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Both obesogenic and isocalorically-controlled diets drive changes in microbiota composition in a sex-dependent manner in mice

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ABSTRACT

We have observed the loss of structure and function of olfactory circuits following consumption of excess dietary fat as maintained by isocaloric feeding. Because changes in microbiome composition have not been examined during this type of diet that does not induce overt obesity, we performed isocaloric feeding of a moderately high-fat (MHF) diet, and assessed gut microbiome changes over 5 months by sequencing the 16s V4 region in fecal samples. MHF diet similarly reduced alpha diversity in both isocaloric and *ad libitum* fat-fed animals by 5 months. Bray-Curtis dissimilarity analysis revealed unique beta diversity in each dietary group in female mice at 3 and 5 months, while fat-fed groups of male mice were not different. Elevated abundance of Firmicutes was found in all MHF *ad libitum* fed male and female mice. Bacteroidetes was reduced in all MHF-fed male mice, but only female mice fed the MHF diet *ad libitum. Allobaculum* abundance was positively associated with MHF *ad libitum* feeding, while *Muribaculaceae* abundance exhibited a negative association. Notable graded-abundance associations between *ad libitum* control-fat fed, isocalorically fat-fed, and *ad libitum* fat-fed mice were observed for bacteria such as *Lactobacillus salivarius*; providing insight into effects of dietary fat consumption vs. overconsumption. Transient abundance changes, observed with *Ruminococcaceae,* for example, capture microbial dynamics reflective of diet-induced obesity onset that were not sustained in chronic obesity. We conclude that the gut microbiome is differentially modulated by consumption of excess dietary fat vs. overconsumption of excess dietary fat leading to obesity.

1. Introduction

Obesity is a chronic inflammatory disease characterized by excess body fat and associated with comorbidities including type 2 *diabetes mellitus*, cardiovascular disease, metabolic dysfunction-associated steatohepatitis, and hypertension [1–10]. Using rodent models to understand how over-nutrition affects the structure and function of the olfactory system, we discovered that diet-induced obesity (DIO) causes a variety of physiological and behavioral effects associated with this sensory system [11,12]. Mice with DIO were found to have reduced electroolfactogram amplitude in response to odor stimulation; a loss of olfactory sensory neurons and axonal projections to their central targets; changed morphology of mitochondria within the olfactory bulb, irregular action potential firing frequency in the output neurons of the olfactory bulb, and insensitivity to insulin signaling in the olfactory bulb; and poor odor discrimination and cognitive flexibility when behaviorally trained in an olfactometer requiring operant learning paradigms [13–16]. To test whether excess fat in the diet vs. overt adiposity was driving these deleterious effects in olfactory anatomy and function, we designed isocaloric pair-feeding experiments that prevented significant gain in body weight when mice were maintained on moderately high-fat diets but only permitted to consume the caloric equivalent of mice eating control fat diets. The moderately high-fat diet contains 32% kcal fat and 51% kcal carbohydrate in the form of condensed milk. These studies demonstrated that overconsumption of fat in the diet, but not only obesity *per se*, was capable of enhancing

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inflammatory markers and driving loss of olfactory sensory neurons and their associated olfactory circuits [16].

Given the fact that consumption of increased fat in the diet causes loss of neuronal abundance and olfactory circuitry, which was associated with inflammation, we wanted to examine if mice administered these same isocaloric pair-feeding designs had similar changes in the gut microbiome as those challenged with DIO. Changes in the gut microbiome are known to drive inflammation, neuronal loss, and behavioral deficits [17–23], but the potential changes to the microbiome associated with isocaloric diets under these physiological and behavioral deficits is unknown. We hypothesized that over consumption of fat in the diet should have similar gut microbiota changes as DIO because both evoked loss of olfactory sensory neurons with elevated inflammation, which could result from gut dysbiosis. Diets high in fat induce gut microbiome dysbiosis exacerbating inflammation, gut permeability, diabetes, and weight gain [24]. Time-restricted feeding and caloric restriction are two behavioral interventions for obesity that affect the gut microbiome (reviewed by [25,26]). The effect on gut microbiota in response to increasing the percent of dietary fat, while retaining a standard level of daily calories, has not been well studied. Because we observe a disruption in olfactory neural abundance and associated circuits with such an isocaloric diet, investigation into potential gut microbiota changes, and their comparison with the state of obesity, was warranted.

Herein, we report the first longitudinal gut microbiome study in both male and female mice isocalorically fed a moderately high-fat diet (isocaloric treatment or ISO). By collecting feces prior to treatment onset and at staggered time points during dietary treatment, we were able to explore microbiota changes attributed to the development of obesity vs. that driven by a chronic excess of fat/carbohydrate in the diet (poor diet). We report distinct phylum- and family-level changes in bacteria that vary with sex and according to the means of administration of the fat diet (isocaloric restricted vs. *ad libitum*). Overall, our data provide insight into MHF diet-induced changes in the gut microbiome that may occur in the absence of overt obesity and in a sex-dependent manner, thus providing a context to gut microbiome dysbiosis with chronic consumption of poor diet.

2. Material and methods

2.1. Animal care

All mice were obtained from The Jackson Laboratory (Catalog #000664; Bar Harbor, ME) and were C57BL/6J background strain. Experiments were approved by the FSU Institutional Animal Care and Use Committee (IACUC) under protocol #202000036 and were conducted in accordance with the American Veterinary Medicine Association (AVMA) and the National Institutes of Health (NIH). Mice were bred in-house and maintained singly-housed in conventional style open cages with two sources of enrichment (nesting square and house) in the Florida State University (FSU) *vivarium*. Here, mice were maintained on a reverse 12/12-hour dark/light cycle, and cages were changed once per week. Mice were observed daily by FSU Laboratory Animal Resource technicians throughout their life, in addition to daily observations throughout the 5-month feeding intervention by laboratory members. Abnormal skin conditions, excessive itching/grooming, and pica were not observed associated with modified diets. Mice were weaned at postnatal day (PND) 24, following which they were randomly assigned to one of three dietary treatment groups. A total of 24 male and 22 female mice were used in our study. Following final fecal collections, mice were euthanized by isoflurane-drop anesthesia and decapitated [27]. Use of the ARRIVE guidelines for reporting animal research was followed in the design of our manuscript [28].

2.2. Isocaloric pair feeding

Mice were randomly assigned to a dietary treatment group at PND 28 and were maintained on this daily feeding regimen for 20 weeks (Fig. 1). Mice were fed either control-fat chow (CF, 13.6% kcal/fat, 28.9% kcal/protein, 57.5% kcal/carbohydrates; Lab Diet 5001, St. Louis, Missouri, USA) or moderately high-fat chow (MHF, 31.8% kcal/fat, 16.8% kcal/protein, 51.4% kcal/carbohydrates; Research Diets D12266B, New Brunswick, New Jersey, USA). Mice were pair fed according to the number of calories consumed by a leader animal on CF diet to prevent overconsumption of the MHF diet, as done previously [16]. This dietary regimen was manually maintained daily for 5 months. The first treatment group (leader animal) was provided *ad libitum* access to control-fat chow (CF; n=8 male, n=9 female). Animals in the second treatment group were provided the same number of calories (isocaloric) consumed by their designated CF leader mouse the previous day, but they were provided their calories as moderately high-fat chow (ISO; n=9 male, n=7 female). Company-reported caloric values were used to compute the applied conversion (CF: 3.35 kcal/g, MHF: 4.41 kcal/g). The third group was provided *ad libitum* access to moderately high-fat food (MHF; n=7 male, n=6 female).

2.3. Fecal sample collection

Fecal samples were collected from mice for gut microbiome analyses. Collections were made prior to dietary assignment (designated as month 0), and at 3- and 5-month time points. Samples were collected at the onset of the dark cycle ± 2 hours, by placing a mouse into a disinfected, standard cage bottom without bedding, and without access to food and water for the sampling duration. Mice were kept in this isolated mouse cage until fecal pellets were expelled. Pellets were handled with individual, autoclaved wooden sticks and stored at -80°C until processing.

2.4. DNA extraction and 16s sequencing

DNA was extracted from fecal pellets (1-2 pellets/sample) using Qiagen QIAamp PowerFecal Pro DNA Kit (Catalog #51804; Hilden, Germany) as per manufacturer's instructions. Extracted DNA was processed for sequencing at the Florida State University Department of Biology Molecular Core Facility. Briefly, the V4 hypervariable region of the 16s rRNA gene was amplified by PCR using region-specific, barcoded primers and targeted for paired-end (2×251 base pair) sequencing by Illumina MiSeq (San Diego, California, USA) essentially as described by the Earth Microbiome Project protocol [29]. Sequences were processed in the QIIME2 (Quantitative Insights into Microbial Ecology version 2022.11; [30]) pipeline, demultiplexed, and denoised (soft cutoff of median quality score of 20) using DADA2 [31], and then assigned to taxa using the Greengene database (version 13_8, gg-13-8-99-515-806-nb-classifier) [32]. Data were rarefied to 18,000 reads/sample to retain all samples. Taxonomic data were exported and imported into MicrobiomeAnalyst 2.0 (rarefied to 18,000 reads/sample, without additional filtering, normalization, scaling, or transforming [33]) for alpha diversity, clustering, and Linear Discriminant Analysis Effect Size.

2.5. Statistical analyses

Before performing any statistical comparisons, data were first analyzed with an outlier's test (Grubb's or Dixon) to identify any outliers. In the alpha diversity (6), the phylum fold change (6), and the family fold change (12) data sets outliers were removed. Data were then checked for homogeneity of variance using the Brown-Forsythe test. Where collected data violated homogeneity of variance (failed the Brown-Forsythe test), the selection of applied statistics was changed to the Welch's analysis of variance (ANOVA). An ordinary one-way (1-w) ANOVA was applied to normally distributed data using dietary treatment as the factor (reported as F statistic (degrees of freedom of the numerator, degrees of freedom of the denominator)) followed by Tukey's multiple comparisons test. A Welch's ANOVA was applied to

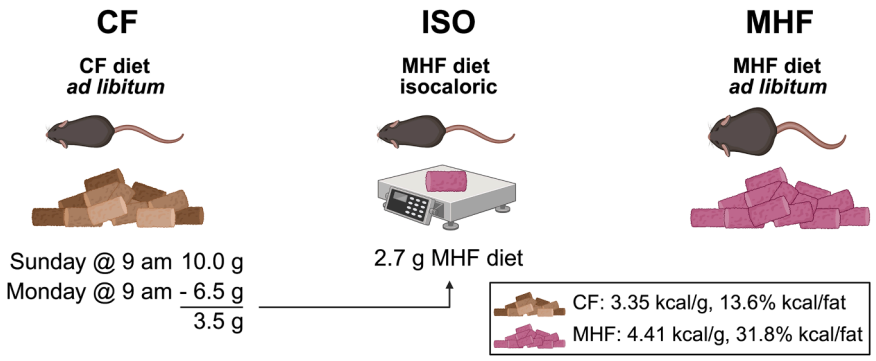

Fig. 1. Schematic of isocaloric pair-feeding regimen. Mice consumed either control-fat diet (13.6% kcal/fat) or moderately high-fat diet (31.8% kcal/fat). Mice were assigned to one of three groups: control-fat diet *ad libitum* (CF), moderately high-fat diet *isocaloric* (ISO), or moderately high-fat diet *ad libitum* (MHF). CF mice serve as leaders, and their food intake is measured at the same time each day. Food intake of control-fat chow is measured in grams, converted to kilocalories, and the equivalent kilocalories of moderately high-fat diet is provided to an isocalorically-paired mouse. Thus, the ISO animals are provided the same number of calories consumed the previous day by the CF animals, but in MHF diet. Created in BioRender. Loeven, A. (2025) https://BioRender.com/5bymev1.

data with unequal variances (reported as W statistic (degrees of freedom of the numerator, degrees of freedom of the denominator)) followed by Dunnett's T3 multiple comparisons test. Statistical significance was determined at the 95% confidence level ($\alpha \leq 0.05$) unless otherwise noted. Values are reported as the mean ± standard deviation (SD). A majority of statistical analyses and graphing were performed using Prism software (Version 10; GraphPad, Boston, Massachusetts).

Alpha diversity was assessed in terms of Chao 1 index [34] and Shannon's index [35], where beta diversity was assessed using the Bray-Curtis Dissimilarity Index [36] Principal Coordinate Analysis [37]. Chao 1 Index and Shannon's Index were analyzed with a 1-w ANOVA. Bray-Curtis Dissimilarity Index was analyzed using a pairwise-PERMANOVA calculated in MicrobiomeAnalyst 2.0 (reported as F statistic (number of groups - 1, n)). Reported p values applied the Benjamini-Hochberg (BH) correction for multiple comparisons controlling the false discovery rate (FDR). Taxonomic data were exported from QIIME2 and relative abundances were computed and analyzed using a nonparametric Kruskal-Wallis test (within sex, within time) attributed to the percentage data being not normally distributed (reported as H statistic (number of groups - 1, n)), followed by Dunn's multiple comparisons test. To compute changes in taxa over time, Log 2 fold-changes from baseline (Month 0) were applied for normalization as described previously [38]. Because some samples lacked a particular phylum or family under baseline conditions, +0.5 was added to all raw counts in a data set to allow for application of statistics that don't allow zero values [39]. Post hoc multiple comparisons test results were represented on graphs as follows: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

3. Results

3.1. Dietary fat consumption reduces diversity in male and female mice

To understand if the gut microbiome was modulated by the consumption of excess dietary fat, mice were assigned to one of three dietary groups for 5 months as described in the methods and depicted in Fig. 1. Because body weight, food intake, fat and lean tissue mass, glucose tolerance, energy expenditure and respiratory exchange ratio have been previously reported with this paradigm [16], we focused our work strictly on the gut microbiota. Prior to dietary treatment, both male (Fig. 2A) and female (Fig. 3A) mice had gut microbiota that demonstrated equivalent alpha diversity according to both Shannon's Index and Chao1 Index (male, Shannon's: $F(2, 20) = 1.306$, $p = 0.2931$; and Chao1: $F(2, 21) = 0.2349$, $p = 0.7927$; female, Shannon's: $F(2, 19) = 0.1010$, $p = 0.9044$ and Chao1: $F(2, 19) = 1.271$, $p = 0.3033$, 1-w ANOVA)

By 3 and 5 months (Fig.s 2C, 3C and Fig.s 2E, 3E; respectively), alpha diversity exhibited differences across groups in both sexes (3 months: male, Shannon's $W(2.000, 10.51) = 47.26$, $p < 0.0001$ and Chao1 $F(2, 21) = 11.75$, $p = 0.0004$; female, Shannon's $W(2.000, 8.556) = 44.54$, $p < 0.0001$ and Chao1 $F(2, 18) = 17.27$, $p < 0.0001$; 5 months: male, Shannon's $F(2, 20) = 5.113$, $p = 0.0161$ and Chao1 $F(2, 21) = 7.039$, $p = 0.0046$; female, Shannon's $F(2, 19) = 5.116$, $p = 0.0167$ and Chao1 $F(2, 18) = 6.471$, $p = 0.0076$, 1-w or Welch ANOVA). However, post hoc comparisons demonstrated some sex-specific and index-comparative differences. Following 3 months of dietary treatment, the Shannon's index indicated a stepwise reduction in alpha diversity and significant differences between all three dietary groups in male mice (Fig. 2C left). In female mice, however, using the Shannon's index, the MHF group had significantly lower alpha diversity compared to those in the CF and ISO groups at month 3, and the CF and ISO groups were not significantly different from each other (Fig. 3C left). By the 5-month time point, MHF groups of both sexes had lower alpha diversity compared to those in the CF group, with no difference in the ISO group (Fig. 2E left, Fig. 3E left). This indicates that MHF *ad libitum* fed male mice have a transient lower diversity than the ISO group at 3 months, that is lost with longer dietary treatment by the 5-month time point (Fig. 2C left vs. Fig. 2E left). Using the Chao1 index, the pattern of alpha diversity reduction was different across sex, with males having no significant difference between ISO and MHF dietary groups either at 3 or 5 months (Fig. 2C right, Fig. 2E right). Female mice, however, exhibited different alpha diversity patterns at 3 and 5 months. At 3 months, the MHF group had lower Chao1 index than the CF and ISO groups, but by 5 months both fat-fed groups had lower Chao1 index compared to CF fed mice (Fig. 3C right vs. Fig. 3E right). Shannon's Index and Chao 1 index estimate community diversity and richness, thus the distinct patterns observed by these two indices is likely derived from different weighting of rare taxa.

In terms of baseline beta diversity, neither male (Fig. 2B: $F(2, 24) = 2.1291$, $p = 0.025$, PERMANOVA; post hoc Pairwise PERMANOVA FDR adjusted, $p > 0.05$) nor female mice (Fig. 3B; $F(2, 22) = 0.47411$, $p = 0.958$) demonstrated any difference between the 3 dietary groups as measured by Bray-Curtis Dissimilarity. By 3 months, both male fat-fed groups had signatures that were significantly different from CF mice (Fig. 2D: $F(2, 24) = 11.105$, $p = 0.001$), with similarities to one another that persisted at 5 months (Fig. 2F: $F(2, 24) = 6.7437$, $p = 0.001$). Female mice exhibited near complete overlap at month 0, with each dietary group exhibiting unique features at 3 (Fig. 3D: $F(2, 22) = 13.899$, $p = 0.001$) and 5 months (Fig. 3F: $F(2, 22) = 9.2393$, $p = 0.001$) of feeding.

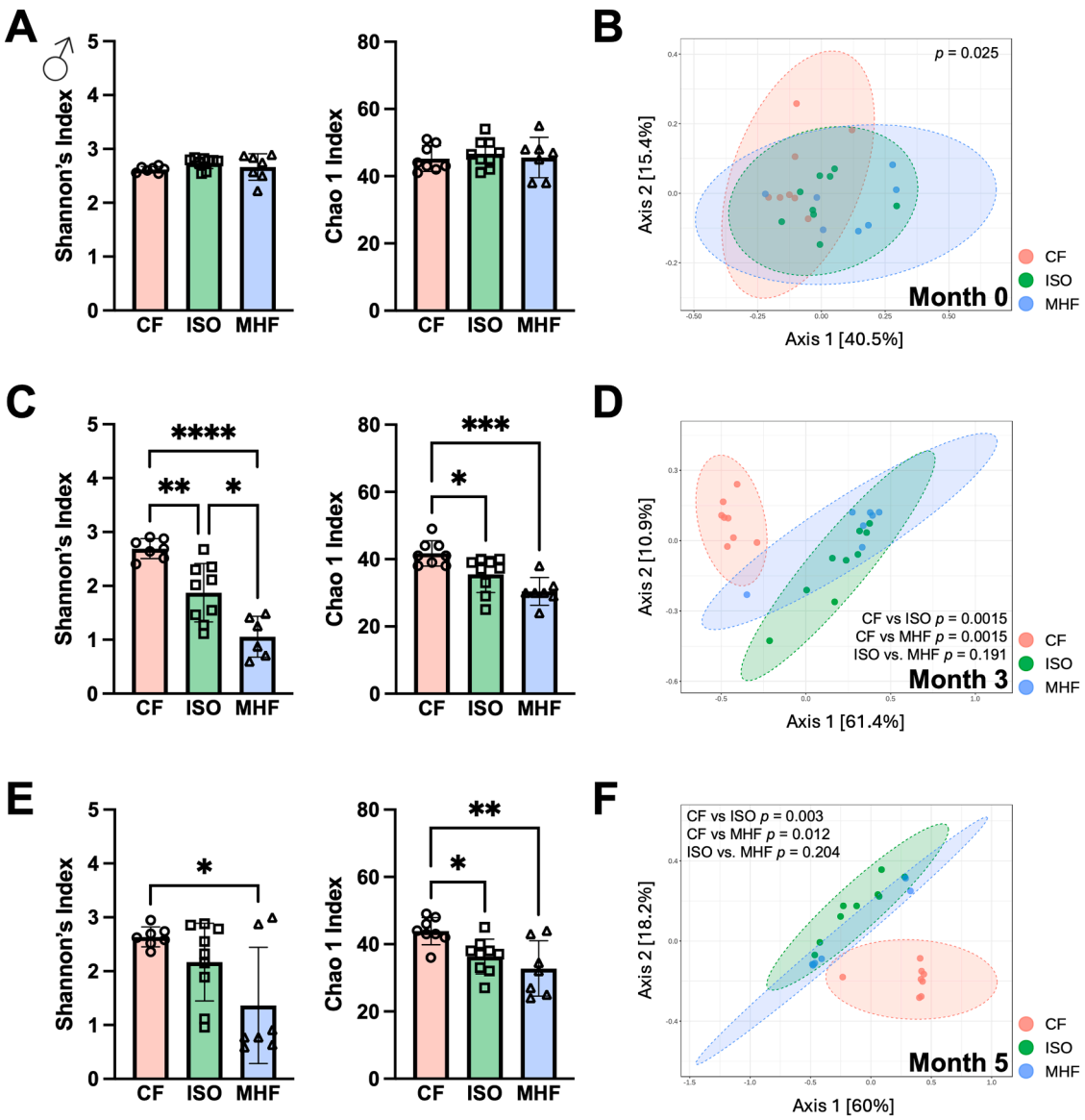

Fig. 2. Alpha and beta diversity are disrupted by dietary fat consumption, whether isocaloric or *ad libitum* in male mice. A, C, and E. Bar plots of Shannon's Index (left) and Chao 1 Index (right) at 0, 3, and 5 months, respectively. Data are plotted as mean ± SD. B, D, and F. Bray-Curtis Dissimilarity index principal coordinate analysis plots at 0, 3, and 5 months, respectively. Alpha diversity data were analyzed using an ordinary one-way ANOVA with Tukey's multiple comparisons test, or Welch ANOVA with Dunnett's T3 multiple comparisions test. Beta diversity data were analyzed with a PERMANOVA and pairwise PERMANOVA post-hoc test.

3.2. Dysbiosis at the phylum level in mice fed a fatty diet isocalorically or ad libitum

Phylum-level differences in microbiota are reported across time (0, 3, and 5 months) and separated by sex (male – Fig. 4; female – Fig. 5). Relative abundance of phyla within each individual replicate in a dietary group is first plotted as raw data, and then the mean abundance for the dietary group is plotted. Next the bacteria phyla enrichment or depletion by dietary treatment is compared across time (Log 2 fold-change from month 0 baseline). Finally, the relative abundance of individual phyla (mean ± SD) per dietary group for a given time point is reported in a bar graph in an associated supplementary figure (male - Supplementary Fig. 1; female - Supplementary Fig. 2). Here, data were analyzed with a Kruskal-Wallis test with H values, with post-hoc results displayed on figures.

For male mice, despite no difference in alpha and beta diversity (Fig. 2), there was a pre-treatment difference in abundance of 3 phyla in the mice randomly assigned to the MHF treatment group (Fig. 4A-B). At baseline, Firmicutes and Proteobacteria were less abundant, and Bacteroidetes more abundant, in the MHF group of male mice compared to the CF group of male mice despite all mice being fed CF food up to this time (Supplementary Fig. 1A-C: Firmicutes H (2, 22) = 8.565, p = 0.0088; Bacteroidetes H (2, 23) = 9.302, p = 0.0096; Proteobacteria H (2, 24) = 6.950, p = 0.0310). No baseline differences in Actinobacteria, Tenericutes, or Deferribacteres abundances were observed (Supplementary Fig. 4D-F: Actinobacteria H (2, 24) = 0.8817, p = 0.6435; Tenericutes H (2, 24) = 2.265, p = 0.3223; Deferribacteres H (2, 23) = 1.115, p = 0.5728).

Interestingly, after 3 months of diet maintenance, Firmicutes was more abundant in the male MHF group compared to the CF group (Fig. 4C-D, Supplementary Fig. 1G: H (2, 23) = 12.16, p = 0.0023). In contrast, Bacteroidetes was less abundant in mice maintained on MHF and ISO diets when compared to those on the CF diet (Supplementary Fig. 1H: H (2, 23) = 12.80, p = 0.0017). These abundance patterns persisted at 5 months of treatment (Fig. 4E-F; Supplementary Fig. 1M-N) - Firmicutes H (2, 24) = 8.152, p = 0.0170; Bacteroidetes H (2, 23) =

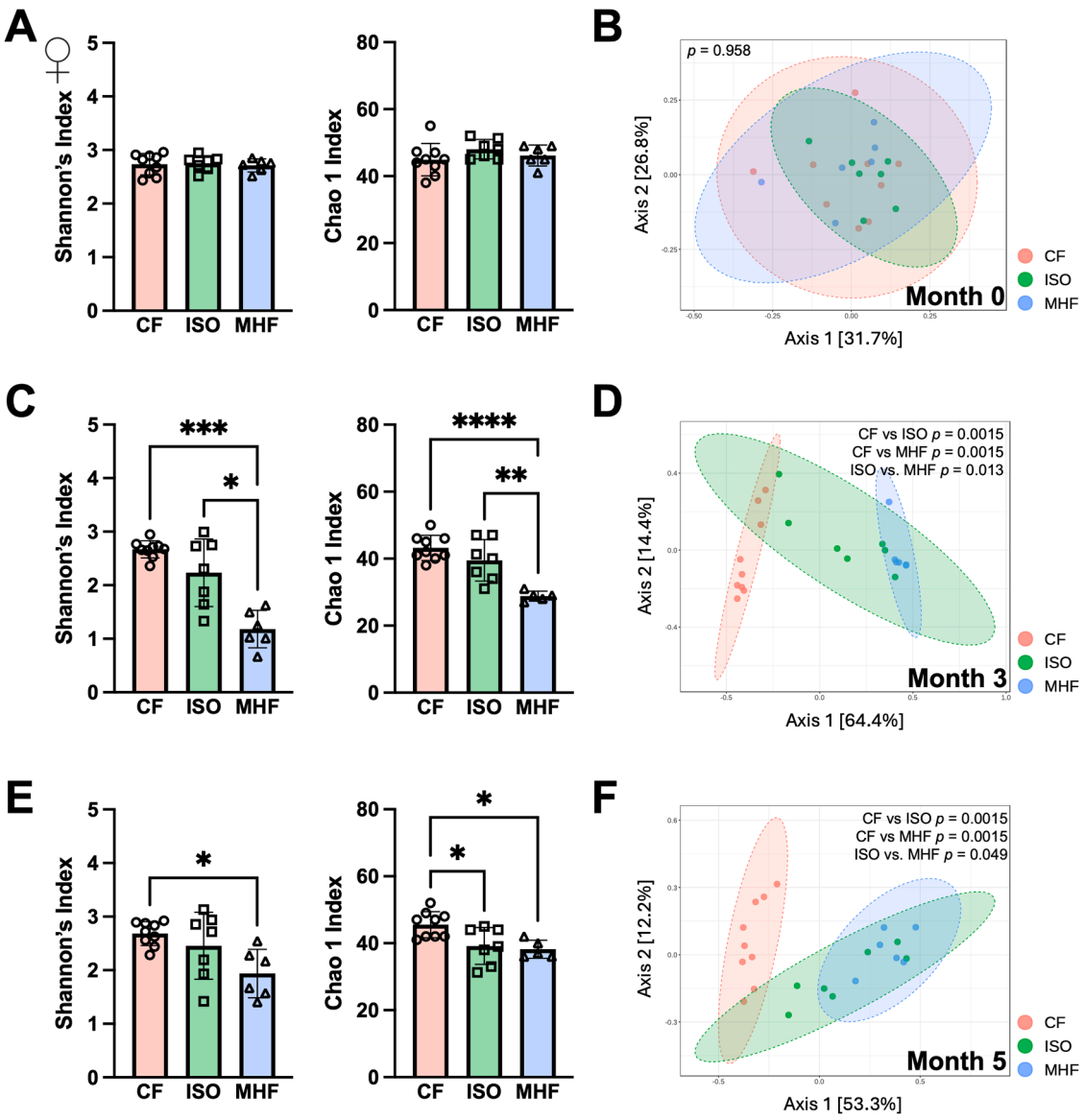

Fig. 3. Alpha and beta diversity are disrupted by dietary fat consumption, whether isocaloric or *ad libitum* in female mice. Layout, analyses, and notations same as Figure 2, but for female mice.

12.28, $p = 0.0022$. Transient changes in microbiome composition were also observed. Proteobacteria was more abundant in the CF group compared to the ISO group at 3 months, while no differences between groups were measured at 5 months (Supplementary Fig. 1I, O: 3 months H (2, 24) = 8.002, $p = 0.0183$; 5 months H (2, 23) = 2.500, $p = 0.2865$). Actinobacteria was more abundant in both fat-fed groups at 3 months (Fig. 4C-D); however, this abundance persisted only in the ISO group at 5 months (Fig. 4E-F, Supplementary Fig. 1J, P: 3 months H (2, 23) = 12.20, $p = 0.0022$; 5 months H (2, 22) = 6.854, $p = 0.0261$). Tenericutes abundance was reduced by fat-feeding at 3 months (Fig. 4C-D), however, this was not observed in the ISO group at 5 months (Fig. D-E, Supplementary Fig. 1K, Q: 3 months H (2, 24) = 16.98, $p = 0.0002$; 5 months H (2, 24) = 10.71, $p = 0.0047$). Deferribacteres was unchanged by diet regardless of the duration of the treatment (Supplementary Fig. 1L, R: 3 months H (2, 22) = 2.109, $p = 0.3609$; 5 months H (2, 21) = 2.245, $p = 0.3380$).

Abundance changes over time were quantified using Log 2 (Fold Change) from month 0 to investigate longitudinal trends and compare across dietary treatments (Fig. 4G-H, 1-w ANOVA). Firmicutes abundance was increased by fat feeding in male mice at both 3 and 5 months, while Bacteroidetes and Tenericutes abundances were reduced by fat feeding at both time points (Fig. 4G-H, Firmicutes: 3 months: F (2, 21) = 13.17, $p = 0.0002$, 5 months: F (2, 21) = 6.591, $p = 0.0060$; Bacteroidetes: 3 months: F (2, 21) = 16.82, $p < 0.0001$, 5 months: F (2, 21) = 13.45, $p = 0.0002$; Tenericutes: 3 months: F (2, 20) = 75.67, $p < 0.0001$, 5 months: F (2, 20) = 15.17, $p < 0.0001$). Abundance of Proteobacteria and Deferribacteres were unchanged by dietary treatment (Fig. 4G-H, Proteobacteria: 3 months: F (2, 21) = 0.7656, $p = 0.4776$, 5 months: F (2, 21) = 0.7189, $p = 0.4989$; Deferribacteres: 3 months: F (2, 21) = 0.01848, $p = 0.9817$, 5 months: F (2, 21) = 1.466, $p = 0.2535$). Actinobacteria exhibited increased abundance by 3 months for fat feeding in male mice, however, this trend was absent at 5 months (Fig. 4G-H, Actinobacteria: 3 months: F (2, 20) = 9.306, $p = 0.0014$, 5 months: F (2, 21) = 1.798, $p = 0.1901$). Male mice exhibited phylum level shifts at 3 months of dietary treatment that were largely persistent at 5 months.

Female mice exhibited no baseline composition differences between groups at the phylum level (Fig. 5A-B, Supplementary Fig. 2A-F: Firmicutes H (2, 21) = 0.3321, $p = 0.8577$; Bacteroidetes H (2, 21) = 0.1447, $p = 0.9374$; Proteobacteria H (2, 21) = 0.1319, $p = 0.9428$; Actinobacteria H (2, 22) = 1.354, $p = 0.5285$; Tenericutes H (2, 20) =

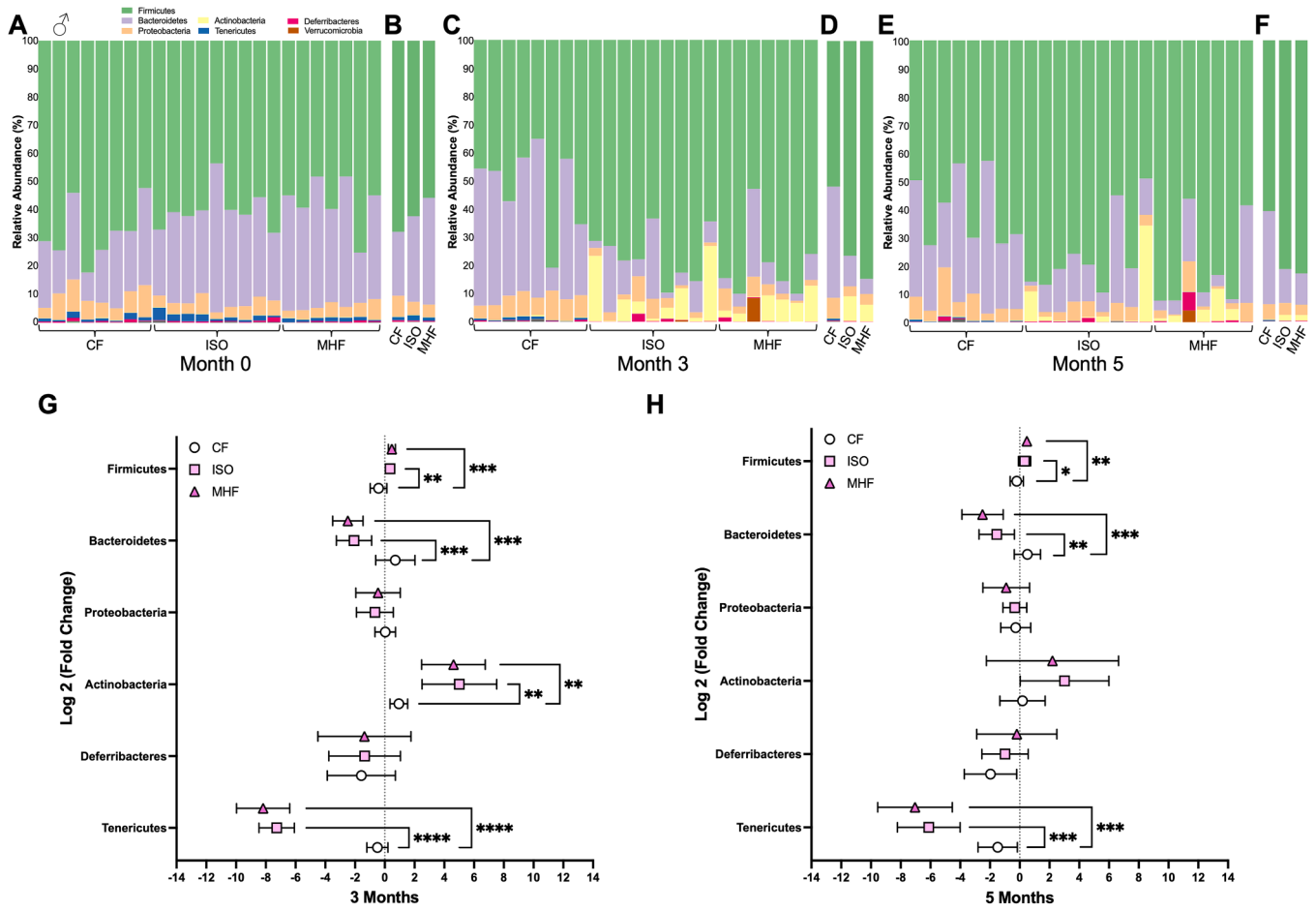

Fig. 4. Dietary fat induces gut microbiome dysbiosis at the phylum level in male mice. Taxa bar plots of phylum composition display individual animals within each dietary condition (A month 0, C month 3, E month 5) and the average (excluding outlier values, B month 0, D month 3, F month 5). G, and H. Line graphs showing bacterial phyla that were significantly enriched or depleted by dietary treatment (Log 2 fold- changes from month 0 baseline). Denotes significant differences between groups: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. Also see **Supplementary Fig. 1** for absolute bar plots of bacteria abundance sorted per family across dietary treatment and sorted by months.

3.045, $p = 0.2246$; Deferribacteres H (2, 21) = 2.237, $p = 0.3399$). After 3 months of feeding, elevated abundance of Firmicutes was attributed to *ad libitum* consumption of a fatty diet, while elevated Actinobacteria abundance was attributed to consumption of excess dietary fat in both ISO and MHF groups of female mice compared to that of the CF group (Fig. 5C-D, Supplementary Fig. 2G, J: Firmicutes H (2, 22) = 13.02, $p = 0.0002$; Actinobacteria H (2, 21) = 15.97, $p < 0.0001$). Lower abundance of Bacteroidetes, Proteobacteria, and Tenericutes were observed in MHF *ad libitum* fed female mice (Supplementary Fig. 2, H-I, K: Bacteroidetes H (2, 21) = 12.39, $p = 0.0003$; Proteobacteria H (2, 21) = 10.42, $p = 0.0020$; Tenericutes H (2, 19) = 8.620, $p = 0.0078$). Deferribacteres abundance was unchanged by dietary intervention similar to male mice (Supplementary Fig. 2L: H (2, 20) = 0.1512, $p = 0.9363$). Patterns of abundance were persistent after 5 months of dietary maintenance in Firmicutes, Bacteroidetes, Proteobacteria, and Deferribacteres (Fig. 5E-F, Supplementary Fig. 2M-O, R: Firmicutes H (2, 22) = 6.362, $p = 0.0353$; Bacteroidetes H (2, 21) = 8.150, $p = 0.0109$; Proteobacteria H (2, 20) = 7.081, $p = 0.0222$; Deferribacteres H (2, 19) = 5.225, $p = 0.0685$). Like male mice, female mice that were isocalorically fed a MHF diet had an abundance of Firmicutes that was not significantly different from that of the CF, and only those maintained on MHF ad libitum had elevated abundance, at both 3 and 5 months of treatment (Supplementary Fig. 2G, M). Elevated Actinobacteria abundance attributed to consumption of excess dietary fat at 3 months persisted at 5 months only in female mice consuming excess dietary fat *ad libitum* (Supplementary Fig. 2P: H (2, 19) = 13.12, $p < 0.0001$), while lower abundance of Tenericutes was observed in all fat-fed female mice at 5 months (Supplementary Fig. 2Q: H (2, 21) = 14.09, $p < 0.0001$).

Beyond analysis at each collection time point, phyla changes were analyzed over time (Log 2 fold-change from Month 0; Fig. 5G-H, 1-w ANOVA). Female mice fed a MHF diet *ad libitum* had increased abundance of Firmicutes and reduced abundance of Proteobacteria by 3 months of maintenance (Fig. 5G, Firmicutes: F (2, 19) = 4.683, $p = 0.0222$; Proteobacteria: F (2, 19) = 4.483, $p = 0.0254$). Consumption of excess dietary fat increased abundance of Actinobacteria (Fig. 5G, F (2, 19) = 20.68, $p < 0.0001$). Bacteroidetes abundance was reduced in mice consuming the MHF diet, with the ISO group falling intermediate to both the CF and MHF groups at 3 months (Fig. 5G, F (2, 19) = 16.19, $p < 0.0001$). By 3 months of diet maintenance, female mice fed a MHF diet exhibited reduced Tenericutes abundance and unchanged Deferribacteres abundance, which was sustained at 5 months (Fig. 5G-H, 3 months: Tenericutes: F (2, 19) = 5.240, $p = 0.0154$, Deferribacteres: F (2, 19) = 0.2948, $p = 0.7480$; 5 months: Tenericutes: F (2, 19) = 8.712, $p = 0.0021$, Deferribacteres: F (2, 19) = 1.358, $p = 0.2811$). Differences in Firmicutes and Proteobacteria abundance at 3 months were transient (Fig. 5G-H, 5 months: Firmicutes: F (2, 18) = 1.225, $p = 0.3170$; Proteobacteria: F (2, 19) = 1.546, $p = 0.2387$). The stepwise reduction in Bacteroidetes abundance observed at 3 months persisted only in the MHF group at 5 months (Fig. 5G-H, 5 months: F (2, 19) = 5.839, $p = 0.0106$). Similarly, the elevated Actinobacteria abundance in the ISO group at 3 months was not observed at 5 months (Fig. 5G-H, F (2, 19) = 6.875, $p = 0.0057$). Overall, female mice exhibited more transient

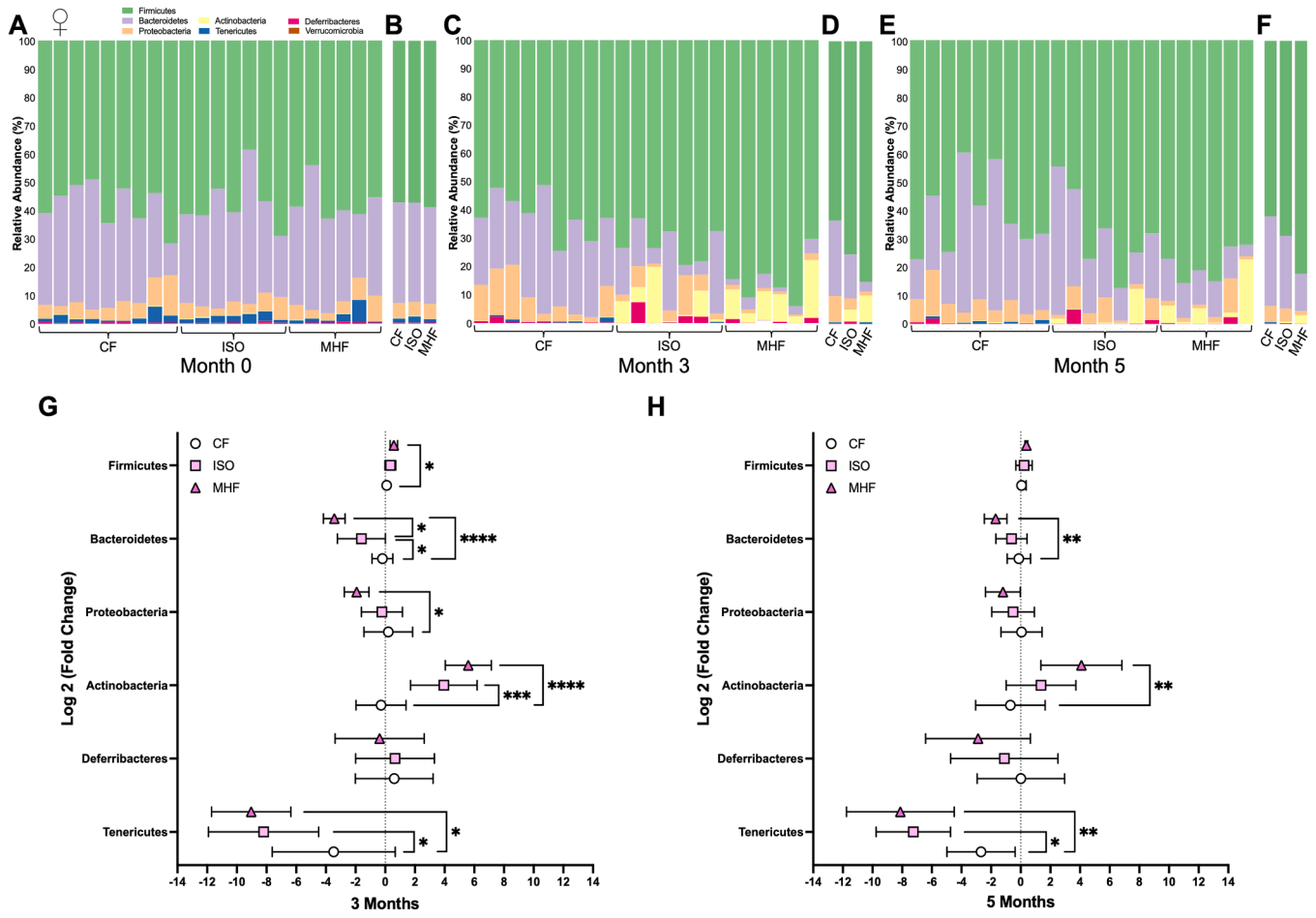

Fig. 5. Consumption of excess dietary fat disrupts the gut microbiome at the phylum level in female mice. Layout, notation, and analysis same as in Fig. 4, but for female mice. Also see **Supplementary Fig. 2** for absolute bar plots of bacteria abundance sorted per phyla across dietary treatment and sorted by months.

changes in phyla abundance due to dietary manipulation than male mice.

3.3. Bacterial family composition is disrupted by the introduction of a moderately high-fat diet but largely remains stable over time

Family-level differences in microbiota are reported across time (0, 3, and 5 months) and separated by sex (male – Fig. 6; female – Fig. 7). Relative abundance of family within each individual replicate in a dietary group is first plotted as raw data, then the mean abundance for the dietary group is plotted. Next the bacteria family enrichment or depletion by dietary treatment is compared across time (Log 2 fold-change from month 0 baseline). Finally, the relative abundance of individual families (mean ± SD) per dietary group for a given time point is reported in a bar graph in an associated supplementary figure (male - Supplementary Fig. 3; female - Supplementary Fig. 4). Here, data were analyzed with a Kruskal-Wallis test as described previously.

Male mice exhibited relatively consistent family-level composition of the gut microbiome across individuals and treatment group means (Fig. 6A-B). No differences in *Erysipelotrichaceae, Lachnospiraceae, Lactobacillaceae, Rikenellaceae, Ruminococcaceae, Helicobacteraceae, Bifidobacteriaceae, Prevotellaceae, Clostridiaceae,* and *Deferribacteraceae* were observed at baseline (Supplementary Fig. 3A-B, D, F-L: *Erysipelotrichaceae* H (2, 24) = 0.3751, $p = 0.8290$; *Lachnospiraceae* H (2, 24) = 4.931, $p = 0.0849$; *Lactobacillaceae* H (2, 24) = 2.180, $p = 0.3362$; *Rikenellaceae* H (2, 24) = 5.083, $p = 0.0788$; *Ruminococcaceae* H (2, 24) = 4.822, $p = 0.0897$; *Helicobacteraceae* H (2, 24) = 5.714, $p = 0.0574$; *Bifidobacteriaceae* (all values 0); *Prevotellaceae* H (2, 24) = 0.6151, $p = 0.7353$; *Clostridiaceae* H (2, 22) = 4.497, $p = 0.1036$; *Deferribacteraceae* H (2, 23) = 1.115, $p = 0.5728$). The baseline elevation of Bacteroidetes in MHF fed male mice was partially driven by *Muribaculaceae* and *Bacteroidaceae* (Supplementary Fig. 3C, E: *Muribaculaceae* H (2, 24) = 7.936, $p = 0.0189$; *Bacteroidaceae* H (2, 22) = 6.567, $p = 0.0318$).

After 3 months of fat-feeding, *Erysipelotrichaceae* dominated the gut microbiome of male mice fed a MHF diet, regardless if isocaloric or *ad libitum* (Fig. 6C-D, Supplementary Fig. 3M: H (2, 22) = 17.18, $p < 0.0001$). *Bifidobacteriaceae* was not detected prior to dietary onset, however, abundance was elevated after 3 months of fatty diet feeding (Supplementary Fig. 3U: H (2, 24) = 14.36, $p = 0.0008$). Lower abundance of *Lachnospiraceae, Rikenellaceae, Helicobacteraceae*, and *Prevotellaceae* resulted from moderately high-fat diet feeding (Supplementary Fig. 3N, R, T, V: *Lachnospiraceae* H (2, 23) = 15.73, $p = 0.0004$; *Rikenellaceae* H (2, 23) = 14.05, $p = 0.0009$; *Helicobacteraceae* H (2, 22) = 12.96, $p = 0.0002$; *Prevotellaceae* H (2, 24) = 21.81, $p < 0.0001$). The elevation of *Muribaculaceae* at month 0 in the MHF group of male mice was reversed, with lower abundance of *Muribaculaceae* in both fat-fed groups of male mice (Supplementary Fig. 3O: H (2, 23) = 14.35, $p = 0.0008$). Notably, abundances of *Lactobacillaceae, Bacteroidaceae*, and *Ruminococcaceae* were lower only in male mice consuming moderately high-fat chow *ad libitum* at 3 months (Supplementary Fig. 3P-Q, S: *Lactobacillaceae* H (2, 23) = 14.28, $p = 0.0008$; *Bacteroidaceae* H (2, 23) = 7.770, $p = 0.0206$; *Ruminococcaceae* H (2, 22) = 11.04, $p = 0.0013$). *Clostridiaceae* was more abundant in the ISO group only (Supplementary Fig. 3W: H (2, 23) = 14.39, $p = 0.0007$), and *Deferribacteraceae* abundance was unchanged by diet (Supplementary Fig. 1X: H (2, 22) = 2.109, $p = 0.3609$).

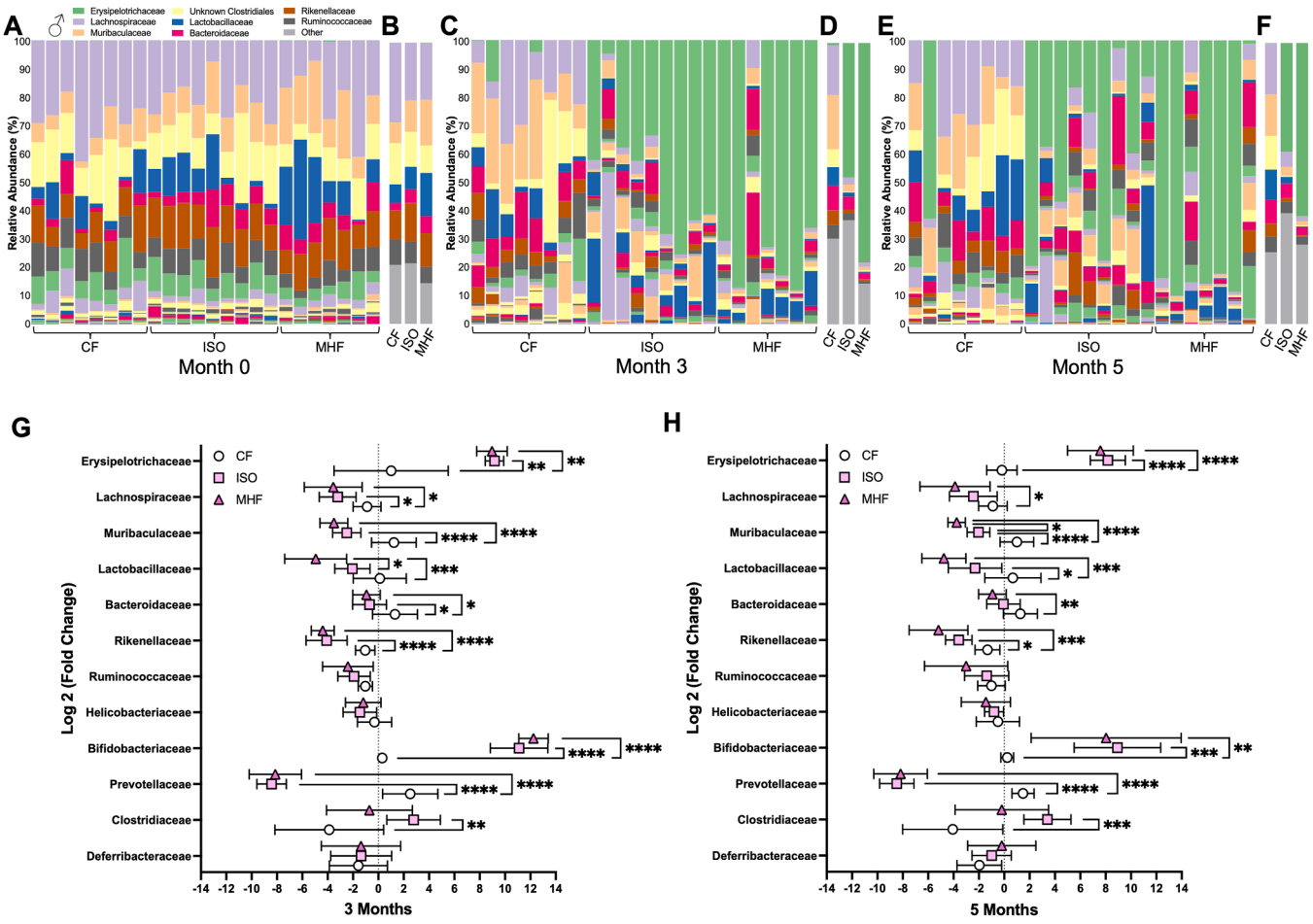

Fig. 6. Dietary fat induces gut microbiome dysbiosis at the family level in male mice. Taxa bar plots of family composition display individual animals within each dietary condition (A month 0, C month 3, E month 5) and the average (excluding outlier values B month 0, D month 3, F month 5). G, and H. Line graphs showing bacterial family that were significantly enriched or depleted by dietary treatment (Log 2 fold- changes from month 0 baseline). Denotes significant differences between groups: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. Also see **Supplementary Fig. 3** for absolute bar plots of bacteria abundance sorted per family across dietary treatment and sorted by months.

Gut microbiome composition was markedly similar at 3 and 5 months of dietary maintenance in male mice (Fig. 6C-F, Supplementary Fig. 3Y, A'-D', G'-J': *Erysipelotrichaceae* H (2, 23) = 11.22, $p = 0.0037$; *Muribaculaceae* H (2, 23) = 15.52, $p = 0.0004$; *Lactobacillaceae* H (2, 23) = 13.92, $p = 0.0009$; *Bacteroidaceae* H (2, 22) = 6.727, $p = 0.0346$; *Rikenellaceae* H (2, 23) = 13.62, $p = 0.0011$; *Bifidobacteriaceae* H (2, 22) = 11.89, $p = 0.0009$; *Prevotellaceae* H (2, 23) = 21.08, $p < 0.0001$; *Clostridiaceae* H (2, 22) = 15.98, $p < 0.0001$; *Deferribacteraceae* H (2, 21) = 2.245, $p = 0.3380$). Lower *Lachnospiraceae* and *Helicobacteraceae* abundances in fat-fed male mice at 3 months were only maintained by *ad libitum* fat-fed mice at 5 months (Supplementary Fig. 3Z, F': *Lachnospiraceae* H (2, 23) = 13.16, $p = 0.0014$; *Helicobacteraceae* H (2, 23) = 9.151, $p = 0.0103$). In contrast, lower *Ruminococcaceae* abundance in male MHF-fed mice at 3 months was transient, with no dietary effects on abundance observed at 5 months (Supplementary Fig. 3E': H (2, 24) = 2.685, $p = 0.2612$).

Longitudinal analysis (Log 2 fold-change from month 0) revealed shifts in family abundance due to both diet and feeding regimen (Fig. 6G-H, 1-w or Welch ANOVA). Consumption of excess dietary fat elevated abundance of *Erysipelotrichaceae* and *Bifidobacteriaceae*, and decreased abundance of *Lachnospiraceae, Muribaculaceae, Bacteroidaceae, Rikenellaceae*, and *Prevotellaceae* in male mice after 3 months (Fig. 6G: *Erysipelotrichaceae* W (2.000, 9.900) = 11.98, $p = 0.0023$; *Bifidobacteriaceae* W (2.000, 8.653) = 371.4, $p < 0.0001$; *Lachnospiraceae* F (2, 21) = 6.132, $p = 0.0080$; *Muribaculaceae* F (2, 21) = 26.11, $p < 0.0001$; *Bacteroidaceae* F (2, 21) = 5.851, $p = 0.0096$; *Rikenellaceae* F (2, 21) = 18.95, $p < 0.0001$; *Prevotellaceae* F (2, 21) = 94.04, $p < 0.0001$). *Lactobacillaceae* abundance was reduced in the MHF group of male mice compared to both the CF and ISO groups (Fig. 6G, F (2, 21) = 12.31, $p = 0.0003$). Interestingly, *Clostridiaceae* abundance was increased by 3 months of isocaloric fatty diet consumption (Fig. 6G, F (2, 21) = 8.480, $p = 0.0020$). *Ruminococcaceae, Helicobacteriaceae*, and *Deferribacteraceae* abundances were unchanged by dietary treatment after 3 months (Fig. 6G: *Ruminococcaceae* F (2, 20) = 1.745, $p = 0.2002$; *Helicobacteriaceae* F (2, 21) = 1.650, $p = 0.2160$, *Deferribacteraceae* F (2, 21) = 0.01847, $p = 0.9817$).

Shifts in *Erysipelotrichaceae, Rikenellaceae, Ruminococcaceae, Helicobacteriaceae, Bifidobacteriaceae, Prevotellaceae, Clostridiaceae,* and *Deferribacteraceae* abundances observed after months of dietary manipulation were maintained at 5 months (Fig. 6G-H; *Erysipelotrichaceae* F (2, 20) = 49.97, $p < 0.0001$; *Rikenellaceae* F (2, 20) = 12.17, $p = 0.0003$; *Ruminococcaceae* F (2, 21) = 1.801, $p = 0.1897$; *Helicobacteriaceae* F (2, 21) = 0.7749, $p = 0.4735$; *Bifidobacteriaceae* F (2, 21) = 12.78, $p = 0.0002$; *Prevotellaceae* F (2, 20) = 102.2, $p < 0.0001$; *Clostridiaceae* F (2, 21) = 11.36, $p = 0.0005$; *Deferribacteraceae* F (2, 21) = 1.466, $p = 0.2535$). Reduced abundances of *Lachnospiraceae* and *Bacteroidaceae* due to fatty diet consumption at 3 months were observed at 5 months only in mice consuming the fatty diet *ad libitum* (Fig. 6G-H, *Lachnospiraceae* F (2, 21) = 4.231, $p = 0.0286$; *Bacteroidaceae* F (2, 21) = 5.860, $p = 0.0095$). *Muribaculaceae* abundance exhibited a stepwise reduction at 5 months

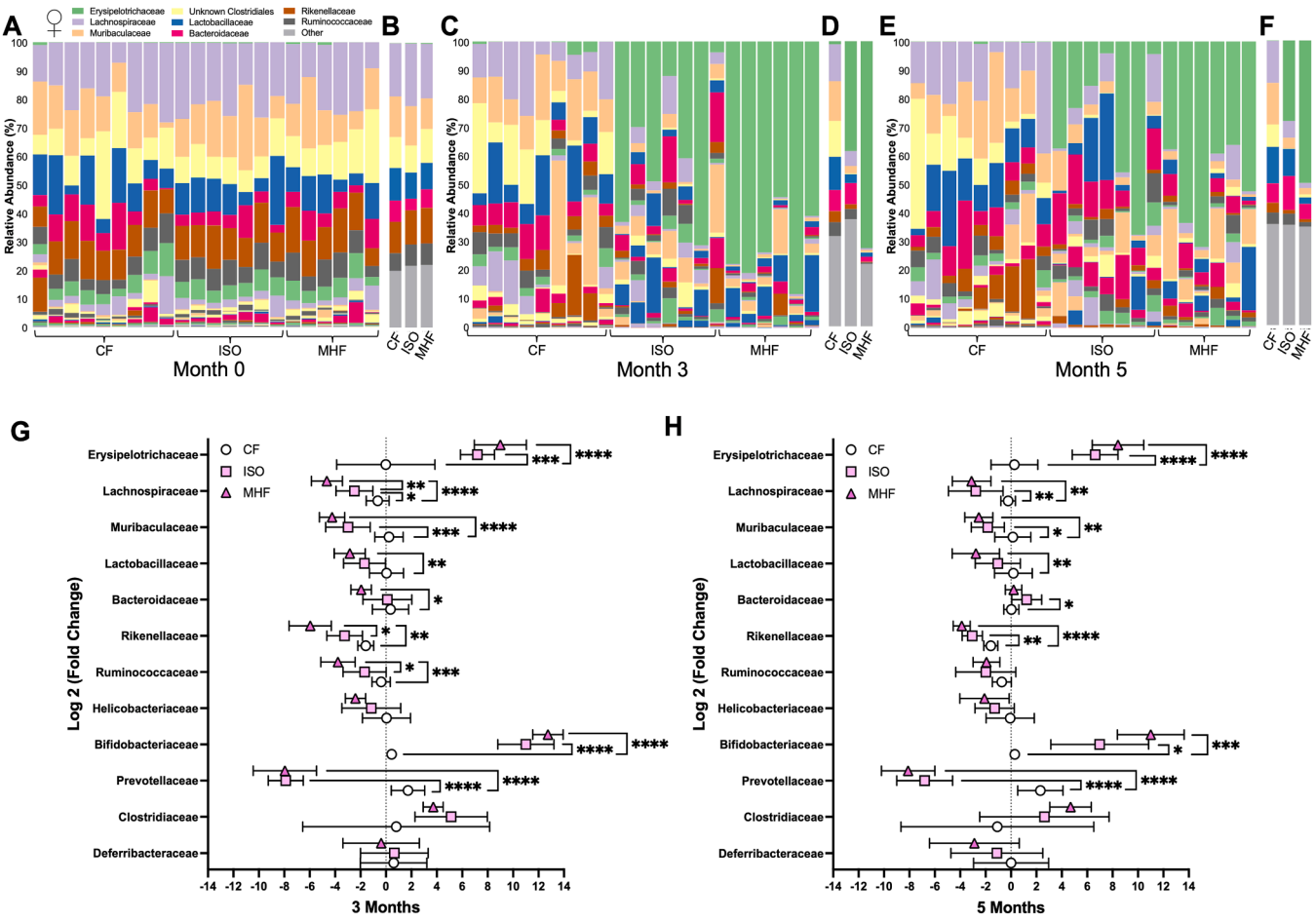

Fig. 7. Consumption of excess dietary fat disrupts the gut microbiome at the family level in female mice. Layout, notation, and analysis same as in Fig. 6, but for female mice. Also see **Supplementary Fig. 4** for absolute bar plots of bacteria abundance sorted per family across dietary treatment and sorted by months.

(Fig. 6H, F (2, 20) = 39.36, p < 0.0001), and *Lactobacillaceae* abundance was reduced by consumption of excess dietary fat (Fig. 6H, F (2, 21) = 13.34, $p = 0.0002$). Overall, compositional shifts induced by dietary manipulation were largely persistent in male mice.

Consistent with no baseline composition differences at the phylum level, no differences were observed prior to treatment at the family level in female mice (Fig. 7A-B; Supplementary Fig. 4A-L: *Erysipelotrichaceae* H (2, 21) = 2.254, $p = 0.3359$; *Lachnospiraceae* H (2, 22) = 1.203, $p = 0.5654$; *Muribaculaceae* H (2, 19) = 3.789, $p = 0.1521$; *Lactobacillaceae* H (2, 21) = 0.6133, $p = 0.7559$; *Bacteroidaceae* H (2, 21) = 3.505, $p = 0.1762$; *Rikenellaceae* H (2, 22) = 1.573, $p = 0.4716$; *Ruminococcaceae* H (2, 22) = 1.762, $p = 0.4288$; *Helicobacteraceae* H (2, 19) = 0.6853, $p = 0.7292$; *Bifidobacteriaceae* (all values 0); *Prevotellaceae* H (2, 21) = 0.6136, $p = 0.7506$; *Clostridiaceae* H (2, 22) = 0.1220, $p = 0.9453$; *Deferribacteraceae* H (2, 21) = 2.237, $p = 0.3399$). After 3 months of dietary treatment, shifts in family composition are prominent (Fig. 7C-D; Supplementary Fig. 4M-X). Similar to male mice, elevated abundance of *Erysipelotrichaceae* and *Bifidobacteriaceae*, and reduced abundance of *Muribaculaceae* and *Prevotellaceae* were driven by consumption of dietary fat, while diet did not alter abundance of *Deferribacteraceae* (Supplementary Fig. 4M, O, U-V, X: *Erysipelotrichaceae* H (2, 22) = 16.99, $p < 0.0001$; *Bifidobacteriaceae* H (2, 21) = 17.32, $p < 0.0001$; *Muribaculaceae* H (2, 22) = 16.50, $p < 0.0001$; *Prevotellaceae* H (2, 21) = 18.57, $p < 0.0001$; *Deferribacteraceae* H (2, 20) = 0.1512, $p = 0.9363$). Unlike male mice, female mice exhibited lower abundance of *Lachnospiraceae, Lactobacillaceae, Bacteroidaceae, Rikenellaceae, Ruminococcaceae*, and *Helicobacteriaceae* in response to *ad libitum* consumption of excess dietary fat (Supplementary Fig. 4N, P-T: *Lachnospiraceae* H (2, 21) = 13.96, $p < 0.0001$; *Lactobacillaceae* H (2, 21) = 14.71, $p < 0.0001$; *Bacteroidaceae* H (2, 22) = 9.792, $p = 0.0036$; *Rikenellaceae* H (2, 22) = 15.14, $p < 0.0001$; *Ruminococcaceae* H (2, 21) = 10.66, $p = 0.0016$; *Helicobacteriaceae* H (2, 22) = 7.708, $p = 0.0154$). Of note, *Clostridiaceae* was significantly elevated in isocalorically moderately high-fat fed female mice when compared to CF fed mice (Supplementary Fig. 4W: H (2, 19) = 6.232, $p = 0.0374$).

Family composition of the gut microbiome in female mice was stable from 3 months to 5 months of diet maintenance for *Erysipelotrichaceae, Lachnospiraceae, Lactobacillaceae, Helicobacteraceae, Prevotellaceae*, and *Deferribacteraceae* (Fig. 7C-F; Supplementary 4 Y-Z, B', F', H', J': *Erysipelotrichaceae* H (2, 21) = 15.34, $p < 0.0001$; *Lachnospiraceae* H (2, 20) = 10.47, $p = 0.0017$; *Lactobacillaceae* H (2, 22) = 8.101, $p = 0.0119$; *Helicobacteraceae* H (2, 20) = 9.568, $p = 0.0038$; *Prevotellaceae* H (2, 21) = 18.09, $p < 0.0001$; and *Deferribacteraceae* H (2, 19) = 5.225, $p = 0.0685$). Lower abundance of *Muribaculaceae* and elevated abundance of *Bifidobacteriaceae* was observed only in *ad libitum* moderately high-fat fed female mice at 5 months (Supplementary Fig. 4A', G': *Muribaculaceae* H (2, 21) = 14.71, $p < 0.0001$; *Bifidobacteriaceae* H (2, 20) = 16.05, $p < 0.0001$). *Bacteroidaceae* abundance was higher in the ISO group compared to the MHF group, and *Rikenellaceae* had lower abundance in all fat-fed mice (Supplementary Fig. 4C'-D': *Bacteroidaceae* (2, 21) = 6.656, $p = 0.0294$; *Rikenellaceae* H (2, 20) = 11.41, $p = 0.0008$). The lower *Ruminococcaceae* abundance in MHF-fed female mice at 3 months was not observed at 5 months (Supplementary Fig. 4E': H (2, 21) = 3.564, $p = 0.1708$). The elevated abundance of *Clostridiaceae* at 3 months in the ISO group compared to the CF group was transient, as the MHF group had elevated abundance of *Clostridiaceae* compared to the CF

group after 5 months of diet maintenance (Supplementary Fig. 4I': H (2, 21) = 6.316, $p = 0.0361$).

Longitudinal analysis (Log 2 fold-change from month 0) revealed shifts in family abundance due to both diet and feeding regimen in female mice (Fig. 7G-H). Increased abundance of *Erysipelotrichaceae* and *Bifidobacteriaceae*, and decreased abundance of *Muribaculaceae* and *Prevotellaceae*, were driven by consumption of excess dietary fat over 3 months of dietary maintenance (Fig. 7G: *Erysipelotrichaceae* F (2, 19) = 22.27, $p < 0.0001$; *Bifidobacteriaceae* W (2.000, 7.769) = 342.4, $p < 0.0001$; *Muribaculaceae* F (2, 19) = 23.34, $p < 0.0001$; *Prevotellaceae* F (2, 18) = 76.17, $p < 0.0001$). Reduced abundance of *Lactobacillaceae* and *Bacteroidaceae* was driven by consumption of a fatty diet *ad libitum* compared to the CF group, while reduced abundance of *Rikenellaceae* and *Ruminococcaceae* was driven by consumption of a fatty diet *ad libitum* compared to both the CF and ISO groups (Fig. 7G: *Lactobacillaceae* F (2, 19) = 7.881, $p = 0.0032$; *Bacteroidaceae* F (2, 19) = 4.863, $p = 0.0197$; *Rikenellaceae* W (2.000, 8.771) = 20.07, $p = 0.0005$; *Ruminococcaceae* F (2, 19) = 12.95, $p = 0.0003$). *Lachnospiraceae* exhibited a stepwise reduction in abundance due to consumption of a moderately high-fat diet (Fig. 7G: F (2, 19) = 20.74, $p < 0.0001$). *Helicobacteriaceae, Clostridiaceae*, and *Deferribacteraceae* abundance was not impacted by dietary intervention (Fig. 7G: *Helicobacteriaceae* F (2, 19) = 3.227, $p = 0.0622$; *Clostridiaceae* W (2.000, 10.11) = 1.447, $p = 0.2801$; *Deferribacteraceae* F (2, 19) = 1.358, $p = 0.2811$).

Abundance shifts at 3 months were sustained at 5 months for *Erysipelotrichaceae, Muribaculaceae, Helicobacteriaceae, Lactobacillaceae, Bifidobacteriaceae, Prevotellaceae, Clostridiaceae, and Deferribacteraceae* in female mice (Fig. 7H: *Erysipelotrichaceae* F (2, 19) = 40.40, $p < 0.0001$; *Muribaculaceae* F (2, 19) = 8.710, $p = 0.0021$; *Helicobacteriaceae* F (2, 19) = 2.391, p = 0.1185; *Lactobacillaceae* F (2, 19) = 5.544, $p = 0.0127$; *Bifidobacteriaceae* W (2.000, 7.329) = 54.92, $p < 0.0001$; *Prevotellaceae* F (2, 18) = 57.96, $p < 0.0001$; *Clostridiaceae* F (2, 19) = 1.944, $p = 0.1706$; *Deferribacteraceae* F (2, 19) = 0.2949, $p = 0.7480$). Reduced *Ruminococcaceae* abundance at 3 months in the MHF group was not observed at

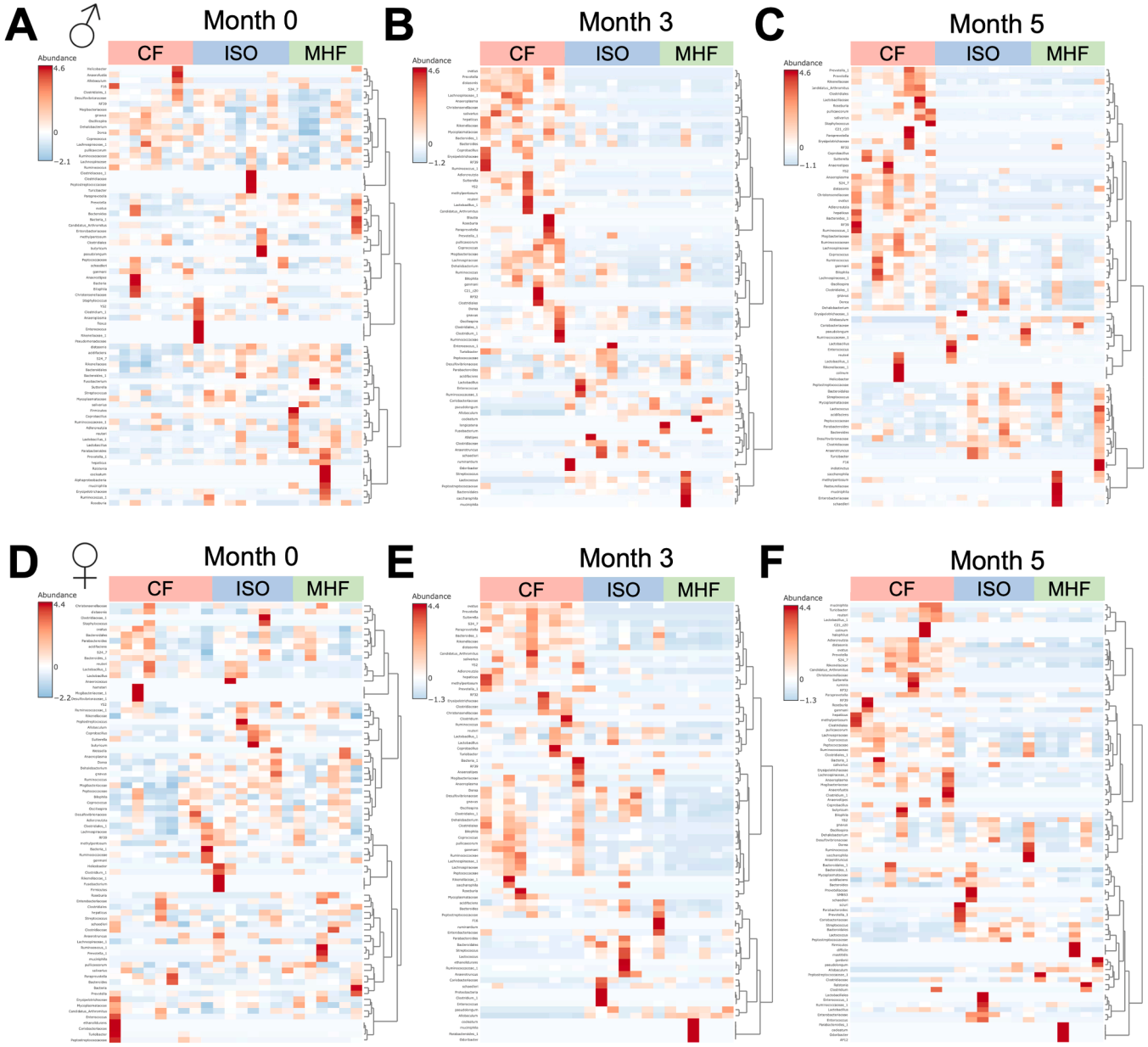

Fig. 8. Dietary fat induces microbiota dysbiosis that persists through month 5 in male (A-C) and female (D-F) mice. Heatmap visualizations of feature abundance among diet groups at A,D. month 0, B,E. month 3, and C,F. month 5. Please note that taxonomic features along the y axis of each heatmap are not consistent across time.

5 months (Fig. 7H: W (2.000, 9.668) = 3.275, $p = 0.0820$). The stepwise reduction in *Lachnospiraceae* abundance in fat-fed female mice at 3 months was not sustained at 5 months, with both fat-fed groups exhibiting reduced abundance of *Lachnospiraceae* compared to the CF group (Fig. 7H: F (2, 19) = 8.872, $p = 0.0019$). *Bacteroidaceae* was selectively elevated in the ISO group compared to the CF group at 5 months (Fig. 7H: F (2, 17) = 4.124, $p = 0.0347$), and *Rikenellaceae* was reduced in the ISO and MHF groups compared to the CF group at 5 months (Fig. 7H: F (2, 19) = 22.72, $p < 0.0001$). Overall, many compositional shifts observed at 3 months were sustained at 5 months, while others were transient.

3.4. *Feature heatmaps and LEfSe analysis reveal unique microbial signatures due to excess dietary fat consumption and modulated by feeding regimen*

Hierarchical clustering analyses of bacterial feature abundance demonstrates no clustering at month 0, with strong segregation of abundance patterns due to consumption of dietary fat and feeding regimen at 3 and 5 months of diet maintenance in both male (Fig. 8: A month 0, B month 3, C month 5) and female (Fig. 8: D month 0, E month 3, F month 5) mice. A Linear Discriminant Analysis Effect Size (LEfSe) revealed top taxa unique to dietary groups (Fig. 8: A-B males, C-D females; $p < 0.05$). Both male and female mice fed a fatty diet *ad libitum* had significant enrichment of *Allobaculum* at months 3 and 5 of feeding with the ISO group falling intermediate between the MHF and CF groups (Fig. 9). *Muribaculaceae*, Clostridiales, *Lactobacillus salivarius* and *Bifidobacterium pseudolongum* were typically more abundant in CF mice (Fig. 9). Interestingly, *Prevotella* were more abundant in CF fed mice compared to both groups of fat-fed mice (Fig. 9A-B, D).

4. Discussion

Our study reveals the first analysis of longitudinal changes in the gut microbial composition in male and female mice fed a moderately high-fat diet that was isocalorically matched versus one administered *ad libitum*. We report that gut microbiome composition is largely stable after 3 and 5 months of dietary maintenance. However, differences emerge across sex and method of diet administration (isocalorically (ISO) vs. *ad libitum* (MHF)). Fatty diet consumption reduced alpha diversity similarly in isocaloric and *ad libitum* fat-fed male mice, but only *ad libitum* fat-fed female mice showed significant reduction in alpha diversity. Moreover, beta-diversity analysis in female mice revealed unique signatures of each dietary group, while fat-fed groups of male mice were not significantly different from one another. Phylum-level shifts in composition were largely consistent in male mice, which exhibited an increase in Firmicutes and a decrease in Bacteroidetes in both MHF *ad libitum* and isocaloric fed mice. However, isocaloric fed female mice did not show this compositional shift. Unique features consistent across male and female MHF *ad libitum* fed mice included positive association with *Allobaculum* and negative association with *Muribaculaceae*. Interesting graded-changes in abundance between

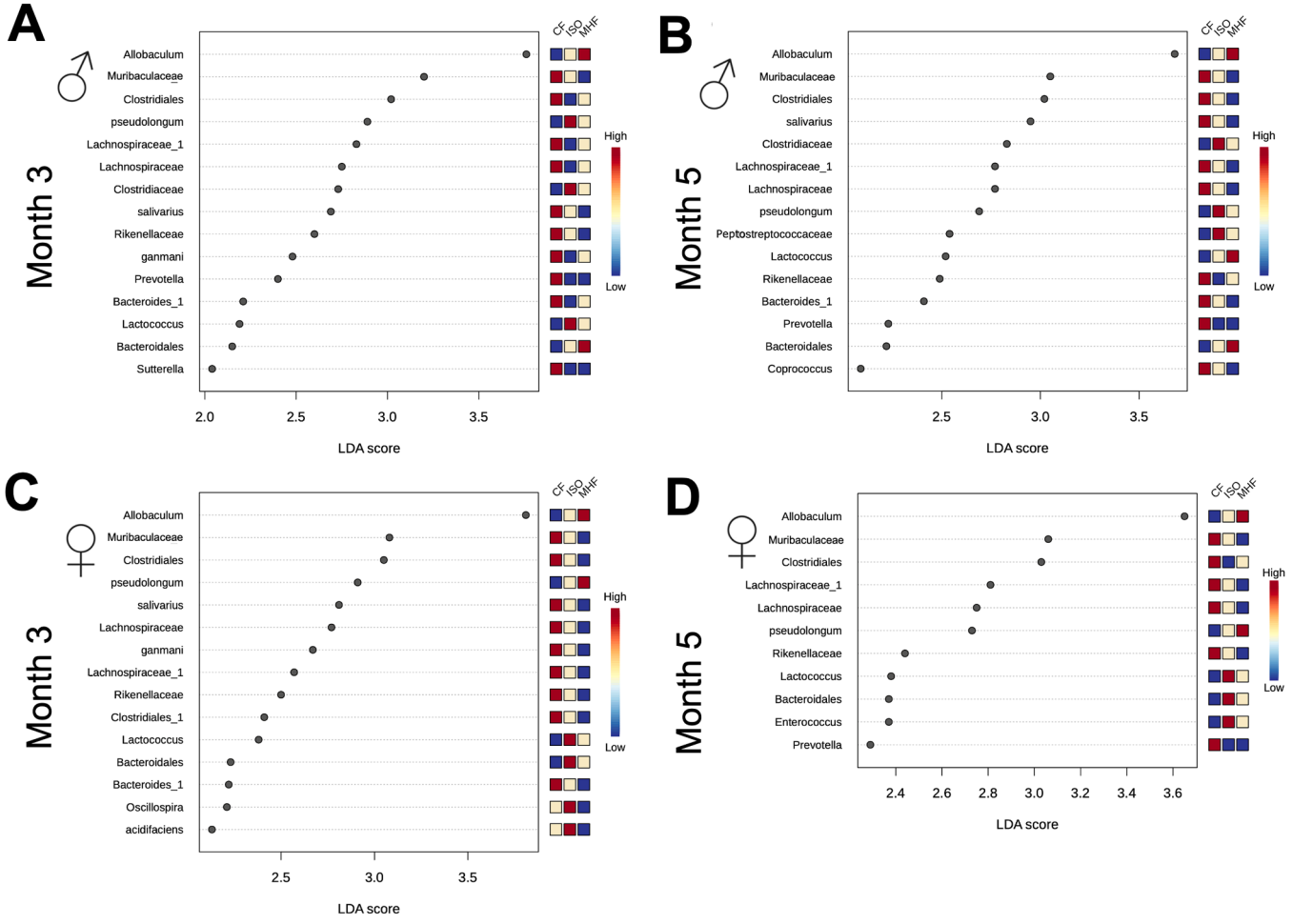

Fig. 9. Gut microbiome signatures emerge after 3 and 5 months of moderately high-fat diet feeding in male (A,B) and female (C,D) mice. Linear Discriminant Analysis (LDA) Effect Size (LEfSe) dot plot displaying features differentially abundant between CF, ISO, and MHF male mice after 3 months (A,C) and 5 months (B,D) of feeding.

control fed, isocaloric fat-fed, and *ad libitum* fat-fed mice in bacteria such as *Lactobacillus salivarius* provide insight into effects of dietary fat consumption vs. dietary fat overconsumption. Additionally, transient shifts like those observed for *Ruminococcaceae* capture microbial dynamics at the onset of diet-induced obesity that are not sustained in chronic obesity.

At baseline, no groups exhibited differences in alpha or beta diversity. However, the CF and MHF groups of male mice had significantly different abundances in 3 specific phyla. This was surprising, as all mice were maintained on control-fat chow until this point. Specifically, there were reduced abundances of Firmicutes and Proteobacteria, and elevated abundance of Bacteroidetes (*Bacteroidaceae, Muribaculaceae*) in the MHF group compared to the CF group. These trends did not persist, but instead reversed, as after 3 months of dietary maintenance Firmicutes abundance was elevated and Bacteroidetes abundance was reduced in male MHF fed mice compared to that in CF-fed male mice. Both male and female MHF groups of mice at 3 and 5 months had elevated abundance of Firmicutes compared to that of the CF group. Concomitant reduced abundance of Bacteroidetes was observed in both fat-fed groups of male mice at 3 and 5 months compared to the CF group, while only the female mice fed the fatty diet *ad libitum* had reduced Bacteroidetes compared to the CF group.

Both ISO and MHF groups of male mice exhibited reduced alpha diversity (Chao 1 index) and beta diversity after 3 and 5 months of feeding, while not differing from one another. Female mice follow the same alpha diversity trend. However, the ISO and MHF groups of female mice are distinct from one another at 3 and 5 months of feeding. The observed sex differences in beta diversity may result from known interactions between sex hormones on gut microbiota (reviewed by [40]).

Consistent across both male and female mice, Actinobacteria display an interesting transient elevated abundance due to dietary fat feeding at 3 months, which is ameliorated at five months; elevated Actinobacteria abundance persisted in the male ISO group and the female MHF group, only. Actinobacteria do not have a clear association with obesity and metabolic syndrome. However, this trend may be driven by age-related microbial changes instead of obesity as female mice do not become obese when maintained on a fatty diet [16].

Notably, *Erysipelotrichaceae* are highly abundant in fat-fed male and female mice at both 3 and 5 months of diet maintenance. Members of the phylum Firmicutes, Gram-positive *Erysipelotrichaceae* have been associated with inflammatory disorders such as irritable bowel syndrome and Crohn's disease, but these data are variable [41–44]. Similarly, studies of fatty diet feeding vary in association with *Erysipelotrichaceae*. High-fat diet fed rats have increased abundance of *Erysipelotrichaceae* [20], while *Erysipelotrichaceae* was most abundant in low-fat fed mice in a study of diet-induced and genetic obesity [45]. We observed elevated *Erysipelotrichaceae* in both sexes and both moderately high-fat fed groups at both time points. Combined with the present data these inconsistencies suggest that effects may be fat-source specific, as different sources of dietary fat impact microbial composition differently [46].

It has been shown that diet affects gut microbiota differently in male and female vertebrates [47]. *Lactobacillus, Alistipes, Lachnospiraceae*, and *Clostridium* are reported to be more abundant in male mice fed a fatty diet versus control diet, while these were less abundant in fat-fed female mice versus control diet [47]. However, Bolnick *et al.* report that male and female mice maintained on the same diet exhibit largely similar trends in microbes compared to other vertebrates studied (stickleback, perch and humans) [47]. Nagpal *et al.* report higher abundance of *Lachnospiraceae* in genetic-induced obese male mice [45]. We observed decreased abundance of *Lachnospiraceae* in the MHF male and female groups after both 3 and 5 months of fat feeding. Interestingly, the ISO male group has reduced abundance of *Lachnospiraceae* only at 3 months; abundance of *Lachnospiraceae* in the ISO groups at 3 months in female mice and 5 months in both male and female mice is not different from that of the CF or MHF groups. Members of *Lachnospiraceae*, as producers of short-chain fatty acids, are associated with metabolic improvement [48] as well as reducing inflammation and improving glucose tolerance [49]. Thus, the retained abundance of *Lachnospiraceae* in the ISO group at 5 months of diet, potentially due to isocaloric feeding preventing overconsumption, may have protective effects against diet-induced obesity, glucose intolerance, and metabolic syndrome.

Muribaculaceae is consistently highly associated with CF-fed mice, intermediately associated with ISO fat-fed mice, and poorly associated with MHF-fed mice, across both sexes at 3 and 5 months. MHF-fed male mice initially have elevated abundance of *Muribaculaceae*, but by 3 months abundance of *Muribaculaceae* is reduced in both ISO fat-fed and MHF-fed mice. In female mice, *Muribaculaceae* abundance is reduced in fat-fed mice at month 3 yet at month 5 this only persists in MHF *ad libitum* fed mice. This reduction in *Muribaculaceae* in MHF-fed mice is consistent with other reports of rodent *ad libitum* fatty diet consumption [20,45,50].

Prevotella and *Bacteroides* are Gram-negative members of Bacteroidetes (order Bacteroidales). An increase in *Prevotella/Bacteroides* ratio has been reportedly associated with obesity [51], while another study reported that the *Prevotella/Bacteroides* ratio is a predictor of weight-loss intervention success [52]. Abundance of *Prevotellaceae* was significantly reduced by dietary fat-feeding in both male and female mice consistently at 3 and 5 months of maintenance, and *Prevotella* were highly associated with CF diet treatment. *Prevotella* was the only feature consistently highly associated with CF diet group while having poor association with both fatty-diet fed groups, suggesting that *Prevotella* abundance is reduced by consumption of dietary fat regardless of feeding paradigm. Despite consistency of *Prevotella* across both fat-fed groups, Bacteroides was positively associated with the CF male group at 3 and 5 months and the CF female group at 3 months. Association, however, with the ISO and MHF groups was not consistent. These differences may result from emergence of diet-induced obesity as body weights between ISO and MHF male mice diverge [16].

The genus *Allobaculum* (Gram positive, family *Erysipelotrichaceae*) was consistently the strongest feature associated highly with the MHF group, intermediately with the ISO group, and poorly with the CF group. Members of this genus have been associated with intestinal inflammation [53], and fatty diet feeding has been associated with elevated *Allobaculum* [54]. The intermediate association of *Allobaculum* in our ISO group reveal the nuanced dysbiosis between mice consuming fatty diets isocalorically or *ad libitum*.

Notable sex differences are revealed by patterns of *Bifidobacteriaceae* differential abundance. *Bifidobacteriaceae* was low or undetected in fecal samples from control-fat fed mice, similar to that reported by Yang *et al.* [55]. *Bifidobacterium* are considered part of a normal gut microbiome of healthy mice (typically 0.5% levels and less) and are elevated in resilient animals following a stressor [55]. Perhaps there are not high stressors in our control groups (they are singly housed with 2 sources of enrichment) that would induce elevation of this species. The fact that it becomes elevated in diet-modified groups at 3 and 5 months may then reveal a type of stress of the diet, or dietary source of substrates metabolized by *Bifidobacterium* species. *Bifidobacterium* species are associated with human milk oligosaccharide consumption, thus the presence of milk fats in the moderately high-fat diet likely drive the elevation of these bacteria observed. Interestingly, male mice consuming a moderately high-fat diet exhibit elevated *Bifidobacteriaceae* at both 3 and 5 months of feeding, while female mice exhibit this trend at 3 months, but at 5 months, only the MHF group of female mice has elevated *Bifidobacteriaceae* abundance. *Bifidobacterium pseudolongum* was most highly associated with the ISO male group at both 3 and 5 months of feeding, with low association in the CF group. These data support abundance of *B. pseudolongum* driven by isocaloric feeding. Although not inherently a caloric restrictive diet, isocaloric feeding does involve feeding a discrete amount of food at the start of the dark cycle. Rodents consume the majority of their food during the dark cycle [56], so isocaloric feeding may involve fasting periods. Consequently, where infrequent binge consumption was suspected in a few mice, we split up food consumption

into 2 to 3 feedings to prevent any long inter-meal interval. Potentially *B. psuedolongum* is sensitive to the interaction between the feeding behaviors of the isocalorically fed group and the moderately high-fat diet itself. Female mice exhibit different association trends; the MHF female group had the highest association with *B. pseudolongum*, with lowest association in the CF group, which is consistent at 3 and 5 months of feeding. As female mice do not become diet-induced obese, this may drive the strong association between MHF *ad libitum* feeding that is absent in male mice. *B. pseudolongum* strains exhibit strain-specific differences in inflammation, with unique carbohydrate metabolism capabilities and persistence in the gut microbiome [57], and exerts protective effects against non-alcoholic fatty liver disease-associated hepatocellular carcinoma [58].

Lactobacillus salivarius is a well-studied probiotic member of Firmicutes known for therapeutic properties such as antagonizing bacterial pathogens and having probiotic properties (reviewed in [59]), regulating body weight and feed conversion ratio in broiler chickens [60], improving liver lipid metabolism in laying hens [61], and improved exercise performance in mice [62]. Studied from broiler chickens and rodents to human populations, *L. salivarius* treatment is commonly regarded as safe and beneficial. Our data support association of *L. salivarius* with the CF groups of mice with graded reduction of association in the ISO and the MHF groups. It would be interesting to evaluate how moderately high-fat diet feeding supplemented with a probiotic such as *L. salivarius* would affect obesity phenotype and metabolic syndrome.

The microbiota-gut-brain axis [63] has gained recognition for its potential role in neurodegenerative diseases such as Alzheimer's disease and Parkinson's disease [64–67]. Direct (enteroendocrine cells, vagus nerve) and indirect (bacterial metabolites, inflammation/immune modulation) linkages connect the gut with the brain, and may be modulated by gut bacteria. We postulate that the olfactory system is damaged by chronic consumption of a moderately high-fat diet [14,16] via factors such as lipotoxicity, gut microbial metabolites, lipopolysaccharides, systemic inflammation, glucose intolerance, and/or metabolic syndrome. This present study provides a basis of how this feeding model can be applied to further *in vivo* investigations of the functional consequences of the observed microbial change.

5. Conclusion

Overall, there are distinctions between the gut microbiota of isocaloric and *ad libitum* moderately high-fat fed mice. Our data emphasize the effects of not only diet, but also the feeding regimen, on the gut microbiome. Sex differences highlight the role of sex hormones and the necessity to explore obesity physiology in female mice, despite the resistance to diet-induced obesity.

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CRediT authorship contribution statement

Ashley M. Loeven: Writing – review & editing, Writing – original draft, Visualization, Validation, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Amber N. Brown:** Writing – review & editing, Methodology, Investigation, Conceptualization. **Francis U. Ebuara:** Writing – review & editing, Visualization, Data curation. **Kathryn M. Jones:** Writing – review & editing, Supervision, Data curation. **Debra Ann Fadool:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Resources, Project administration, Funding acquisition, Data curation, Conceptualization.

Declaration of competing interest

The authors have no competing interest financial or scientific.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.physbeh.2025.115062.

Data availability

Data will be made available on request.

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