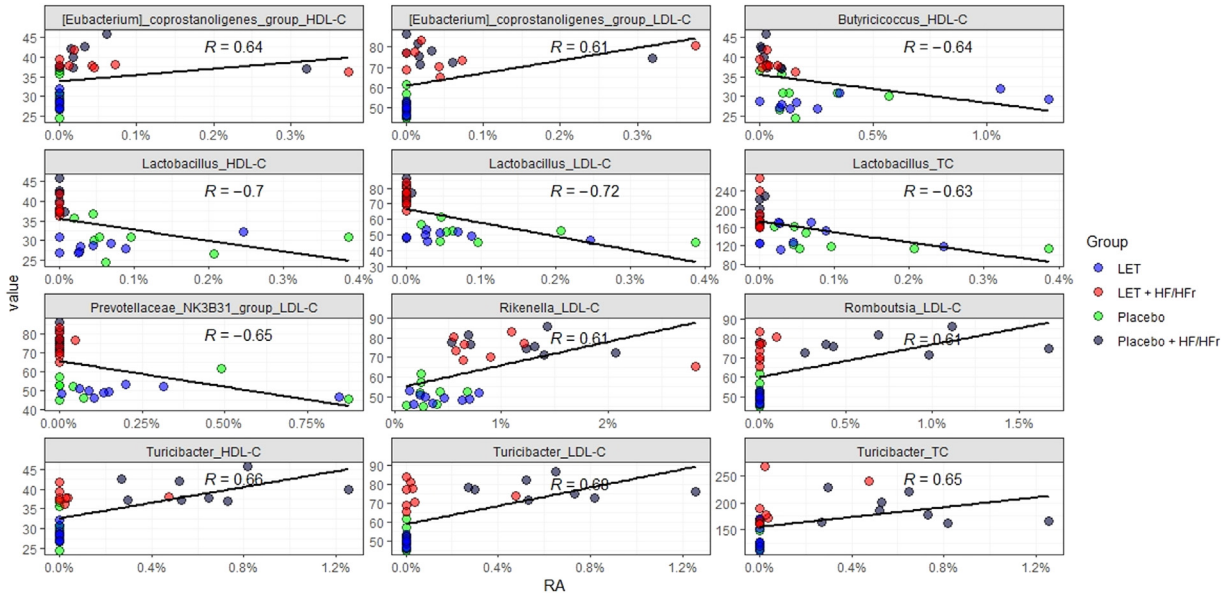

Fig. 5. Spearman correlations between abundances of genera and lipid metabolism parameters associated with PCOS. The significance of all the correlations is < 0.01. TC, total cholesterol; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol.

(R = 0.52, q < 0.05), *Turicibacter* (R = 0.52, q < 0.05), and testosterone concentration. On the other hand, the *Prevotellaceae_NK3B31_group* was negatively associated with testosterone concentration (R = -0.58, q < 0.05). In addition, a positive association between *Bilophila* abundance and body weight was also noted (R = 0.52, q < 0.05) (all data shown in Supplementary Figure S3).

Discussion

Contrary to expectations, alpha diversity proved to be significantly higher in the Placebo + HF/HFr and LET + HF/HFr groups than in the Placebo and LET groups fed the standard diet. Several studies [24,25] have shown that the alpha diversity of intestinal microbiota is associated with the health of the host, with lower values being associated with metabolic or endocrine disorders like PCOS [5]. On the other hand, some authors have also emphasized that the expectation that high alpha diversity of intestinal microbiota is always beneficial is an overly simplistic one [26]. Very complex correlations exist between the diversity of gut microbiota and its stability and functionality [27]. Moreover, some studies [28,29] have indicated that lower alpha diversity does not always mean a poorer community or poorer health [27]. Our results may in fact suggest that alpha diversity is not a universal parameter that can be analyzed alone, without taking into account the composition and functioning of the intestinal microbiota [27].

Similarly to us, Aho et al. [30] found higher alpha diversity and lower levels of SCFAs in people suffering from Parkinson's disease compared to healthy subjects; this is probably due to a rearrangement in the composition of the intestinal microbiota. Indeed, in both our groups fed the HF/HFr diet (Placebo + HF/HFr and LET + HF/HFr), significantly lower concentrations of individual SCFAs in the feces were observed than in the Placebo group fed the standard diet; this is consistent with the results of Sulistyowati et al. [31], who confirmed that there was a negative impact of HFD on the ability of microbiota to produce metabolites. Moreover, the LET group that was fed the standard diet did not differ significantly from the other groups in terms of SCFA concentrations, while Zhang et al. showed that women with PCOS have reduced SCFAs level compared to healthy women [32]. A reduction in the abundance of bacteria producing SCFAs, especially butyric acid, may negatively affect the integrity of the intestinal barrier and mucosal immunity [33]. Indeed, our LET + HF/HFr group also had significantly higher LPS concentrations than either group fed a standard diet (placebo and LET), which suggests that only the combination of improper diet and letrozole caused damage to the intestinal barrier. Moreover, it is known that the LPS produced by Gram(-) bacteria enters the bloodstream and may cause inflammation, insulin resistance, and obesity [3]. We noted in our previous article that only the LET + HF/HFr group showed significantly higher body weight gain and developed carbohydrate metabolism disorders, while the remaining three groups—LET, Placebo and Placebo + HF/HFr—did not [15].

We noted no significant differences on the phylum level, except in the case of *Actinobacteria*, which was present only in the Placebo + HF/HFr group. Zheng et al. [6], however, noted a greater abundance of this phylum in a group combining letrozole with HFD. Although it has been suggested that the abundance of *Actinobacteria* is related to the amount of fat in the diet [34] and to excess body weight [28], the study of Lindheim et al. [35], showed the reduced relative abundance of this phylum in PCOS patients. This result may partly explain why these bacteria were not observed in the LET + HF/HFr group.

More pronounced differences were observed at the genus level, with *Lactobacillus* abundance being significantly lower in the groups fed HF/HFr (Placebo + HF/HFr and LET + HF/HFr groups) than in the groups fed a standard diet (Placebo and LET). It can be unequivocally concluded that the abundance of this genus is related to the diet, and other studies have also shown that the HFD diet significantly reduces the abundance of bacterial taxa from this genus [36]. We also observed a negative correlation between *Lactobacillus* and lipid profile, particularly relating to TC and LDL-C. Furthermore, in the mice fed HF/HFr (the Placebo + HF/HFr and LET + HF/HFr groups), significantly higher abundances of *Rikenella* and *Parasuterella* were observed. It has been suggested that *Rikenella* is strongly associated with serum triglyceride concentrations [37]. We also noted that the abundance of this genus correlates with elevated LDL-C levels. Moreover, *Parasuterella* has been found in the microbiota of people with excess body weight [38] and

associated metabolic disorders [39]. Indeed, significant metabolic abnormalities have been observed in both groups fed HF/HFr (the Placebo + HF/HFr and LET + HF/HFr groups), in which *Parasuterella* was enriched—further exacerbated by letrozole [15].

Equally interesting is the significantly higher abundance of certain genera, such as *Muribaculum, Romboutsia, Turicibacter,* and *Clostridium_sensu_stricto _1*, in the Placebo + HF/HFr group, as compared to the other three groups, including even the LET + HF/HFr group. Also, when considering the beta-diversity parameter, we can observe that only the Placebo + HF/HFr group differs from the other groups. Unlike Zheng et al. [6], we did not observe any synergistic effect of the HF/HFr diet with letrozole here. The age of PCOS induction with letrozole should be taken into account: Torres et al. noted that administration of letrozole at a prepubertal age was associated with the development of marked metabolic abnormalities, but with only minor changes in the composition of the gut microbiota, as compared to mice in which PCOS was induced in adulthood [40]. Both human and animal studies have showed the dependence of the intestinal microbiota on the organism's maturity, and this applies particularly to the complexity of microbiota, which increases with age [41,42]. This is probably due to the developing hormonal system, although the mechanism is not yet known [43].

Indeed, we noted only a few changes in the LET group, such as the significantly higher abundance of *Prevotellaceae_UCG-001* than in the Placebo group and the significantly higher abundance of *Prevotellaceae_NK3B31_group* than in the LET + HF/HFr group. An increase in the number of different genera from the Prevotellaceae family has been observed among women with PCOS, and this is often associated with increased inflammation in the organism [44,45]. However, these reports are inconclusive, as Zeng et al. noted a dramatic decrease in the abundance of *Prevotellaceae* in women with PCOS [39]. Interestingly, we observed a negative correlation between *Prevotellaceae_NK3B31_group* and testosterone and LDL-C, which is consistent with the results of Zeng et al. [39].

It can thus be suggested that our induction of PCOS at prepubertal age (in 4-wk old animals) led to us no noting many significant differences between the microbiota composition of the LET group and that of the other groups; neither did we observe any intensification in these differences as a result of the interaction of letrozole and the HF/HFr diet. Furthermore, Paris et al. [46] noted that diet has a stronger effect on the composition of the intestinal microbiota than PCOS pathology *per se*, which is consistent with our results.

However, diet may also affect gut microbiota favorably, though this depends on the relative identity and abundance of the constituent bacterial populations [47]. It has been suggested that positive regulation of gut microbiota composition is mediated mainly by phenolic acids released from plant polyphenols and SCFAs derived from the fermentation of dietary fiber by commensal bacteria in the gut [48]. Both phenolic acids and SCFAs have anti-inflammatory properties [49], which also act beneficially in PCOS [50]. Moreover, it has been suggested that probiotic supplementation may also alleviate PCOS-associated hormonal and metabolic disorders [51].

Our study has some limitations. First of all, we assessed the composition of the microbiota at only one point in time, so we did not observe any potential fluctuating disorders that might have resulted from an improper diet or the presence of PCOS. In addition, the composition of the microbiota was assessed from intestinal contents, while the level of SCFAs was determined from the feces, on account of the limited amount of intestinal content that could be obtained from the mice.

Furthermore, we are also aware of the occurrence of coprophagous behavior in experimental mice. First of all, it should be noted that this phenomenon affects the composition of the microbiota in the small intestine, but has a much less pronounced effect on the microbiota in the large intestine [52]. There are several ways to prevent coprophagia, for example using cups placed under the tail of the mice. However this is not useful for female mice, due to their anatomical structure and the accumulation of urine in the cups [53]. In addition, young animals, due to their high activity, would need to be kept in isolation to prevent the cup from being moved or bitten. However, mice are social animals and the need to maintain their well-being precludes their isolation [54]. There are also numerous reports that inhibiting coprophagous behavior in animals adversely affects their body weight, height, and biochemical parameters, and promotes the development of inflammation, which may affect experimental results [55–57].

This study also possesses a number of strengths. To the best of our knowledge, ours is the first study to assess differences in the composition of gut microbiota resulting from a HF/HFr diet and LET-induced PCOS in prepubertal mice. Moreover, our assessment of the composition of the microbiota was performed on the basis of the intestinal content, which is considered a more precise method than assessment based on feces. It has also been suggested that cecal microbiota might be more revealing of HF/HFr diet-induced proinflammatory stimuli (such as LPS and bacterial DNA), because some differences have been detected on the taxonomic level in the fecal microbiota and the caecum microbiota [58].

Conclusions

The HF/HFr diet provided to the experimental animals had a much stronger effect on the composition of the intestinal microbiota of prepubertal mice than did the letrozole itself. However, the age of PCOS induction may be a key factor in our results. Moreover, the correlations we observed indicate that the composition of the gut microbiota has a significant effects on parameters associated with PCOS, and particularly on the correlation between the *Turicibacter* and *Lactobacillus* genera and the lipid profile. However, these require observations further exploration in human studies, which will allow the development of macrobiotic profiles along with individualized diets and probiotic therapy adapted to them.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

CRediT authorship contribution statement

Joanna Maria Pieczyńska-Zając: Writing – original draft, Methodology, Formal analysis, Data curation, Conceptualization. **Anna Maria Malinowska:** Writing – review & editing, Methodology, Formal analysis, Data curation. **Ewa Pruszyńska-Oszmałek:** Writing – review & editing, Methodology, Formal analysis, Data curation. **Paweł Antoni Kołodziejski:** Writing – review & editing, Methodology, Formal analysis, Data curation. **Sławomira Drzymała-Czyż:** Writing – review & editing, Methodology, Data curation. **Joanna Bajerska:** Writing – review & editing, Formal analysis, Conceptualization.

Institutional review board statement

The study was approved by the Local Ethical Commission under permission no. 51/2021 and was performed in line with the ARRIVE 2.0 guidelines for animal research [17].

Supplementary materials

Supplementary material associated with this article can be found in the online version at doi:10.1016/j.nut.2024.112450.

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