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Gut microbiome-driven regulation of sex hormone homeostasis: a potential neuroendocrine connection

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ABSTRACT

The gut microbiome is known to have a bidirectional relationship with sex hormone homeostasis; however, its role in mediating interactions between the primary regulatory axes of sex hormones and their productions is yet to be fully understood. We utilized both conventionally raised and gnotobiotic mouse models to investigate the regulatory role of the gut microbiome on the hypothalamic-pituitary-gonadal (HPG) axis. Male and female conventionally raised mice underwent surgical modifications as follows: (1) hormonally intact controls; (2) gonadectomized males and females; (3) gonadectomized males and females supplemented with testosterone and estrogen, respectively. Fecal samples from these mice were used to colonize sex-matched, intact, germ-free recipient mice through fecal microbiota transplant (FMT). Serum gonadotropins, gonadal sex hormones, cecal microbiota, and the serum global metabolome were assessed. FMT recipients of gonadectomized-associated microbiota showed lower circulating gonadotropin levels than recipients of intact-associated microbiota, opposite to that of FMT donors. FMT recipients of gonadectomized-associated microbiota also had greater testicular weights compared to recipients of intact-associated microbiota. The gut microbiota composition of recipient mice differed significantly based on the FMT received, with the male microbiota having a more concerted impact in response to changes in the HPG axis. Network analyses showed that multiple metabolically unrelated pathways may be involved in driving differences in serum metabolites due to sex and microbiome received in the recipient mice. In sum, our findings indicate that the gut microbiome responds to the HPG axis and subsequently modulates its feedback mechanisms. A deeper understanding of interactions between the gut microbiota and the neuroendocrine-gonadal system may contribute to the development of therapies for sexually dimorphic diseases.

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Introduction

The gastrointestinal tract contains trillions of microorganisms that influence host development, physiology, and health.[1,2] Onset of puberty and sextual maturation, where drastic increases in gonadal sex steroids production occur, coincides with a shift in the gut microbiome.[3] Evidence from mice born and raised in a sterile environment without any microorganisms (i.e., germ-free mice) highlights the direct contribution of the microbiome on sex hormone homeostasis.[4] Specifically, germ-free mice exhibit impaired reproductive capacity, and it has been shown that bacterial colonization restored some aspects of their fertility.[4] This includes normalization of estrous cycles in females, greater sperm motility in males, and significant increases in both copulation frequency and implantation rates.[4] The lack of gut microbiome also impacts reproductive development and impairs blood-testis-barrier integrity.[5] Interestingly, transplantation of male gut microbiome into female mice increased the production of testosterone, sufficient enough to affect disease predisposition.[6]

In mammals, reproductive function is regulated by the hypothalamic-pituitary-gonadal (HPG) axis, a tightly controlled feedback loop. This loop begins with the hypothalamus releasing gonadotropin-releasing hormone (GnRH), which prompts the gonadotrophic cells of the anterior pituitary to

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secrete luteinizing hormone (LH) and follicle stimulating hormone (FSH). LH and FSH then enter systemic circulation to promote steroidogenesis and gamete development within the gonads. Sex hormones, estrogen from ovaries and testosterone from testes, regulate the upstream components of the HPG axis to achieve homeostasis. Although commonly referred to as the "reproductive axis", the HPG axis naturally fluctuates throughout various stages of life – such as puberty, menstruation, pregnancy, menopause, and aging to maintain homeostatic status of the sex hormones.[7]

Disruption of sex hormone homeostasis can increase the risk and progression of numerous diseases, such as breast cancer, endometriosis, prostate cancer, infertility, and cardiometabolic disease.[8–11] The influence of the gut microbiome on sex hormone production through direct modifications of the steroidal molecules has been more extensively studied than its effects on the downstream components of the HPG axis.[12–14] Additionally, sex steroid supplementation in rodents and humans suggest that sex steroids may influence disease predisposition by interacting with the gut microbiota.[15,16] While literature is limited, there is evidence that the gut microbiome interacts with the upstream components of the HPG axis. For instance, germ-free mice have lower levels of gonadotropins, both FSH and LH, compared to conventionally raised mice that harbor a natural microbiome.[5] The use of a GnRH analog has been shown to lower the relative abundance of *Enterobacteriaceae* in the gut microbiome of rats, and injections of lipopolysaccharides (LPS), a component of the bacterial cell wall, lowered GnRH expression in both rats and sheep.[17–19] Additionally, dietary supplementation with a probiotic *Bacillus licheniformis* DSM5749 elevated the GnRH expression in laying hens and improved laying performance.[20] Furthermore, supplementation of short-chain fatty acids, which are bacterial fermentative end products, elevated gonadotropins compared to non-supplemented controls in sheep.[21]

Despite these findings, the gut microbiome's role in mediating interactions between the HPG axis and sex hormone regulation remains poorly understood. In this study, we aimed to assess the interactions between the gut microbiome and the HPG axis using a combination of conventionally raised and gnotobiotic mouse models. Additionally, we aimed to identify gut microbiome-driven features that are responsive to the loss and gain of gonadal sex steroids. We hypothesized that the gut microbiome could respond to changes in sex hormone homeostasis and subsequently impact the production of gonadal sex hormones and gonadotropins. To investigate this, we introduced the gut microbiome via fecal microbial transplant (FMT) from gonadectomized conventionally raised donor mice – either with or without sex hormone supplementations into sex-matched, HPG axis intact germ-free recipient mice. This approach allows us to assess the causal effects of the gut microbiota on the HPG axis.

Results

Gut microbiota from donor mice with altered sex hormone status induced alterations of the HPG axis in FMT recipient mice

Sex hormone status of 8-week-old conventionally raised "microbiota donor" mice were altered through gonadectomy and subcutaneous implantation of a sex hormone pellet, resulting in six treatment groups: (1) hormonally intact/unaltered male sham controls (INT-M); (2) orchiectomized males (ORX-M); (3) orchiectomized males with testosterone supplementation (ORX+T-M); (4) hormonally intact/unaltered female sham controls (INT-F); (5) ovariectomized females (OVX-F); (6) ovariectomized females with 17Beta-estradiol supplementation (OVX+E-F);. Sex steroid pellets used were designed to release steady, physiologically relevant levels of hormones over an 8-week period, serving as a positive control to suppress gonadotropin levels in microbiota donors.[22,23] Eight weeks after the hormonal alterations, at the age of 16 weeks, fecal samples were collected from these microbiota donor mice and used to colonized 6-week-old, sex-matched germ-free mice of the same genetic strain through FMT ("FMT recipients") (Figure 1). Four weeks after colonization, FMT recipient mice were euthanized. The 8-week post-surgical intervention timeframe was selected based on our previous work showing that the gut microbiota differ between ovariectomized mice and sham controls

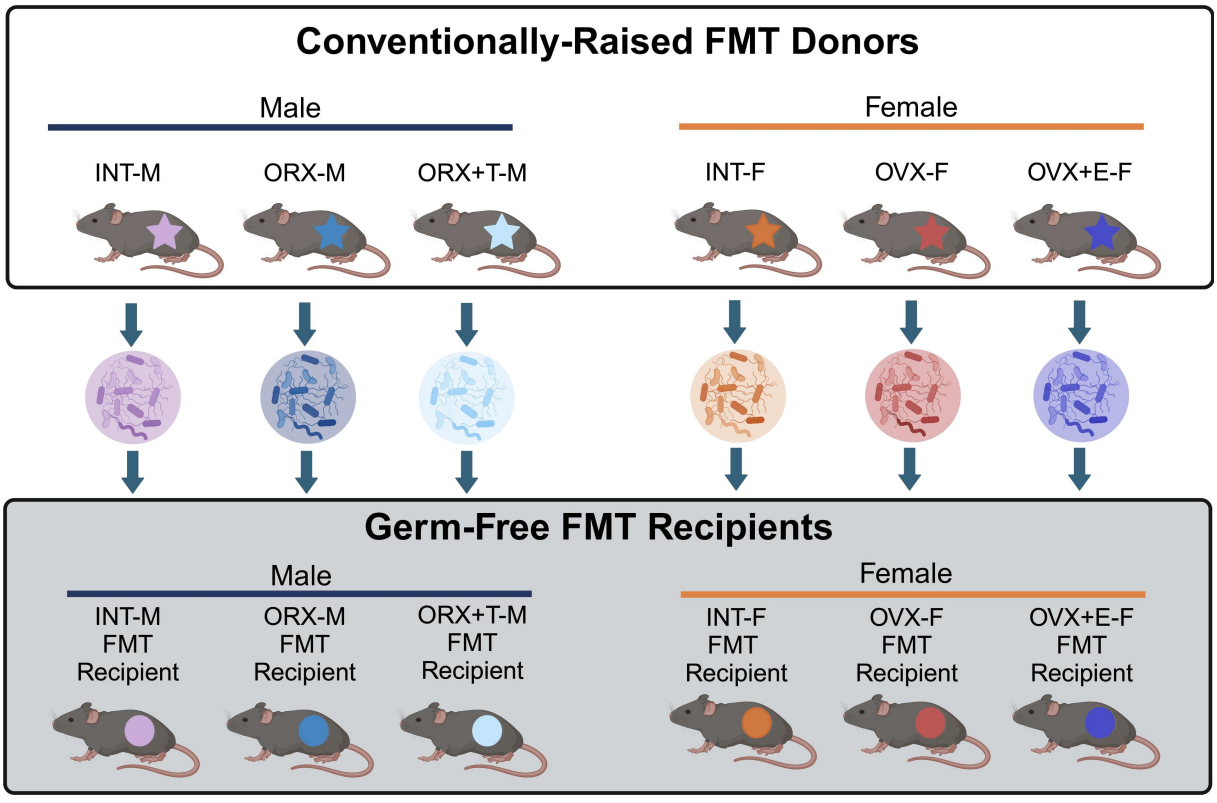

Figure 1. *Study schema.* 8-week-old C57BL/6J fecal microbial transplant (FMT) donor mice had sex hormone status altered through gonadectomy and subcutaneous hormone pellet implantation resulting in six donor groups: (1) intact male controls (INT-M, $n = 13$); (2) orchiectomized males (ORX-M, $n = 13$); (3) orchiectomized males with testosterone (12.5 mg/pellet 60-day release) supplementation (ORX+T-M, $n = 10$); (4) intact female controls (INT-F, $n = 7$); (5) ovariectomized females (OVX-F, $n = 8$); (6) ovariectomized female with 17Beta-estradiol (0.1mg/pellet 60-day slow release) supplementation (OVX+E-F, $n = 8$); Eight weeks after surgery, fecal samples were collected from donor mice and used to colonize 6-week-old germ-free C57BL/6J mice via FMT resulting in six FMT recipient groups: INT-M FMT recipient ($n = 8$); ORX-M FMT recipient ($n = 8$); and ORX+T FMT recipient ($n = 9$); INT-F FMT recipient ($n = 7$); OVX-F FMT recipient ($n = 6$); and OVX+E FMT recipient ($n = 8$). Recipient mice were sex matched to donor mice and were euthanized 4 weeks after colonization.

8 weeks after the surgery.[24] Serum FSH and LH were determined to assess the alterations of sex hormone homeostasis in the microbiota donor and FMT recipient mice. As expected, gonadectomy in donor mice resulted in significantly higher ($p < 0.05$) levels of FSH and LH compared to hormonally intact controls in both sexes (Figure 2(a–d)). Sex steroid supplementation in the gonadectomized microbiota donor mice (ORX+T-M and OVX+E-F) significantly lowered ($p < 0.05$) circulating gonadotropin levels compared to the gonadectomized mice. However, sex steroid supplementation resulted in larger variance in hormonal levels than intact mice.

Four weeks after colonization, FMT recipient mice were euthanized and serum gonadotropins, testes weight and uterine, and intragonadal sex hormones were assessed to determine the status of the HPG axis. This colonization timeframe was selected based on prior data indicating that it is sufficiently long enough to detect physiological changes driven by gut microbiome.[25] In males, FMT recipients of ORX-M-associated microbiota had significantly lower ($p < 0.05$) levels of both FSH and LH with large effect sizes of 1.34 and 1.81, respectively, compared to FMT recipients of INT-M-associated microbiota (Figure 2(e,f)). FMT recipients of ORX-M-associated microbiota had significantly greater ($p < 0.05$) testicular weight than FMT recipients of INT-M (Figure 2(g)); however, statistical differences in intratesticular testosterone were not detected among male FMT recipients (Figure 2(h)). Female FMT recipient animals did not exhibit statistically significant differences in serum gonadotropins, uterine weight, or ovarian estradiol (Figure 2(i–l)). However, the effect size,

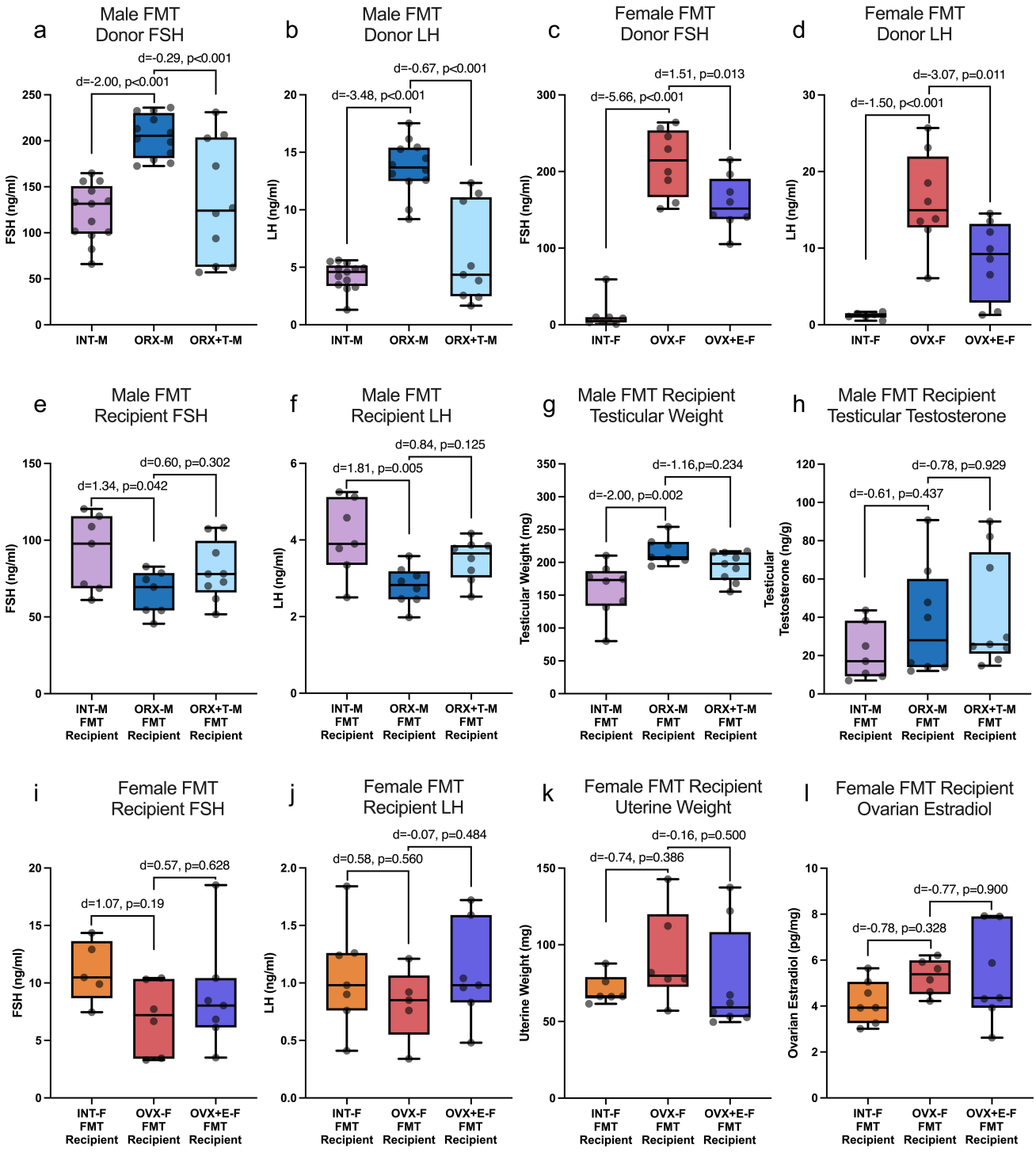

Figure 2. Circulating gonadotropins, follicle-stimulating hormone (FSH) and luteinizing hormone (LH), were measured in the serum of male and female microbiota donor mice with surgical alteration of sex hormone status (a–d) and in gnotobiotic hormonally intact recipient mice (Male E&F; Female I&J). Testicular weight was measured in male gnotobiotic recipients (g) and uterine weight female gnotobiotic recipients (k). Intragonadal sex hormone levels were evaluated in gnotobiotic recipients (h&l). Statistics were performed using direct contrasts comparisons of gonadectomized FMT donors and recipients with respective controls: intact-associated microbiota donors and recipients, and gonadectomized donors and recipients supplemented with sex steroids. P-values were corrected using the Sidak methodology and shown directly above the comparison. Effect sizes (d) were calculated using Cohen's d to quantify the strength of the observed effects.

as measured by Cohen's d, indicated a strong biological impact of gonadectomized-associated microbiota on HPG axis signaling, which appeared to act in opposition to the signaling observed in the FMT donors. For instance, the effect sizes between FMT recipients of INT- and OVX-associated microbiota ranged from 0.58 to 1.07, highlighting a notable biological effect. These findings suggest that while the biological signal is evident, larger sample sizes may be required to achieve statistical significance. Together, our data indicate that the gut microbiome from mice with sex hormone alterations via gonadectomy and sex hormone supplementation may be able to impact HPG axis homeostasis when transplanted into germ-free recipients.

Gut microbiota features of FMT recipient mice may be contributing to the observed shifts in HPG axis hormones

Community-level differences of the cecal microbiota were assessed in donor and recipient mice using 16S rRNA gene sequencing. In both male and female donor mice, gonadectomy (ORX-M and OVX-F) resulted in significant shifts in gut microbial communities compared to sex-matched intact controls as assessed by principal coordinate analysis (PCoA) ($q = 0.007$ and $q = 0.038$, respectively) (SI Fig. S1a, b). In male donor mice, gonadectomy with hormone supplementation (ORX+T-M) also significantly ($q = 0.001$) shifted microbial communities compared to ORX-M (SI Fig. S1a). In FMT recipient mice, microbial communities clustered significantly differently ($q < 0.05$) based on the microbiota received in both sexes (Figure 3(a,b)).

Adjacency matrices were constructed to identify the most important relationships between bacterial taxa within the gut microbiota, potentially critical in driving alterations in the HPG axis signaling. These matrices capture pairwise interactions by assessing the degree of co-occurrence or exclusion among taxa. A co-occurrence network was then built to visualize these relationships (Figure 3(c,d)). Nodes within the network represent individual bacterial taxa, while edges represent significant relationships. To further delineate and emphasize the strongest relationships within the microbial communities, a minimum spanning tree (MST) was constructed from the network (Figure 3(e,f)). This method allows for the visualization of only the most important relationships within the microbial community.[26,27] Interestingly, networks constructed from male microbiota (Figure 3(c)) formed more firm connections than those constructed from females (Figure 3(d)), suggesting a more concerted impact on male gut microbiota in response to changes in HPG axis hormonal signaling. In male recipient mice, the node having the greatest interconnectedness belonged to the genus *Akkermansia* (Bact-5) (Figure 3(e) and SI Table s1). In females, the node identified to have the greatest interconnectedness belonged to the genus *Lachnospiraceae* NKA126 (Bact-16) (Figure 3(f) and SI Table s2), a major producer of short-chain fatty acids.[28]

Differential abundance analysis was performed using Kruskal-Wallis followed by Dunn's post-hoc test to compare recipients of gonadectomized-associated microbiota against controls and discern specific bacterial taxa within the community that may be contributing to gut microbial interactions with the HPG axis. In males, differential abundance analysis showed that FMT recipients of ORX-M-associated microbiota had significantly lower ($p < 0.05$) levels of an uncultured genus from the family *Lachnospiraceae* as well as *Oscillibacter* compared to FMT recipients of INT-M (Figure 3(g,h)). FMT recipients of ORX-M-associated microbiota had significantly greater ($p < 0.05$) *Tuzzerella* compared to recipients of ORX+T-M (Figure 3(i)). In females, FMT recipients of OVX-F-associated microbiota had significantly lower ($p < 0.05$) relative abundance of an uncultured genus in the *Oscillospiraceae* family compared to FMT recipients of INT-F (Figure 3(k)). Additionally, FMT recipients of OVX-F-associated microbiota exhibited significantly higher ($p < 0.05$) proportions of *Erysipelatoclostridium* compared to FMT recipients of OVX+E-F (Figure 3(l)). The genus *Clostridium Sensu Stricto 1* was found to be significantly higher ($p < 0.05$) in FMT recipients of OVX-F compared to FMT recipients of INT-F (Figure 3(m)). In addition to genus level, differential abundance of amplicon sequence variants (ASV) of the gut microbiota were assessed and shown in SI Table s3, s4. Many of these aforementioned bacterial taxa have previously been described to either be directly

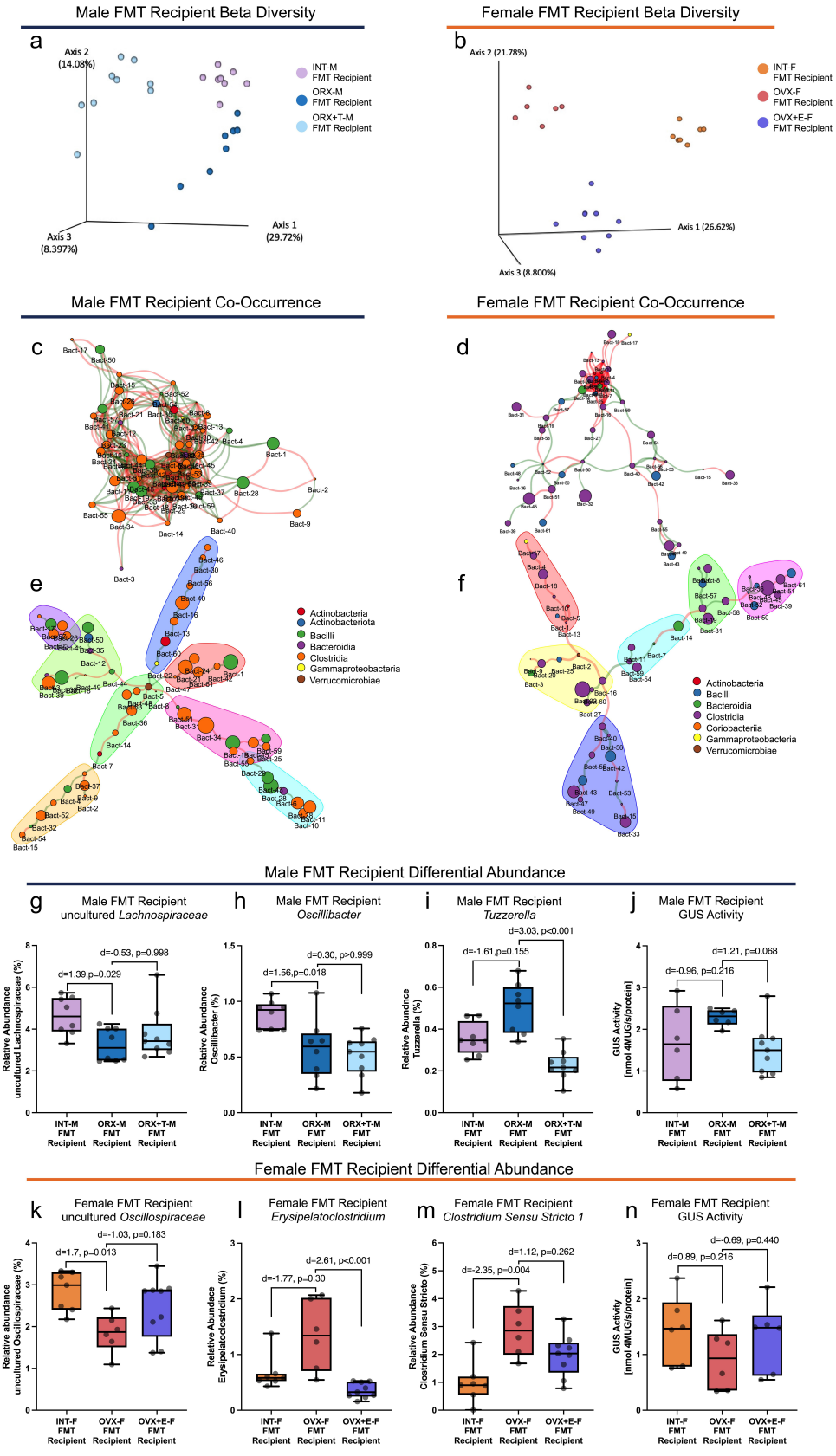

Figure 3. Principal coordinate analysis (PCoA) plots of cecal microbiota in gnotobiotic recipients are shown using the Jaccard distance metric (a&b). Co-occurrence networks were created for both male and female cecal microbiota from adjacency matrices (c&d) and further delineated with minimum spanning analysis, which builds a network with the fewest necessary edges, highlighting the most critical relationships within the gut microbiota (e&f). Red lines denote negative relationships between nodes, while green lines denote

involved in sex hormone metabolism in *in vitro* studies or be altered in diseases with disrupted sex hormone homeostasis such as polycystic ovary syndrome.[29–31]

A gut bacterial enzyme known to deconjugate sex steroid to increase its availability does not fully explain the microbiome-driven phenotype observed

Bacterial beta-glucuronidase (GUS) activity has previously been reported to be a critical mechanism by which the gut microbiota may influence host sex steroid homeostasis.[32] Glucuronidation, which occurs mostly in the liver, facilitates the biliary excretion of the sex hormones via feces. As these glucuronidated sex hormones travel through the intestinal tract lumen, bacterial beta-glucuronidase can cleave off the glucuronide molecule and release free sex steroids. These liberated hormones then have greater availability to bind to local receptors or be reabsorbed into circulation, influencing systemic hormone levels through enhancing enterohepatic recirculation.[31,32] To determine whether this deconjugation was involved and contributed to the phenotypes observed, GUS enzyme activity was measured in the cecal content of FMT recipients. Statistical differences were not observed among FMT recipient groups (Figure 3(j,n)). Given that alterations in GUS enzyme activity were not observed, we posit that the observed alterations in sex hormone status of FMT recipients were not exclusively driven by GUS activity.

Circulating metabolomic features significantly shifted in recipient mice and may suggest potential gut microbiome-mediated mechanisms in regulating the HPG axis

Discovery-based untargeted metabolomics was performed using the serum of gnotobiotic FMT recipient mice and germ-free mice to investigate potential metabolic mechanisms of gut microbial modification of sex hormone homeostasis. Principal component analysis (PCA) utilizing all the metabolomic data showed that the largest factor driving metabolomic profile differences was sex (Figure 4(a)). When elastic net feature selection was utilized to preselect only the inputs that were not related to sex, the PCA showed that an important factor driving differences in metabolome profile was colonization status (e.g., FMT colonized vs. GF mice) manifesting along PC1 (Figure 4(b)). Moreover, microbiota received via FMT (i.e., FMT recipients of intact vs. gonadectomized vs. gonadectomized with sex hormone supplement-associated microbiota) was the largest driver of variation shown across PC2, indicating potential substantial metabolomic changes driven by the gut microbiome. To visualize the relationship between metabolites, a MST of metabolite abundance correlation network was constructed using both male and female experimental groups. Both males and females were utilized to construct this analysis framework to identify shared metabolite features. Within the framework of the MST, the metabolites that were most impacted by the microbiome received were assessed and denoted on the tree (Figure 4(c)). We discovered that metabolites that differed among FMT groups were located across multiple branches of the tree, suggesting that the gut microbiota may be able to drive alterations in the sex hormone regulation through a variety of metabolically distinct pathways that are unrelated. Interestingly, several of the metabolites observed in minimum spanning tree analysis are derived from microbial metabolism, such as butyrate, valerate, indolepropionate, and p-cresol glucuronide.

Alterations in microbially derived metabolites such as short-chain and branched-chain fatty acids were observed in our study. In males, FMT recipients of ORX-M-associated microbiota had significantly higher ($p < 0.05$) butyrate, isovalerate, and valerate compared to FMT recipients of INT-M-associated microbiota (Figure 4(d–f)). Similarly,

positive relationships. Branches of minimum spanning tree are highlighted with shadowing. Differential abundance analysis was performed at the genus level in male (g–i) and female fecal microbial transplant (FMT) recipient mice (k–m). Enzyme activity of beta-glucuronidase (GUS) was determined in the cecal content of male (j) and female (n) recipient mice. Differential abundance was assessed by Kruskal Wallis with Dunn's post hoc test. Effect sizes (d) were calculated as Cohen's d to quantify the strength of the observed effects.

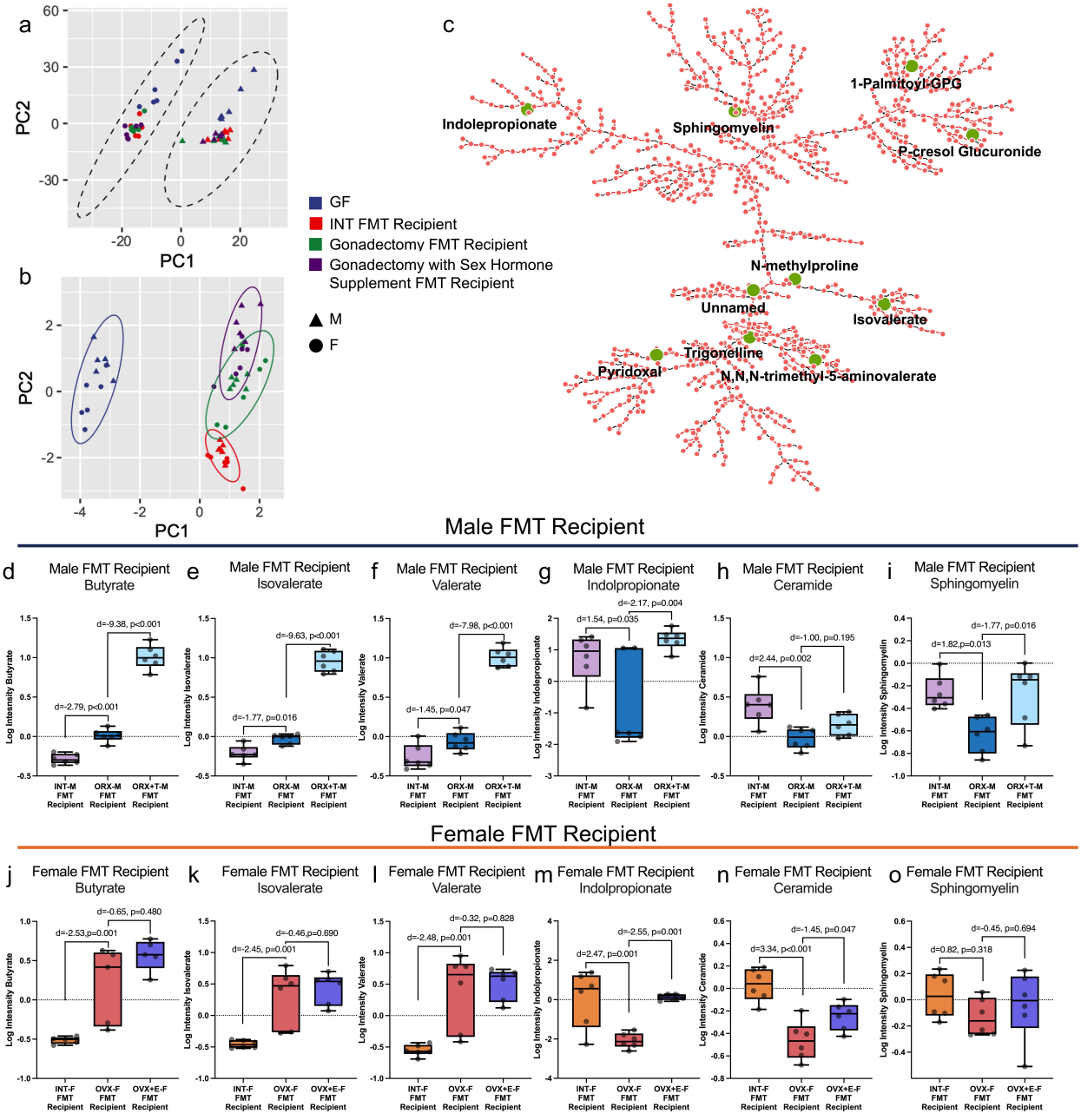

Figure 4. Untargeted metabolomics was performed in serum of gnotobiotic recipient mice along with germ-free controls. Principal coordinate analysis (PCA) was used to assess global metabolomic profile, revealing the largest factor driving metabolomic profile to be sex (a). Elastic net feature selection was then used to eliminate for features that vary in relation to sex (b). Metabolomic data from gnotobiotic recipient groups were used to construct a network using minimum spanning tree algorithms (c). Points shown in green represent metabolites most impacted by microbiota received via fecal microbial transplant (FMT). Differentially abundant metabolites of interest were determined in male(d–i) and female (j–o) FMT recipient groups and assessed by direct contrast comparisons of gonadectomized FMT donors and recipients with respective controls: intact-associated microbiota donors and recipients, and gonadectomized donors and recipients supplemented with sex steroids. P-values were corrected using the Sidak method and shown directly above comparisons. Effect sizes (d) were calculated as Cohen's d to quantify the strength of the observed effects.

in females, FMT recipients of OVX-F-associated microbiota had greater ($p < 0.05$) serum butyrate, isovalerate, and valerate compared to FMT recipients of INT-F-associated microbiota (Figure 4(j–l)). In addition to short and branched chain-fatty acids, alterations in indolpropionate, a gut bacteria-derived metabolite of tryptophan, were observed. FMT recipients of ORX-M- and OVX-F-associated microbiota exhibited significantly lower ($p < 0.05$) indolepropionate compared to their respective sex-matched FMT recipients of INT-associated microbiota controls and hormone supplemented groups (Figure 4(g,m)). Indolepropionate has been shown to play an important role in gut-brain communication, but their roles in relation to sex hormones have not been reported.[33]

FMT recipients of ORX-M microbiota had lower ($p < 0.05$) levels of ceramide than FMT recipients of INT-M (Figure 4(h)). In females, FMT recipients of OVX-F-associated microbiota exhibited significantly lower ($p < 0.05$) levels of ceramide compared to FMT recipients of INT-F and OVX+E-F (Figure 4(n)). Alterations in sphingomyelin, a sphingolipid related to ceramide, also reached statistical significance in males (Figure 4(i)). Statistical differences of sphingomyelin were not noted in females; however, comparison of FMT recipients of INT-F- and OVX-F-associated microbiota showed a large effect size of 0.82, suggesting that a biological effect is likely present (Figure 4(o)). Ceramide and related sphingolipids have previously been suggested to play an important role in sex hormone regulation and reproductive function.[34,35]

Heatmaps were generated using the top 100 metabolites identified to be differentially present across FMT recipient groups within each sex, illustrating substantial variations dependent upon the microbiota received. Hierarchical clustering revealed significant grouping of each microbiota recipient group in both sexes, indicating a pronounced gut microbiota treatment effect on serum metabolomics that responds to alterations in the HPG axis (Figure 5(a, b), Table s5, s6). Our data collectively indicates that the gut microbiota may impact the HPG axis through shifting the serum metabolome involving multiple metabolically unrelated pathways such as production of short-chain fatty acids, indole-containing compounds, and sphingolipids.

Associative relationships between gut microbial taxa and serum hormones and metabolites

Spearman correlations were computed with false discovery rate (FDR) corrections to assess gut microbial changes associated with hormone levels in the donor mice to identify potential gut microbial drivers or responders to the loss and gain of gonadal hormones. In male FMT donors, circulating FSH was positively associated ($p < 0.05$) with *Lactobacillus* ($\rho = 0.365$) and *[Eubacterium] nodatum* ($\rho = 0.43$) (SI Fig. S2). LH was significantly correlated ($p < 0.05$) with *Lactobacillus* ($\rho = 0.533$) and *Enterococcus* ($\rho = 0.615$). In female FMT donors, circulating FSH significantly associated ($p < 0.05$) with multiple bacteria taxa, including *Lactococcus* (ρ =-0.487) *Lachnospiraceae UCG-004* (ρ =–0.457), and *Erysipelatoclostridium* ($\rho = 0.455$) (SI Fig. S3). Moreover, LH was positively associated ($p < 0.05$) with *Erysipelatoclostridium* ($\rho = 0.464$), *Enterococcus* ($\rho = 0.608$), and an unknown genus in *Clostridia UCG014* family ($\rho = 0.65$).

Spearman correlations were also computed between gut microbial taxa and the top 100 metabolites that were significantly different across FMT recipient groups within each sex. Multiple comparisons were corrected using the FDR. These top 100 metabolites were selected by sorting the p-values from lowest to highest and choosing the first 100 that are statistically different among treatment groups (SI Table s3, s4). In male FMT recipients, butyrate and isovalerate were positively ($p < 0.05$) associated with *Faecalibaculum* ($\rho = 0.614$ and $\rho =$ 0.635, respectively) and *Dubosiella* ($\rho = 0.862$ and $\rho = 0.841$) and negatively associated with *Oscillibacter* (ρ =–0.655 and ρ =–0.668). Additionally, indolepropionate was positively ($p < 0.05$) correlated with *Faecalibaculum* ($\rho = 0.695$) and negatively ($p < 0.05$) correlated with *Lachnoclostridium* (ρ =-0.721) (Figure 6, SI Table s7). In female FMT recipients, both butyrate and isovalerate were positively ($p < 0.05$) associated with *Turicibacter* ($\rho = 0.751$ and $\rho = 0.762$, respectively) and negatively ($p < 0.05$) correlated with an unknown *Erysipelotrichaceae* ($\rho =$ –0.721 and ρ =–0.618). Dimethyl sulfone was negatively ($p < 0.05$) associated with *Clostridium Sensu Stricto 1* (ρ =–0.746) and positively associated with an uncultured *Oscillospiraceae* ($\rho = 0.767$),

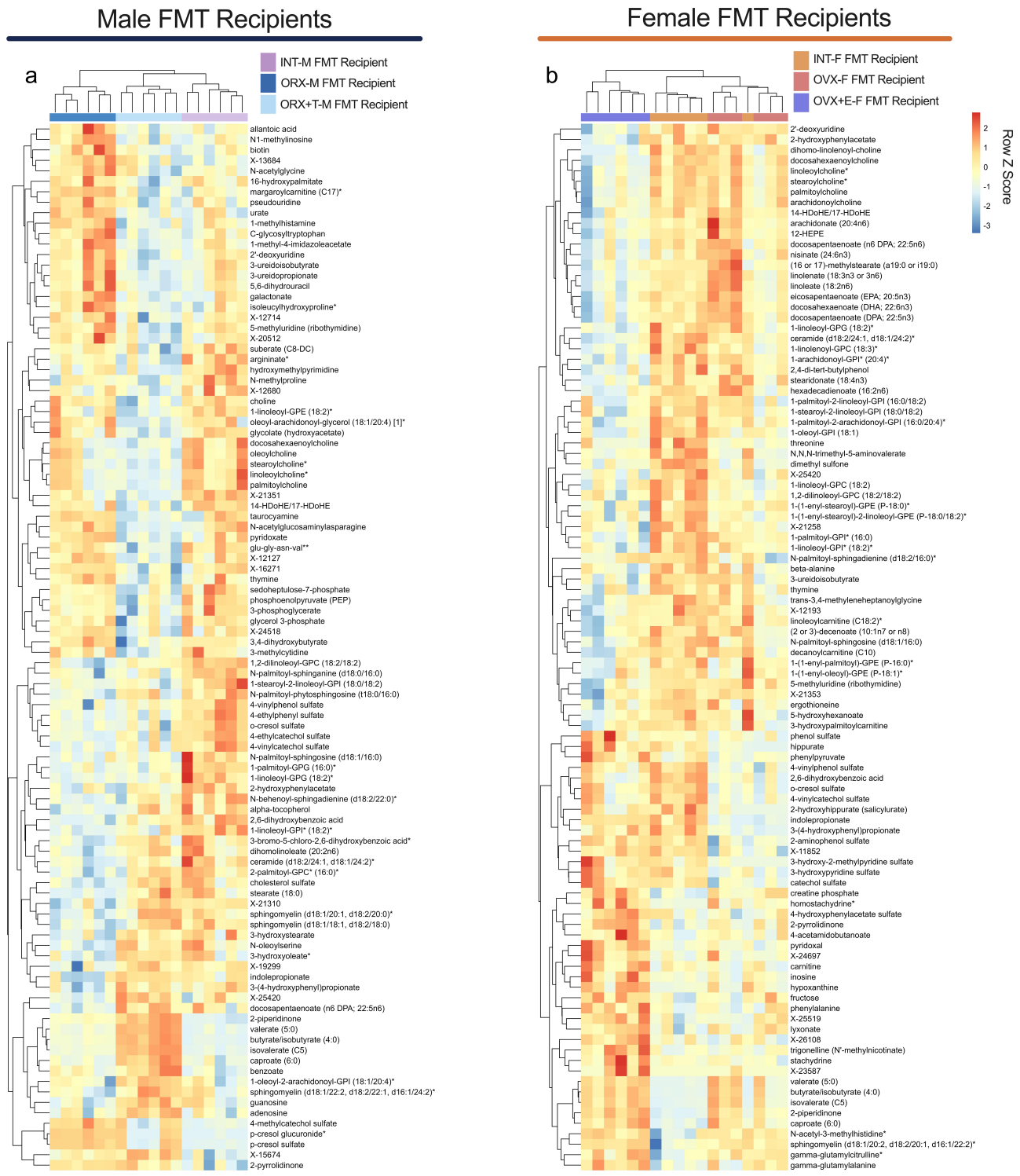

Figure 5. Serum untargeted metabolomics from gnotobiotic recipients of altered-sex hormone associated microbiome. Heatmap of the z scores of the top 100 metabolites determined to be differentially present in females (a) and males (b) with hierarchical clustering based on microbiome received.

uncultured *Lachnospiraceae* ($\rho = 0.641$), and *Lachnospiraceae UCG004* ($\rho = 0.605$) (Figure 7, SI Table s8). These metabolites can be produced exclusively or partially through gut microbial metabolism and were significantly different across FMT recipient groups in this study. These associations demonstrate alterations in gut microbial activity that may influence host-health and sex hormone homeostasis when transplanted into germ-free recipient mice.

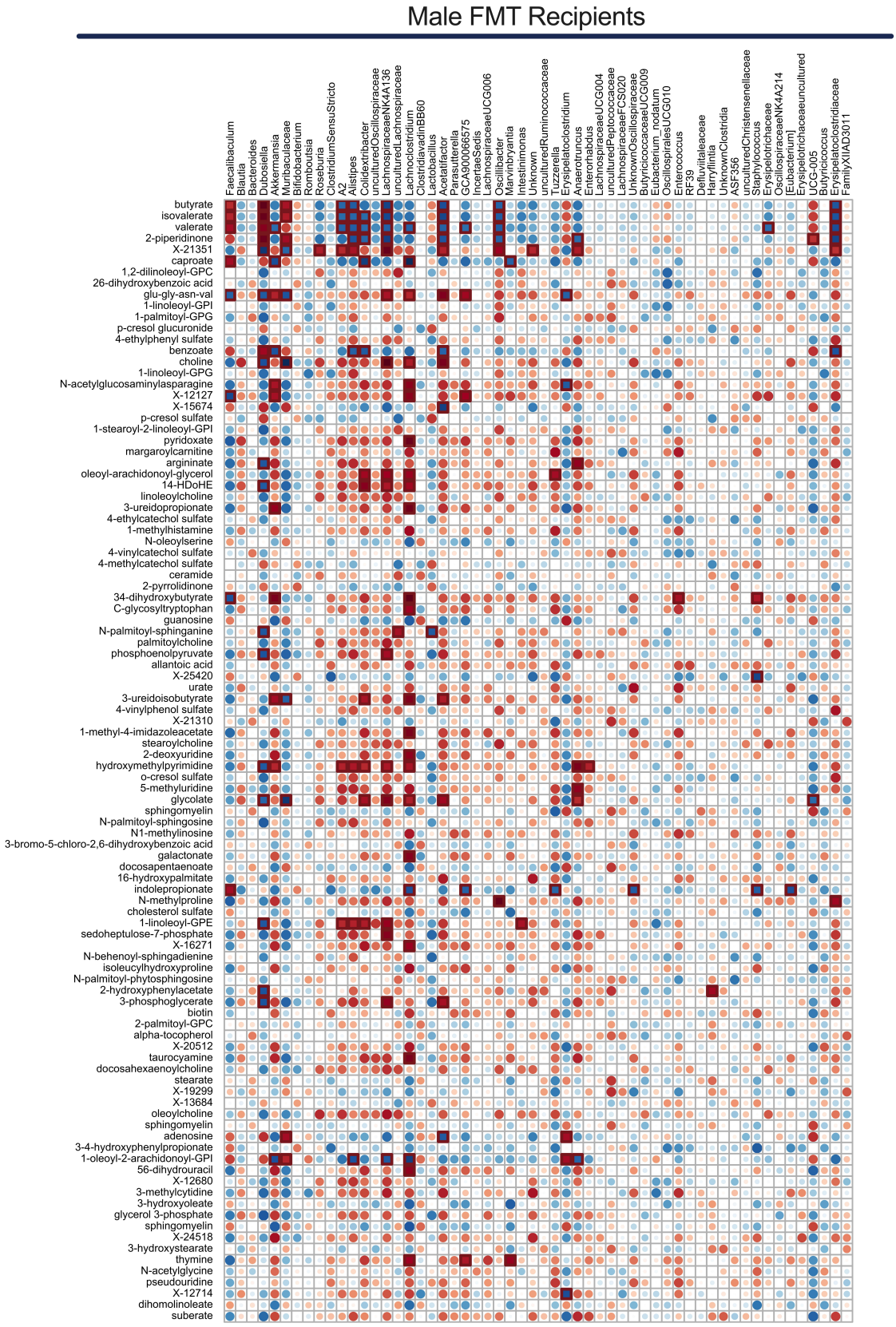

Figure 6. Spearman correlations with FDR corrections between top 100 differentially present serum untargeted metabolomics and gut microbiota at the genus level in male gnotobiotic recipients. Red circle denoted positive associations, and blue circle denotes negative associated with the size of the circle indicating the strength (larger circle represents a stronger association). Statistically significant associations ($p< 0.05$) are shown with red box.

In addition to Spearman correlations, we employed Canonical Correlation Analysis (CCA) to explore the relationships between microbiota composition and metabolite profiles while accounting for group-level differences. Unlike simple pairwise correlations, which assess the relationship between individual variable pairs, CCA uncovers patterns of association between two sets of interrelated variables.[36] To manage the high dimensionality of our datasets, we applied CCA to the first four principal components derived from the taxa data, which were transformed to approximate a Gaussian distribution, and the normalized metabolite matrices. The results (SI Fig. S4) reveal a distinct clustering of treatment groups, highlighting a systematic relationship between bacterial taxa abundances and metabolite levels. This joint variation structure underscores the influence of treatment conditions and, indirectly, the role of hormones. The presence of canonical correlations demonstrates that changes in one dataset – for example, bacterial taxa abundances – can predict or explain corresponding patterns in the other dataset, such as metabolite levels, and vice versa.

Discussion

In this study, we utilized FMT to transfer the gut microbiota from conventionally raised mice with disrupted sex hormone homeostasis into germ-free recipient mice in order to assess gut microbiome-driven impact on the HPG axis. Donor mice underwent one of the three types of surgery: sham-operated controls, gonadectomized, and gonadectomized with sex hormone supplementation. Gonadectomy increased circulating gonadotropins (FSH and LH) compared to intact and gonadectomized with sex steroid supplementation. We observed that recipients of altered-sex hormone-associated microbiota showed opposite patterns in gonadotropins and markers of sex hormone production. Lower circulating gonadotropins were observed in FMT male recipients of gonadectomized-associated microbiota as well as increased markers of sex hormone production compared to those received intact-associated microbiota. Cecal microbiota of the recipient mice clustered differently based on the microbiota received, suggesting that altered gut microbial communities influence sex hormone homeostasis, in part, through interacting with the HPG axis. Our data suggest that the gut microbiome may respond to high levels of gonadotropins or low levels of sex hormones in donor mice and relay the signals to alter sex hormone homeostasis when this microbiome is transplanted into germ-free recipient mice. The link between the gut microbiota and other hypothalamic–pituitary axes such as the HPA axis has been reported and much more studied than the HPG axis.[37] The current report highlights a broader role of the gut microbiome in this gut-brain-endocrine regulation.

We assessed differentially abundant taxa within the gut microbiota to determine potential microbes that may be able to impact HPG axis signaling and, ultimately, sex hormone homeostasis. FMT recipients of gonadectomized-associated microbiota had significantly lower proportions of genera belonging to the family *Oscillospiraceae* compared to FMT recipients of intact-associated microbiota. A previous study showed that the administration of 17beta-estradiol to high-fat diet-fed male mice resulted in reduced relative abundance of taxa belonging to the *Oscillospiraceae* family.[38] In another study administering bacteria capable of breaking down androgens to mice, *Oscillibacter* and another uncultured taxon from the same family as *Oscillibacter* (i.e., *Oscillospiraceae*) were predicted to play an integral role in gut microbial androgen catabolism.[39] If *Oscillibacter* and other taxa within the *Oscillospiraceae* family participate in the catabolism of androgens, the lower abundance observed in our FMT recipients of gonadectomized-associated microbiota may contribute to the greater testes weight observed. Additionally, in obese children aged 9–11, the family *Oscillospiraceae* has been negatively correlated with serum cholesterol, a precursor of sex steroids.[40] Though onset of puberty in obese children can occur at earlier ages compared to lean children, it is likely that the HPG axis is not yet active. However, this data provides evidence by which *Oscillospiraceae* may affect sex hormone homeostasis through modulations of availability of this vital precursor molecule.

Male FMT recipients of ORX-M-associated microbiota exhibited significantly lower

Figure 7. Spearman correlations with FDR corrections between top 100 differentially present serum untargeted metabolomics and gut microbiota at the genus level in female gnotobiotic recipients. Red circle denoted positive associations, and blue circle denotes negative associated with the size of the circle indicating the strength (larger circle represents a stronger association). Statistically significant associations ($p< 0.05$) are shown with red box.

proportions of an uncultured genus from the family *Lachnospiraceae* compared to FMT recipients of INT-M-associated microbiota. Interestingly, reductions of some taxa in the family *Lachnospiraceae* have been shown in mouse models of PCOS. In this hyperandrogenic condition, the conversion of testosterone to estrogen is inhibited, leading to higher circulating testosterone and lower estrogens compared to healthy controls.[41] Additionally, sex differences in relative abundance of *Lachnospiraceae* have been reported with male mice having lower proportions of taxa within this family compared to female mice.[42] In humans, *Lachnoclostridium* (from the *Lachnospiraceae* family) has been negatively associated with circulating testosterone.[43] This evidence suggests that some taxa belonging to the genus *Lachnospiraceae* are responsive to sex hormone levels and may play a role in driving alterations in neuroendocrine regulation of sex hormone homeostasis.

Previous work has supported the notion that a primary facilitator of microbial interactions with sex hormones is the activity of bacterial beta-glucuronidase (GUS), which influences the enterohepatic recirculation of sex steroids; however, we did not observe statistical differences in GUS activity across the treatment groups.[44] Thus, we performed untargeted metabolomics in the serum of FMT recipient animals to assess further potential mechanisms of microbial-driven regulation of sex hormone homeostasis and the HPG axis. MST visualization of the global serum metabolome suggested that multiple metabolically unrelated pathways may be involved in the interactions of gut microbiome and sex hormone homeostasis observed in our study. Importantly, many of the metabolites identified to be differentially present are either partially or exclusively produced by gut-associated bacteria. This evidence highlights potential mechanisms of gut microbially derived metabolite's impact on the HPG axis that need to be elucidated in future studies. Recent work has demonstrated that testosterone supplementation can drive changes in the gut microbiome and metabolome in female and male mice.[44] However, the gut microbiome not only responds to the changes of sex hormones but further impacts the sex hormone homeostasis of the host. Understanding these precise microbial-driven mechanisms can illuminate potential features that can be used as therapeutic targets for various sex hormone-sensitive diseases.

At the individual metabolite level, FMT recipients of gonadectomized-associated microbiota had significantly lower circulating indolepropionate compared to FMT recipients of intact-associated microbiota in both males and females. Indolepropionate is a gut microbially derived metabolite generated from bacterial metabolism of tryptophan.[45] Indolepropionate can readily pass through the blood–brain barrier and may have significant impacts on nerve regeneration and sexually dimorphic neurodegenerative brain conditions such as Parkinson's Disease.[46,47] A higher level of indolepropionate has also been linked with lower inflammation and incidences of type 2 diabetes and hyperlipidemia.[48,49] Additionally, indolepropionate has been identified as a key metabolite involved in intermittent fasting interventions aimed at alleviating type 2 diabetes-associated cognitive decline.[50] Furthermore, a recent study demonstrated that indolepropionate exhibits a critical oncoprotective effect by increasing anti-cancer immune responses partially through the aryl hydrocarbon receptor.[51] Interestingly, crosstalk has been previously described between aryl hydrocarbon receptors and estrogen receptors.[52] Hence, differences observed in the present study suggest that the altered-sex hormone-associated microbiota induces changes in circulating indolepropionate that may be important for gut microbial action on gut-brain communication, risk of sexually dimorphic conditions, and immune function.

We found that FMT recipients of gonadectomized-associated microbiota had significantly lower levels of ceramide compared to recipients of intact-associated microbiota and lower sphingomyelin levels. Additionally, ceramide and multiple other sphingolipids were among the first 100 metabolites impacted by microbiota received in both males and females, suggesting that microbiota-driven alterations in sphingolipid metabolism might exist. *In vitro* studies showed that ceramide and bacterial-derived sphingomyelinase, the enzyme responsible for conversion of

sphingomyelin to ceramide, result in inhibition of progesterone synthesis in FSH-treated granulosa cells, cells responsible for steroidogenesis in ovaries.[53] Moreover, ceramide has been shown to trigger cell arrest and apoptosis of granulosa cells, thereby reducing estrogen production.[54,55] In rats, ceramide injection into the testis hindered steroidogenesis and the expression of steroidogenic protein (StAR), an essential protein for cholesterol movement into the mitochondria for steroid synthesis.[56] Further studies showed that treating Leydig cells with ceramide inhibits testosterone secretion, potentially by reducing expression of StAR proteins.[35,57] Importantly, some gut-associated microbes can produce sphingolipids and contribute these sphingolipids to host circulating levels.[58]

In male FMT recipients, caproate, also known as caproic acid, is a six-carbon medium chain fatty acid that was positively correlated with *Erysipelatoclostridiaceae* and negatively correlated with *Lachnoclostridium* in this study. Interestingly, caproate can be produced from anaerobic fermentation and has been reported to be produced by a cluster of *Clostridium* species co-cultures.[59,60] Supplementation of an eight-carbon medium chain fatty acid, similar to caproate, has previously been associated with increasing steroidogenesis and improved reproductive function in sows.[61] Additionally, caproate has been suggested to be neuroprotective in sexual dimorphic neurodegenerative conditions, such as Parkinson's disease, as it can easily pass through the blood–brain barrier.[62] However, the impact of caproate and the HPG axis has not been reported.

Greater circulating butyrate levels were observed in both male and female FMT recipients of gonadectomized-associated microbiota compared to those of intact-associated microbiota. Butyrate, a short-chain fatty acid, is exclusively produced by the gut microbiota. In an *in vitro* study, butyrate was demonstrated to be capable of increasing steroidogenesis in rat ovarian granulosa cells.[63] Further, dietary sodium butyrate supplementation in roosters has improved semen quality, sperm motility, and increased circulating testosterone.[64] Similar results have been reported in boars, whereby supplementation of inulin and cellulose resulted in increased short-chain fatty acids and improved semen quality, sperm motility, and sperm viability – reproductive factors regulated by the HPG axis.[65] Together, this evidence suggests that increased butyrate produced from the gut microbiota alters sex hormone homeostasis and impacts HPG axis signaling. Further, shifts in the gut microbiota driven by dietary changes may alter reproductive function through butyrate actions on sex hormone homeostasis.

Despite the strengths of our study, there are limitations to consider, as is the case with all research. First, sex steroid supplementation did not fully restore circulating gonadotropin levels to those of intact controls in all mice, leading to increased variability in the data. Although the dosages of estradiol and testosterone were selected based on published literature, pharmacokinetic studies suggest that subcutaneous hormonal pellet release can decline over time, which may have resulted in lower-than-expected systemic sex steroid levels in our animals.[66] Future studies using this model should address this variability by incorporating either larger sample sizes to enhance statistical power or higher dosages of hormonal pellets to maintain consistent systemic levels. Second, we were only able to assess beta-glucuronidase activity due to limited cecal content size from mice, limiting the scope of our enzymatic analysis. Other microbial enzymes, such as sulfatases, reductases, and oxidases, are also likely to play significant roles in modulating sex steroid metabolism and activity.[67,68] Future investigations should examine a broader range of microbiota-derived enzymatic activities, particularly those involved in steroid metabolism, to provide a more comprehensive understanding of the regulatory roles of gut microbiome on systemic sex steroid levels and their downstream effects on host physiology.

In summary, our study demonstrates that gut microbiota modulates sex hormone homeostasis and interacts with the HPG axis. We observed gut microbiota-driven differences in HPG axis hormone signaling in both male and female recipients, indicating the critical role of the gut microbiome in reproductive physiology. Our data indicate that gut microbiota may influence sex hormone homeostasis through multiple metabolic pathways, providing insights for future research aimed at identifying

therapeutic targets for sex hormone-sensitive conditions such as infertility, some forms of cancer, and cardiometabolic disease.

Methods

Ethical approval

Procedures performed in this study were approved by the Institutional Animal Care and Use Committee at Purdue University (protocol # 1909001951). All mice were euthanized using methods approved for humane euthanasia for the species, stage of development, and size. Mice were euthanized using carbon dioxide asphyxiation.

Animals and experimental design

Donor Mice: 8-week-old C57BL/6J mice were purchased from Jackson Laboratory (Bar Harbor, ME) and fed a double irradiated semi-purified diet formulated based on Research Diets D12450J with the following modifications: (1) replacing soybean oil with corn oil to eliminate the inclusion of phytoestrogens, (2) replacing lactic casein with mineral acid casein to eliminate contaminations of lactic acid-producing bacteria utilized during processing, and (3) inclusion of 1.5X vitamin mixture to account for nutrient loss during irradiation (Research Diets, New Brunswick, NJ). One week after acclimation to the diet, sex hormone status of mice was altered through gonadectomy and subcutaneous sex steroid pellet implantation, resulting in six groups: (1) intact male controls (INT-M, $n = 13$); (2) orchiectomized males (ORX-M, $n = 13$); (3) orchiectomized males with testosterone (12.5 mg/pellet 60-day release, Innovative Research of America, Sarasota, FL) supplementation (ORX+T-M, $n = 10$) (4) intact female controls (INT-F, $n = 7$); (5) ovariectomized females (OVX-F, $n = 8$); (6) ovariectomized female with 17Beta-estradiol (0.1 mg/pellet 60-day slow release, Innovative Research of America, Sarasota, FL) supplementation (OVX+E-F, $n = 8$); Dosages of the sex hormone pellets were selected to induce circulating concentrations of physiological relevant levels.[22,69] Briefly, orchiectomy was performed by incision on the ventral side of the scrotum and cremaster muscle wall. The testicular fat pad and testicles were externalized through the incision and the testicles were removed via cauterization. Ovariectomy was performed under isoflurane anesthesia by vertical incision through the skin and muscle layer above the ovarian fat pad followed by externalization of the ovary and removal of the ovary by cauterization. Wounds were closed with sterile wound clips. Intact controls underwent sham surgeries and both intact controls and gonadectomized mice received a subcutaneous placebo pellet during surgical anesthesia. After surgical intervention, FMT donor mice were single housed until euthanasia. Eight weeks after HPG axis alterations, mice were fasted for 4 h, euthanized, and blood was collected via cardiac puncture in addition to fecal and cecal samples. Blood was allowed to clot at room temperature for 30 min then placed on ice until serum was collected after centrifugation at 2000 g for 15 min at 4°C.

Fecal microbial transplant in gnotobiotic mice: Within-group fecal samples were pooled and suspended in 5 mL anaerobic sterile PBS. These fecal slurries were then used to colonize 6-week-old germ-free C57BL/6J mice via FMT that were sex matched to donor animals. This resulted in six FMT recipient groups: INT-M FMT recipient ($n = 8$); ORX-M FMT recipient ($n = 8$); ORX+T FMT recipient ($n = 9$); INT-F FMT recipient ($n = 7$); OVX-F FMT recipient ($n = 6$); and OVX+E FMT recipient ($n = 8$). Colonized and germ-free control mice were house 3–4/cage in positive pressure vent rack cages (Allentown, NJ) to maintain sterility. Four weeks after colonization, mice were fasted for 4 h, euthanized, and blood was collected via cardiac puncture in addition to relevant tissue and cecal samples. Age and sex-matched germ-free controls were included for gnotobiotic recipient mice. All mice were euthanized within a 4-h time-period of the day to reduce hormonal variability associated with circadian rhythm. Female mice were not monitored for estrus stage prior to euthanasia due to risk of contamination that may occur during vaginal lavage.

Circulating gonadotropins and intra-gonadal sex hormones

Concentration of gonadotropins, serum follicular-stimulating hormone (FSH) and luteinizing hormone (LH), in both microbiota donor and recipient mice were assessed using a multiplex Mouse

Pituitary Magnetic Bead Panel (EMB Millipore, Burlington, MA) with a reportable detection range of 0.48–300.0 ng/mL of FSH and 0.24–30 ng/mL of LH by the Ligand Core Laboratory at The University of Virginia.

Intra-gonadal estrogen and testosterone were determined in FMT recipients to assess sex hormone status. In brief, ovaries or testicles were homogenized in 100 µl of PBS and 40 µl of 0.1 mol/L HCl. Homogenate was then mixed with 260 µl ethyl acetate and isopropanol (mixed 1:1 volume) and centrifuged at 2000 g for 5 min. The ethyl acetate phase was transferred to a new tube and ethyl acetate was evaporated. Hormones were then resuspended in zero standard steroid-free serum and stored at −80°C until further use. Testosterone was assessed via ELISA with a detection range from 0.083 ng/mL to 16.0 ng/mL and estradiol was assessed via an estradiol sensitive ELISA kit with a detection range from 5.583 pg/mL to 400 pg/mL (DRG International, Marburg, Germany).

Untargeted metabolomics

Discovery-based untargeted metabolomics was performed by Metabolon Inc. on a subset of the FMT recipients ($n = 6$/group) to measure serum metabolites. Ultrahigh-performance liquid chromatography-tandem mass spectroscopy (UPLC/MS/MS) and gas-chromatography-mass spectroscopy (GC-MS) were utilized. Samples were prepared using the MicroLab STAR system (Hamilton Company, Franklin, MA). After addition of internal standard to the samples, metabolites were recovered by precipitating proteins under methanol with shaking (Glen Mills GenoGrinder, Clifton, NJ) and centrifugation. The extract was dried and reconstituted in solvents compatible with reverse-phase UPLC-MS/MS with positive mode electrospray ionization (ESI), reverse-phase UPLC-MS/MS with negative ion mode ESI, and HILIC/UPLC-MS/MS with negative ion mode ESI. Internal standards at fixed concentrations were added to ensure chromatographic consistency. Data was extracted, peaks identified, and QC processed. Compounds were identified by comparison to library entries of purified standards or recurrent unknown compounds based on retention time, mass-to-charge ratio, and chromatographic data. Peaks were quantified using the area under the curve.

DNA extraction and 16S rRNA sequencing

Genomic DNA was extracted from cecal content using a bead-beating protocol.[70] Briefly, cecal content was suspended in a solution containing 500 µL of extraction buffer (200 mm Tris (pH 8.0), 200 mm NaCl, 20 mm EDTA), 210 µL of 20% SDS, 500 µL phenol:chloroform:isoamyl alcohol (pH 7.9, 25:24:1, Invitrogen, Carlsbad, CA), and 1.2 g of 0.1 mm diameter zirconia/silica beads. Samples were mechanically disrupted using a bead beater (BioSpec Products, Bartlesville, OK) maximum setting for 3 min at room temperature, followed by centrifugation at 7,200 g for 3 min at 4°C to separate phases. Nucleic acids in the aqueous phase were precipitated by the addition of isopropanol and sodium acetate. Following pelleting and evaporation of the isopropanol solution, nucleic acids were resuspended in sterile 10 mm Tris/HCl (pH 8.0) and 1 mm EDTA. QIAquick 96-well PCR Purification Kit (Qiagen, Germantown, MD) was used to purify extracted DNA and remove contaminants. Isolated DNA was eluted into sterile 5 mm Tris-EDTA buffer and stored at −80°C until further use.

PCR was performed using universal primers flanking the hypervariable 4 (V4) region of the bacterial 16S rRNA gene. Each reaction contained 25 ng of genomic DNA, 10 µM of each uniquely barcoded primer, 12.5 µl 2X HiFi HotStart ReadyMix (KAPA Biosystems, Wilmington, MA), and water with a final reaction volume of 25 µl. PCR was carried out under the following conditions: denature for 3 min at 95°C, followed by 25 cycles of denaturation for 30 s at 95°C, annealing for 30 s at 55°C and elongation for 30 s at 72°C, and a final elongation step for 5 min at 72°C. The quality of the PCR products was assessed by electrophoresis with 1.5% agarose gels and purified using the QIAquick 96-well PCR Purification Kit (Qiagen, Germantown, MD). Quantification of the PCR products was performed using a Qubit dsDNA Broad Range Assay kit (Invitrogen, Carlsbad, CA). The final pooled libraries were constructed by combining the PCR products in

equimolar amounts. The final pooled libraries were sequenced at the Bindley Bioscience Center at Purdue University using the v2 chemistry of the Illumina MiSeq platform (Illumina, San Diego, CA) to generate 2 × 250 bp pair-end reads.

Cecal GUS activity

Frozen cecal samples collected at the time of euthanasia were mixed with 350 µl assay buffer (100 mm HEPES, 250 mm NaCl, pH 6.0). Bacterial cells were lysed using TissueLyser II (Qiagen, Germantown, MD) for 2 min at 30 hz. Homogenates were sonicated for 3 min at 4°C, then centrifuged for 10 min at 15,000 RCF. Enzyme kinetics were determined in supernatant by monitoring the change in absorbance of 4Methylumbelliferyl-beta-D-glucuronide (4MUG) (Sigma, Burlington, MA). Reactions were conducted in triplicates with blanks as negative controls and purified bacterial beta-glucuronidase as positive controls (Sigma, Burlington, MA). The 50 µl reaction volume consisted of 5 µl cecal homogenate, 20 µl assay buffer, and 25 µl 4MUG (final concentration of 1 mm). Enzyme activity was measured every 1 min for 75 min at 37°C. Initial velocity of each sample was calculated using a custom MATLAB script, which was then normalized to the total protein concentration from the clarified extractant using a BCA protein assay kit (ThermoFisher Scientific, Waltham, MA).

Data analysis

Metabolomics: To remove variability caused by batch, each metabolite's area under the curve was normalized with respect to each instrument batch (batch-normalized) by dividing by the batch median. The minimum value across all batches in the batch-normalized data was imputed for missing values per metabolite. For each sample, the batch-normalized data was divided by the value of the volume (volume-normalized) and re-scaled to have a median of one by dividing the new values by the overall median for each metabolite. This resulting batch and volume-normalized-imputed data was then transformed using natural log resulting in log-normal distribution.

Principal coordinate analysis (PCA) was used to assess the largest contributors to data variance. Additionally, further PCA was performed in a guided setting using a limited subset of features identified by elastic-net feature selection associated with FMT recipient groups. A partial correlation matrix was created utilizing data from both female and male global metabolomic datasets and a minimum spanning tree (MST) was developed to better understand the relationships among metabolites. The networks and the MST were constructed using igraph package for R.[71] Feature selection was conducted utilizing an elastic net regularization logistic regression model, as implemented in the glmnet package in R.[72]

To construct heatmaps of serum global metabolomics, identified metabolites to be included in the analysis were determined by performing ANOVA in female and male groups separately and sorting the p-values from lowest to highest. The first 100 that were statistically different among treatment groups were chosen. Heatmaps of z-scores with hierarchical clustering via complete linkage algorithm were then constructed using Pheatmap package in R.[73] Individual metabolite differences were further assessed using direct contrast statistics to compare recipients of intact-associated microbiota against controls. Effect size was also computed by Cohen's d to quantity the strength of the observed biological effects.

16S rRNA Gene Sequencing: Sequences were processed using Quantitative Insights into Microbial Ecology (QIIME) 2 pipeline (2019.1).[74] Briefly, demultiplexed paired-end sequences were imported using Casava 1.8 format and denoised using DADA2[75] to obtain an amplicon sequence variant (ASV) table. ASV present less than five times per sample and in less than four samples were discarded. A naive Bayes taxonomy classifier was trained on the SILVA[76] reference database (clustered at 99% similarity). This classifier was used to assign taxonomy to ASVs.[77] An even sampling depth (sequences per sample) of 7800 sequences per sample was used for assessing alpha- and beta-diversity measures. Beta diversity was assessed using principal coordinates analysis (PCoA) of unweighted UniFrac, weighted UniFrac, Bray-Curtis, and Jaccard distance metrics. Alpha diversity was assessed using observed ASV and Shannon indices. Distances between treatment groups were tested by pairwise

PERMANOVA.[78] Differential abundance was performed by Kruskal–Wallis, followed by Dunn's post-hoc test.

Co-occurrence networks were generated from a partial correlation adjacency matrix representing the relationship between identified bacterial taxa in each sex including all FMT recipient groups. The co-occurrence network directly describes the adjacency network; however, the network was trimmed by removing some edges (small-weighted connections between nodes, correlation value minimums >0.6) in order to improve visual interpretation. Further, MST was constructed to simplify the network. An MST in this context is the subset of edges that connects all metabolites with the minimum total edge weight, without forming and without forming cycles. The MST represents the most essential relationships between metabolites based on their abundance patterns across conditions.

Phenotypic data: Data analysis was performed using R language for statistical computing, and graphs were generated using Prism version 9.4.1 (GraphPad Software, San Diego, CA).[79] Outliers were assessed using the 1.5 interquartile range (IQR) method. Statistical differences were evaluated using direct contrast statistics, used to test treatment (gonadectomized FMT donors and recipients) against controls (intact and gonadectomized with respective sex steroid supplementation). P-values were adjusted using the Sidak correction to account for multiple testing and control the family-wise error rate. Additionally, effect sizes were calculated using Cohen's d to quantify the magnitude of the observed biological effects, providing further context to the statistical significance. Trending significance was considered at $p < 0.1$, and the statistical significance was set as $p < 0.05$.

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Disclosure statement

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Data availability statement

The raw sequences that support the findings of this study are available at the NCBI sequences read archive at https://www.ncbi.nlm.nih.gov/sra/, reference number BioProject PRJNA1156752.

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