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RESEARCH

# Sex hormones, sex chromosomes, and microbiota: identification of *Akkermansia muciniphila* as an estrogen-responsive bacterium

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## Abstract

Microbiota composition is known to be linked to sex. However, separating sex hormones and sex chromosome roles in gut microbial diversity is yet to be determined. To investigate the sex chromosome role independent of sex hormones, we used the four-core genotype mouse model. In this mouse model, males with testes and females with ovaries have XX or XY sex chromosome complement. In gonadectomized four-core genotype mice, we observed a significant decrease in the levels of estradiol ($P < 0.001$) and progesterone ($P < 0.03$) in female and testosterone ($P < 0.0001$) in male mice plasma samples. Independent of sex chromosome complement, microbial α diversity was increased in gonadectomized female but not male mice compared to sex-matched gonad-intact controls. β diversity analysis showed separation between male ($P < 0.05$) but not female XX and XY mice. Importantly, *Akkermansia muciniphila* was less abundant in gonadectomized compared to gonadal intact female mice ($P < 0.0001$). In the presence of β-estradiol, *A. muciniphila* growth exponentially increased, providing evidence for the identification of a female sex hormone-responsive bacterium ($P < 0.001$).

**Keywords**

- sex
- hormones
- chromosomes
- microbiota
- *Akkermansia muciniphila*

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## Introduction

Sex-specific determinants of health are increasingly recognized as essential contributors to gender-based medicine (Clayton & Collins 2014, Mauvais-Jarvis *et al.* 2020). From a genetic perspective, sex chromosomes are determinants of sex, but from a developmental perspective, sex hormones are determinants of both sex and gender (Mauvais-Jarvis *et al.* 2020). Dissection of the role of sex hormones and sex chromosomes is critical in the development of gender-based medicine, including hypertension and arterial stiffening (Ogola *et al.* 2018, Reue & Wiese 2022).

Gut microbiota plays a vital role in maintaining the homeostasis of the gastrointestinal tract and the host through nutrient digestion, drug metabolism,

and immunomodulation (Wu & Wu 2012, Shimada *et al.* 2022). Decrease in microbial diversity is linked to many disorders and conditions, including metabolic syndrome (Dabke *et al.* 2019). Sex differences in human and rodent microbial composition are documented in cardiovascular disease *Lactobacillus* is shown to be greater in women, providing protective effects than in men (Shastri *et al.* 2015, Razavi *et al.* 2019, Sinha *et al.* 2019, Virwani *et al.* 2023). Sex difference based on sex steroids estradiol and testosterone has shown distinctive impacts on gut microbes with increased Firmicutes–Bacteroidetes ratio in women than in men (Shastri *et al.* 2015). However, the impact of sex chromosomes on shaping the composition of gut microbiota remains unexplored.

Experimental designs for testing genetic contributions are performed by comparing female and male subjects. In contrast, sex hormonal effects is assessed by comparing experimental gonadectomized animals with control animals without gonadectomy or by comparing aged with young human subjects (Horstman *et al.* 2012, Mauvais-Jarvis *et al.* 2020). In the present study, we exploit the four-core genotype (FCG) mouse model wherein gonadal sex is not determined by the sex chromosomes but by the presence or absence of *Sry*, a testis-determining gene established during male development (Arnold 2020). Therefore, XX and XY mice with the *Sry* gene develop testes and are males (XX$^{Sry}$M and XY$^{-Sry}$M). XX and XY mice without the *Sry* gene develop ovaries and are females (XXF and XY-F). These four genotypes allows the use of this model to study the differences in phenotypes caused by sex chromosome complement (XX vs XY), the differential effects of ovarian and testicular hormones, and the interactive effects of sex chromosomes with the sex hormonal effects.

Bilateral gonadectomy impacts gut microbial composition regulated by estrogen and testosterone, indicated by the abundance of *Akkermansia* and *Ruminococcaceae* in female and male mice (Org *et al.* 2016). In a population study, *Akkermansia* is shown to be more abundant in women than men (Naito *et al.* 2018). Additionally, *Akkermansia* is vital in mitigating diet-induced obesity in mice (Everard *et al.* 2013). Whether *Akkermansia* is impacted by sex hormones, or the interaction of sex hormones and chromosomes is unknown. Therefore, dissecting the magnitude of genetic and hormonal sex-conferring mechanisms is needed. Here, we examine the microbiota composition of the FCG model. By superimposing gonadectomy, we further investigate the relative effects of genetic vs sex hormones on their abilities to reshape gut microbiota composition.

## Material and methods

### Animals

Four-core genotype (FCG) mice on the C57BL/6J background were used. Studies were conducted in mice between 15 and 16 weeks old. Male (M) mice XY$^{-Sry}$ acquired from Dr Franck Mauvais-Jarvis (Tulane University) were bred with female (F) breeders (XX) on C57bl/6J (RRID: IMSR_JAX:000664) background purchased from JAX (Bar Harbor, ME, USA). Breeding XY$^{-Sry}$ and XX mice produced the four-core genotypes: XXF, XX$^{Sry}$M, XY$^{-}$F, and XY$^{-Sry}$M. Mice were genotyped using the following primers to PCR amplify the *Sry* locus: transgene forward primer: 5′-AGC CCT ACA GCC ACA TGA TA-3′, transgene reverse primer: 5′-GTC TTG CCT GTA TGT GAT GG-3′, forward: 5′-CTG GAG CTC TAC AGT GAT GA-3′, reverse: 5′-CAG TTA CCA ATC AAC ACA TCA C-3′, internal positive control forward: 5′-CAA ATG TTG CTT GTC TGG TG-3′ and internal positive control reverse: 5′-GTC AGT CGA GTG CAC AGT TT-3′. All mice were housed together as either males or females independent of their sex chromosome complement and maintained in a temperature-controlled vivarium under a 12-h darkness 12-h light cycle with free access to standard chow (PicoLab® Rodent Diet 20 #5053) and drinking water. Animal experiments were in accordance with the NIH Guide for the Care and Use of Laboratory Animals approved and monitored by the Augusta University Institutional Animal Care and Use Committee.

### Gonadectomy

Mice were ovariectomized or castrated at 8 weeks of age followed by 8 weeks to clear endogenous sex hormones. All procedures followed aseptic techniques. Briefly, mice were placed under a heating pad with 3–4% isoflurane–oxygen mixture, eye ointment was applied, and hair was shaved around the incision site. Alcohol (70%) and betadine were used to clean incision sites. This was followed by administration of buprenorphine 0.1 mg/kg. Ovariectomy was performed by cutting a small incision on both sides of the mouse hip below the rib cage. Ovaries were located above the oviduct on the uterine horn and excised. Castration was performed by locating the scrotum and making a small midline cut above the bladder followed by excision of testicles. Muscle tissue was sutured with absorbable suture and the skin was stapled. Mice were left on heating pads to recover and monitored for pain and stress. Awake and alert mice were placed back in their cages with daily monitoring postsurgical monitoring for wound healing and distress.

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## Fecal matter collection

All fecal matter were collected in the morning between 2 and 5 h into the darkness cycle (8:00–11:00 h; CST). Mice were placed in an empty clean cage without bedding and allowed time to roam the cage and deposit fecal matter at will. Two pellets were collected and frozen immediately for 16S rRNA gene sequencing. These frozen samples were shipped to the University of Toledo Microbiome Core for further processing. A total of six to eight mice were used for each group analysis.

## 16S rRNA gene sequencing and analysis of microbiota composition

### 16S PCR library preparation, cleanup, normalization, and pooling

QIAamp Power Fecal Pro DNA kit (QIAGEN) was used to extract gDNA from the fecal pellets (~40–50 mg) from XXM, XYM, XXF, and XYF (intact and gonadectomized) mice. The gDNA was eluted in low TE buffer (0.1 mM EDTA, Tris–HCl buffer, 10 mM, pH 8.5) instead of the AE buffer provided in the kit. DNA concentration was determined using a NanoDrop and samples were diluted to a final concentration of 5 ng/μL in low TE buffer. The Illumina User Guide was followed: '16S Metagenomic Sequencing Library Preparation-Preparing 16S Ribosomal RNA Gene Amplicons for the Illumina MiSeq System (Part # 15044223 Rev. B).' The 16S rRNA gene targeting the V3-V4 region was amplified by PCR using the Illumina sequencing primers: 5'-TCGTCGGCAGCGTC AGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG and 5'-GTCTCGTGGGCTCGGAGATGTGTATAAGAGAC AGGGACTACHVGGGTWTCTAAT. For index PCR, the Nextera XT Index Kit (FC-131-1002) from Illumina was used to attach dual indices. Each 25 μL reaction mixture contained 2.5 μL of 10X reaction buffer (Invitrogen, Thermo Fisher Scientific, Waltham, MA), 0.5 μL of 10 mM dNTPs, 0.75 (for target PCR)/1 μL (for index PCR) of 50 mM $MgCl_2$, 0.1 μL of 5 U/μL of HotTaq polymerase (Invitrogen), 1 μL of each primer (5 μM), and 2.5 μL of 5 ng/μL DNA. All samples were reconstituted in water for a final volume of 25 μL. Thermocycling was performed in a Bio-Rad T100TM thermal cycler and the cycling conditions were as follows: initial denaturation at 95°C for 5 min, followed by 25 cycles of 95°C for 30 s, 58°C for 30 s, 72°C for 30 s, and a final extension at 72°C for 5 min for target PCR. Index PCR was carried out in eight cycles, with an initial denaturation at 95°C for 3 min, followed by 95°C for 30 s, 55°C for 30 s, 72°C for 30 s, and a final extension at 72°C for 5 min. Each PCR amplicon sample was purified in two rounds using AMPure XP beads (Beckman Coulter Inc. Brea, CA, USA). Each concentration of purified index PCR products was measured using the Qubit dsDNA HS Assay kit with Qubit 3.0 fluorometer (Life Technologies). The 4 nM of each amplicon was pooled equally. Pooled library was checked for quality using a 2100 Bioanalyzer (Agilent) before sequencing. Library Denaturing and MiSeq Sample Loading was done according to Illumina User Guide Illumina MiSeq System. The 10 pmol/L denatured and diluted library with 10% PhiX was loaded on an Illumina MiSeq V3 flow cell kit with 2 × 300 cycles.

### Quality filtering, ASV picking, and data analysis

Chimeric sequences were identified and filtered using Quantitative Insights in Microbial Ecology (QIIME II) software package (version 2021.11) with custom scripts for analyzing all samples and for filtering groups based on comparisons. All samples were rarified at 30,500 sequencing reads. The reads were denoized, merged, and chimera filtered with Divisive Amplicon Denoising Algorithm 2 resulting in an amplicon sequence variants (ASV) table (Callahan *et al.* 2016). The taxonomic assignments of ASVs representative sequence were performed with a naive Bayes classifier, which was trained on the SILVA database (version 138.1). Bray–Curtis principal coordinate analysis (PCoA) was performed using the previously reported method (Lu *et al.* 2023). Sankey diagram was generated using the open resource at OmicStudio (https://www.omicstudio.cn).

## Measurement of steroids and statistical analysis

While mice were under isoflurane anesthesia, blood was collected by intracardiac puncture and drawn into a syringe prefilled with 50 μL of 0.5M pH 8.0 EDTA. The samples were centrifuged for 10 min at 1000 ***g***, and plasma supernatant was collected and immediately stored at −80°C. The samples were shipped to Creative Proteomics (Shirley, NY, USA) for steroids analysis using electron spray ionization liquid chromatography–mass spectrometry. Steroids including Δ4, Δ5, 5α-reduced, and conjugated steroids sulfates were assessed. Data were quantitated as ng/mL in plasma.

## Bacterial culture

*Akkermansia muciniphila* BAA-835 was procured from the American Type Culture Collection. This bacterium

was grown in an anaerobic chamber (Bactron, Sheldon Manufacturing, Inc., Cornelius, Oregon, USA) in 15 mL tubes containing yeast casitone fatty acid broth with carbohydrates (YCFAC BROTH; Anaerobe System, Thermo Fisher Scientific) at 37 °C, without shaking. The cultured *A. muciniphila* was confirmed by whole genome sequencing at CD-genomics (Shirley, NY). Eighteen-hour-old culture was used to inoculate YCFAC broth with or without β-estradiol-water soluble (Sigma-Aldrich) to evaluate its effect on *A. muciniphila* growth. Bacterial growth was monitored at different time intervals by recording the optical density (O.D) at 600 nm with a microplate reader (Molecular Devices, San Jose, CA, USA). Concomitantly, the intact cell count was measured using BactoBox (SBT Instruments, Herlev, Copenhagen, Denmark).

### Statistical analysis

Data was analyzed using GraphPad Prism version 9.1 (GraphPad Software). Outliers were identified by ROUT method (Q=1%). Two-way ANOVA was used to compute main effect (sex hormone, sex chromosome, and interaction) followed by Sidak's multiple comparisons test to determine the difference between groups (XX vs XY and gonadal intact versus gonadectomy). Each dot on the bar graph represents the number of samples. $P < 0.05$ was considered significant.

## Results

### Gonadectomy-mediated decline in sex steroids of female and male mice

A gonadectomy was performed to eliminate circulating sex steroids. Gonadal intact females, compared to gonadectomized (GDX) mice, had a significant decrease in estradiol (Fig. 1A; $P < 0.001$). Intact vs GDX mice (XX: 0.17 vs 0.15 ng/mL; $P=0.03$) and (XY: 0.17 vs 0.14 ng/mL; $P=0.02$). There was no significant difference in plasma testosterone (Fig. 1B; $P=0.06$), but increased levels were noted in XYF ($P=0.04$). Similarly, aldosterone was not significantly impacted by ovariectomy (Fig. 1C; $P=0.2$), but gonadectomized XXF had higher levels than XYF ($P=0.03$). Significant decrease in 11-deoxycorticosterone (Supplementary Fig. 1A, see section on supplementary materials given at the end of this article; $P < 0.006$) was indicated in ovariectomized XXF (7 vs 3 ng/mL; $P=0.02$) and XYF (6 vs 2 ng/mL; $P=0.04$) mice. Ovariectomy revealed that 11-deoxycortisol (Supplementary Fig. 1B) was decreased in XXF (5 vs 3 ng/mL; $P=0.02$), while corticosterone (Supplementary Fig. 1C) was reduced in XYF mice (5 vs 3 ng/mL; $P=0.03$). There was no significant difference in dehydroepiandrosterone (DHEA) (Supplementary Fig. 1D; $P < 0.001$). However, the gonadectomy effect was indicated with increased Pregnenolone levels (Supplementary Fig. 1E; $P=0.01$),

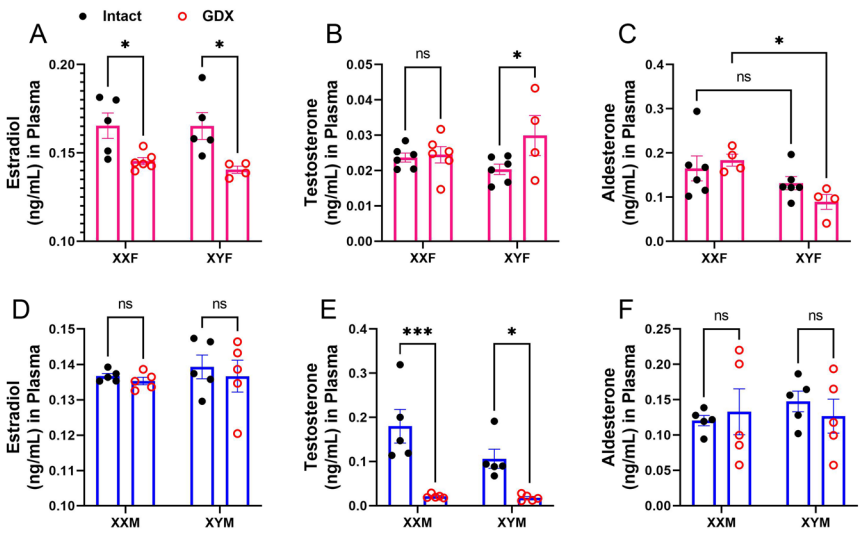


**Figure 1**
Plasma steroid analysis of gonadal intact and gonadectomized mice. Females: (A) Estradiol was significantly decreased by ovariectomy ($P < 0.001$) independent of sex chromosome complement. (B) The main effect of ovariectomy did not impact testosterone levels, but an increase was noted in XYF ($P = 0.04$). (C) There was an overall sex chromosome but not sex hormone effect in Aldosterone levels greater in XXF than XYF ($P < 0.01$). (D) Castrated male mice indicated no significant difference in estradiol levels ($P = 0.5$). (E) However, testosterone was significantly decreased in castrated mice ($P < 0.0001$) independent of the mice genotype. (F) There was no significant effect of castration on aldosterone levels ($P = 0.9$). Data were analyzed as two-way ANOVA to test for the main effect (sex hormone and chromosome), and Sidak's multiple comparisons test was used for the difference between groups. For each group, the dot plot bar graphs indicate sample size ($n = 4$–6 samples). Error bars depict standard error mean. $^{*}P < 0.05$ and $^{***}P < 0.001$.

while progesterone (Supplementary Fig. 1F; $P < 0.001$) was significantly decreased in XXF (0.5 vs 0.3 ng/mL) and XYF (0.5 vs 0.2 ng/mL) mice. Increased levels of testosterone and tetrahydroxyaldosterone (Supplementary Fig. 1G and H) were indicated in XYF mice (0.02 vs 0.03 ng/mL; $P=0.04$) and (0.5 vs 0.8 ng/mL; $P=0.04$), respectively. Tetrahydroxy 11-deoxycortisol (Supplementary Fig. 1I) was not impacted by gonadectomy; however, decreased dehydroepiandrosterone sulfate (DHEAS) was indicated with the elimination of sex hormones (Supplementary Fig. 1J; $P=0.04$). Estriol (Supplementary Fig. 1K) was increased in ovariectomized XYF mice (0.13 vs 0.16 ng/mL; $P=0.03$).

Orchiectomy in male mice indicated no difference in estradiol (Fig. 1D; $P=0.5$), but a substantial decrease in testosterone (Fig. 1E; $P < 0.0001$) was noted in (XX: 0.18 vs 0.02 ng/mL; $P < 0.0002$) and (XY: 0.11 vs 0.02 ng/mL; $P=0.02$) mice. Castration increased 11-deoxycorticosterone in XXM ($P=0.02$), but not XYM mice (Supplementary Fig. 2A). There was no difference observed in aldosterone (Fig. 1F), 11-deoxycortisol, corticosterone, DHEA, and pregnenolone (Supplementary Fig. 2B, C, D, and E; $P > 0.05$). However, progesterone (Supplementary Fig. 1F) was decreased in XYM mice (0.9 vs 0.2 ng/mL; $P=0.02$). A significant increase in tetrahydroxy 11-deoxycortisol (Supplementary Fig. 2G) was indicated in XXM (1.6 vs 4.0 ng/mL; $P < 0.0001$) and XYM (1.6 vs 3.6 ng/mL; $P < 0.001$) mice. However, no substantial changes were noted in tetrahydroxy aldosterone, DHEAS, estradiol, and estriol (Supplementary Fig. 2H, I, J, and K; $P > 0.05$), respectively.

## Gut microbiota was not significantly impacted by sex chromosomes in female mice

Next, the gut microbial composition was evaluated in all eight groups of mice (gonadal intact and gonadectomized male and female mice with either XX or XY sex chromosome complement). Bray–Curtis principal coordinate analysis (PCoA), which is a measure of distance in the bacterial communities between groups, was significantly different between intact male (XY) and intact female (XX) groups (Fig. 2A). Interestingly, there was a trending difference between XY and XX males (Fig. 2B). However, no difference was found between XY and XX females (Fig. 2C). The data suggested that sex chromosomes impacted microbiota composition in males while sex hormones affected females.

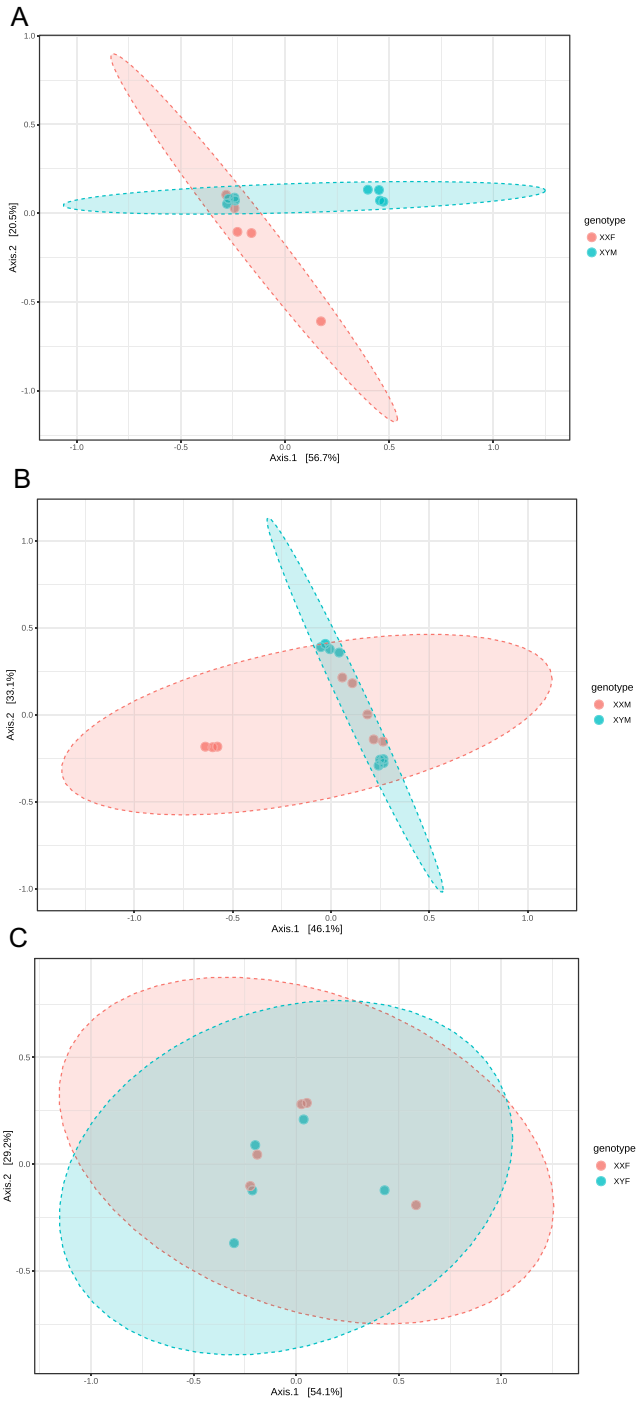


**Figure 2**
Bray–Curtis PCoA analysis of β-diversity of the gut microbiota. (A) Comparison between female intact and male intact, ANOSIM $R = 0.266$, $P < 0.045$. (B) Comparison between male XY and XX ANOSIM $R = 0.157$, $P < 0.057$. (C) Comparison between female XY and XX ANOSIM $R = -0.12$, $P < 0.783$.

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## Gonadectomy increased α-diversity of the gut microbiota in female mice

Next, we evaluated the changes in α-diversity of the gut microbiota in the gonadectomized mice. Remarkably, all four parameters (i.e., richness, evenness, observed taxon, and diversity) were higher in the female gonadectomized mice than in the female intact mice independent of the sex chromosome (Fig. 3A, B, C, and D). This data suggested that female sex hormones impacted the gut microbiota more than sex chromosomes.

## Gonadectomy decreased *Akkermansia* in female mice

We focused on the microbial analysis in the female mice to understand which bacterial taxa were mainly impacted by gonadectomy. Interestingly, we did not observe significant differences in PCoA between intact and gonadectomized female mice with X or Y chromosomes (Supplementary Fig. 3A and B). The Sankey diagram was generated to visualize the significant alterations in bacterial communities at different taxonomic levels. Major shifts in bacterial composition in Verrucomicrobia were found in the female gonadectomized mice, regardless of sex chromosomes complement (Fig. 4A). Additional phyla alterations are indicated in Sankey diagrams of major gut microbiota, including Firmicutes, Bacteroidetes, Proteobacteria, and Actinobacteria (Fig. 4B, C, D, and E), respectively. Analysis of bacterial composition at the genus level showed that *Akkermansia* was significantly reduced in the gonadectomized XX and XY female mice (Table 1). This data suggested that a female sex hormone was positively associated with the abundance of *Akkermansia*. In male mice, there were no significant differences in PCoA between gonadal intact and castrated mice independent of their sex chromosome complement (Supplementary Fig. 4A and B). However, bacterial composition at the genus level indicated *Alloprevotella,* uncultured organism, *Rikenella,* and *Blautia* enrichment in XYM, while *Candidatus Arthromitus, Romboutsia,* and *Turicibacter* were abundant in XXM gonadal intact mice (Supplementary Table 3 and 4).

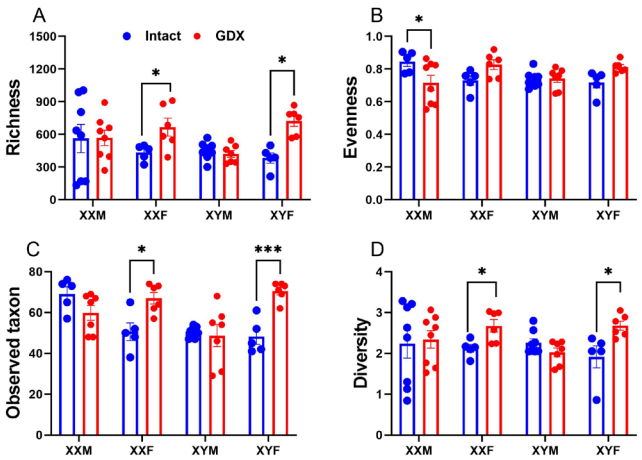


**Figure 3**
Microbial analysis of α-diversity. All groups of samples were analyzed by 16S sequencing. (A) There was an overall effect of gonadectomy ($P = 0.01$) with a significant impact in Richness that was increased in XXF ($P = 0.03$) and XYF ($P < 0.001$). (B) Similarly, evenness was increased in XXF ($P = 0.04$) and XYF ($P = 0.02$). (C) The observed taxon followed a similar trend with a significant impact of gonadectomy ($P < 0.01$) and an increase in XXF ($P < 0.01$) and XYF ($P = 0.0003$). (D) Shannon diversity indicated a similar trend with an increase in XXF ($P = 0.02$) and XYF ($P = 0.02$). Data were analyzed as two-way ANOVA to test for the main effect (sex hormone and chromosome), and multiple unpaired *t*-tests were used to test for differences between groups. Each group had ($n$ = 6–8 samples) indicated by the dot plot bar graphs. Error bars depict standard error mean. *$P < 0.05$ and ***$P < 0.001$.

## β-estradiol promoted the growth of *A. muciniphila*

To evaluate the effect of a female sex hormone on the growth of *Akkermansia*, we cultured *A. muciniphila in vitro* under anaerobic conditions in the presence and absence of β-estradiol. *A. muciniphila* was chosen because it is the most abundant and relevant *Akkermansia* species in humans (Geerlings *et al.* 2018). Growth of *A. muciniphila* was monitored by a linear regression curve to associate bacterial numbers with OD600 values (Fig. 5A). As seen in Fig. 5B, supplementation of β-estradiol, at concentrations of 2.2 µM and 10 µM, had a positive, dose-dependent effect on the growth of *A. muciniphila* over 4 h.

## Discussion

The most significant findings of our study are that (1) we demonstrated that sex hormones override sex chromosomes in shaping gut microbial composition and (2) that we identified *A. muciniphila* as a β-estradiol-responsive bacterium.

Cardiovascular studies focusing on identifying sex differences have been reliant on the primary sex hormones estradiol and testosterone in females and males, respectively (Xue *et al.* 2005). The differences between females and males are not limited to sex steroids but include sex chromosomes XX in females and XY in males (Mauvais-Jarvis *et al.* 2020). Previously, it has been

challenging to identify sexual dimorphism based on sex chromosomes due to limitations on animal models (Reue & Wiese 2022). In this study, we used the four-core genotype mouse to identify gut microbiome and steroid changes in gonadal intact and gonadectomized male and female mice. Our study is the first to evaluate both hormonal and chromosomal effects on gut microbiota characteristics. Consistent with previous publications, the comparison of gut microbial composition between males and females showed significant differences. However, the chromosomal effect was more dominant in the male mice. In contrast, we barely observed differences between XXF and XYF mice, suggesting a diminished chromosomal effect in females. Following this, we evaluated the role of diminishing female sex hormones in gut microbiota and demonstrated that the genus *Akkermansia* in phylum *Verrucomicrobia* was remarkably depleted in the gonadectomized XXF and XYF mice. Importantly, our *in vitro* culture assay provided direct evidence for a stimulatory effect of the female hormone β-estradiol on the growth of *A. muciniphila*.

Sex hormones have a role in shaping the gut microbiome. For example, testosterone exposure to nonobese type 1 diabetic mice is shown to protect male mice from pancreatic islet inflammation that is reversed by androgen receptor antagonism (Markle *et al.* 2013, Yurkovetskiy *et al.* 2013). Several studies have shown protective effects of estradiol in gut microbiome, including more Bacteroidetes and less Firmicutes phyla known to mitigate hypertension and inflammation (Yang *et al.* 2015,

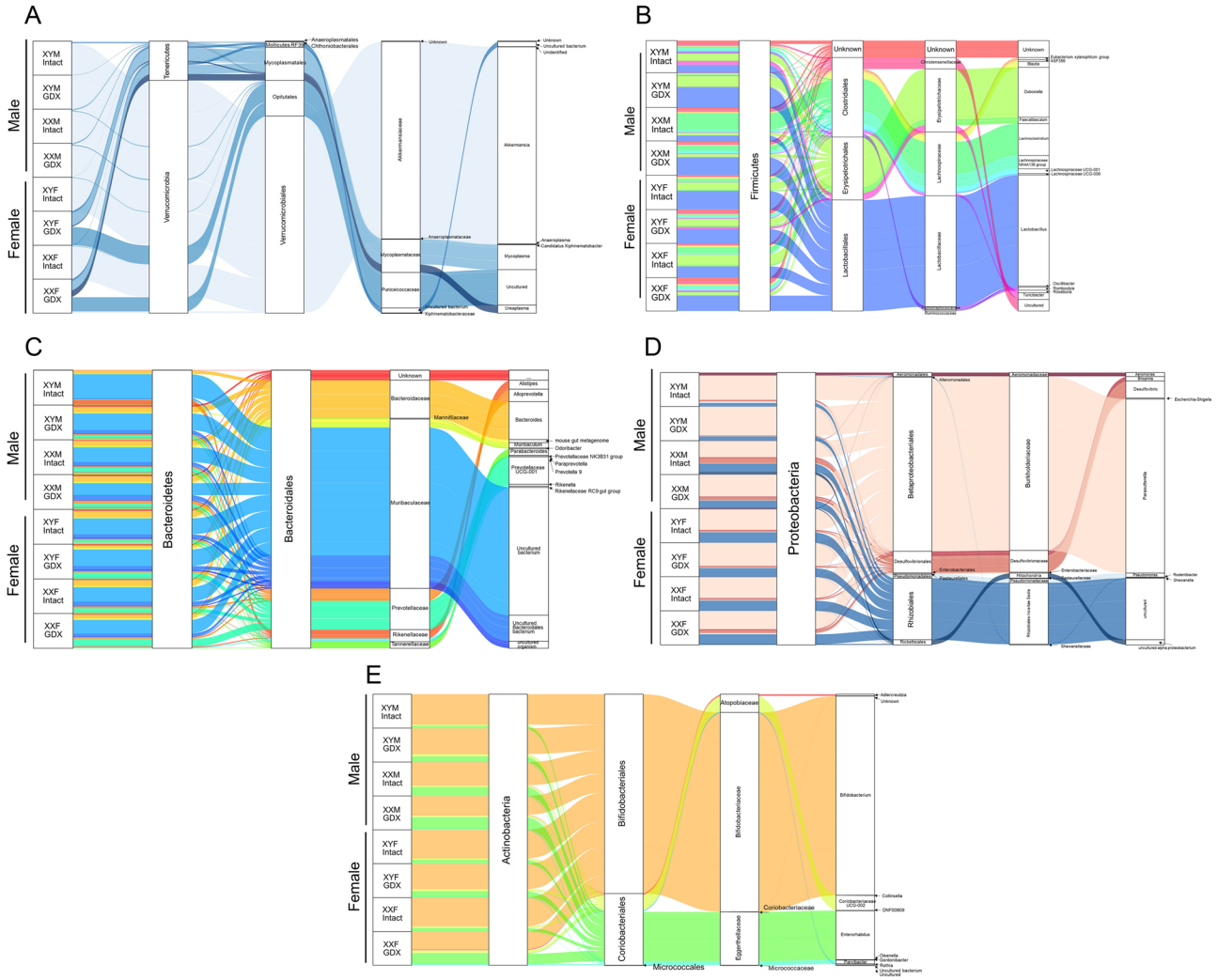


**Figure 4**
Sankey diagrams depicting phyla changes in male and female gonadal intact and gonadectomized mice. (A) Verrucomicrobia, (B) Firmicutes, (C) Bacteroidetes, (D) Proteobacteria, and (E) Actinobacteria.

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**Table 1** Differential bacterial genera between intact and gonadectomized (GDX) female XX and XY mice. EdgeR was performed with adjusted $P < 0.05$.

| EdgeR | Log2FC | Log CPM | *P* | FDR |
|---|---|---|---|---|
| XXF (Intact/GDX) mice | | | | |
| D_5__Akkermansia | 9.506 | 14.45 | 9.42E-06 | 7E-04 |
| D_5__Anaerostipes | 6.641 | 11.24 | 1.86E-05 | 7E-04 |
| D_5__Lachnoclostridium | 5.924 | 17.33 | 0.00073 | 0.012 |
| D_5__Bifidobacterium | 4.278 | 16.82 | 0.00628 | 0.045 |
| D_5__Aeromonas | 4.087 | 7.964 | 0.00054 | 0.011 |
| D_5__Lachnospiraceae_UCG_006 | 4.087 | 10.69 | 0.00241 | 0.023 |
| D_5__uncultured_4 | 3.361 | 10.46 | 0.00267 | 0.023 |
| D_5__Tyzzerella_3 | 3.343 | 9.281 | 0.00383 | 0.03 |
| D_5__Anaerotruncus | 2.653 | 10.47 | 0.00264 | 0.023 |
| D_5__Eubacterium_xylanophilum_group | -4.244 | 11.13 | 0.00021 | 0.006 |
| D_5__Roumboutsia | -4.824 | 11.36 | 0.00161 | 0.021 |
| XYF (Intact/GDX) mice | | | | |
| D_5__Akkermansia | 11.63 | 15.17 | 1.47E-06 | 5.79E-05 |
| D_5__Anaerostipes | 7.264 | 10.8 | 1.30E-05 | 0.00034 |
| D_5__Blautia | 5.324 | 15 | 0.00044 | 0.00701 |
| D_5__Aeromonas | 5.143 | 8.742 | 4.14E-08 | 3.27E-06 |
| D_5__Lachnoclostridium | 4.964 | 15.45 | 0.00071 | 0.00937 |
| D_5__uncultured_4 | 3.316 | 10.05 | 0.00505 | 0.04374 |
| D_5__Parabacteroides | 2.967 | 13.27 | 0.00426 | 0.04211 |
| D_5__Bacteroides | 2.949 | 15.85 | 0.00648 | 0.04642 |
| D_5__Lactobacillus | 2.896 | 18.94 | 0.00705 | 0.04642 |
| D_5__Erysipelatoclostridium | 2.824 | 9.949 | 0.00371 | 0.04187 |
| D_5__Turicibacter | -6.098 | 13.52 | 0.00021 | 0.00411 |

Jang *et al.* 2019). Estrogen-responsive bacteria including *Akkermansia* is shown to play a role in alleviating colitis disease in mouse model of estrogen beta receptor deletion (Naito *et al.* 2018, Ma *et al.* 2022). Our study shows that decline in circulating testosterone levels in male mice tends to decrease richness and diversity in XXM but not in XYM mice suggesting an X chromosome effect. While one can argue that smaller testicular size in XXM than XYM mice contributes to the differential effect, testosterone levels were similar in both groups as previously reported and in our data (Gatewood *et al.* 2006). Castrated mice from three different strains (C57BL/6J, C3H/HeJ, and DBA/2J) fed a regular chow diet indicated altered gut microbiome composition (Org *et al.* 2016). However, we did not observe overt changes in our study. Interestingly, gonadectomy, in addition to a high-fat diet, is shown to impact gut microbiota with increased bile acids in males than females, suggesting protection in female mice (Org *et al.* 2016). In the case of FCG mice, Sry transgene is located on an autosome (Burgoyne & Arnold 2016). Previous studies on sex differences have not shown whether Sry linked to the Y chromosome versus autosome impacts gut composition (Org *et al.* 2016). Our data wherein gonadectomized female mice increased alpha diversity independent of sex chromosome are similar to findings in Sprague–Dawley rats, suggesting that richness and phylogenetic diversity are impacted by loss of sex hormones in fecal samples (Kaliannan *et al.* 2018, Diviccaro *et al.* 2022). Given that we collected fecal matter and not mucosa samples our analysis was limited to changes in the stool. Nevertheless, microbiome diversity in the mucosa is indicated to differ by sex as shown by the enrichment of *Lactobacillus* and *Oscillibacter* in gonadectomized females than in male rats (Diviccaro *et al.* 2022).

Our results are consistent with data from C57BL/6J and DBA/2J strains, indicating that *A. muciniphila* is upregulated in gonadectomized females than male mice (Org *et al.* 2016). *Akkermansia* has been suggested to be linked to female sex hormones and provides beneficial effects in metabolic syndrome by mitigating inflammation and improving intestinal barrier integrity (Santos-Marcos *et al.* 2023). Analysis of 1135 human gut metagenome reported that, after correction for all the variables (i.e., dietary, lifestyle, medication, etc.), *A. muciniphila* was the only bacterial taxon associated with sex, with a higher abundance in females (Sinha *et al.* 2019). Postmenopausal women have a decrease in gut diversity associated with a reduction in estradiol (Menni *et al.* 2018, Peters *et al.* 2022). *Akkermansia muciniphila*, *Clostridium lactatifermentans*, *Parabacteroides johnsonii*, and *Veillonella seminalis* are decreased in postmenopausal women

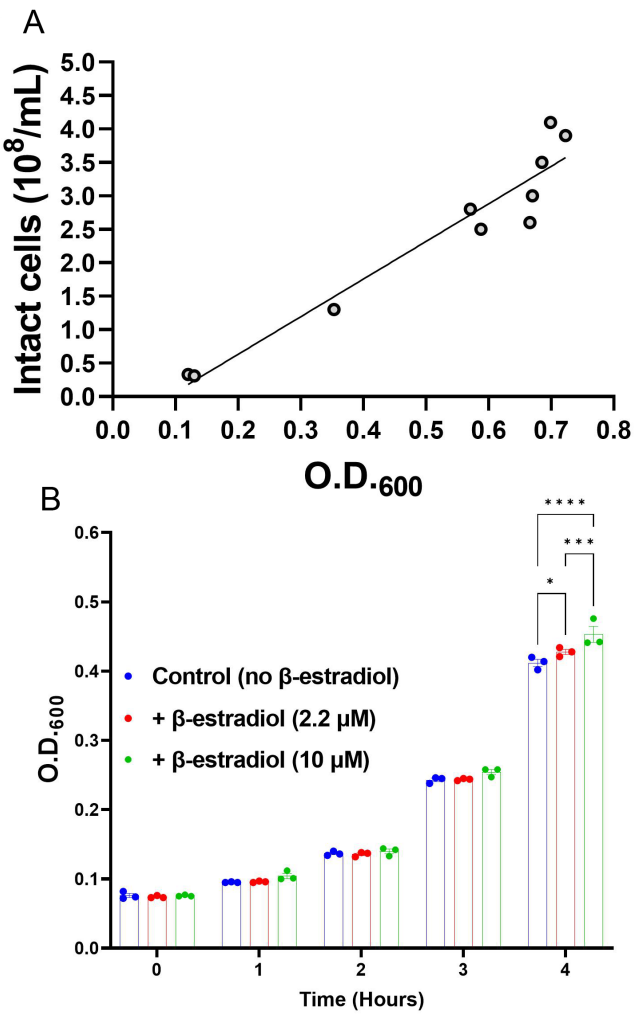


**Figure 5**
Pro-growth effect of β-estradiol on *A. muciniphila*. (A) Linear regression curve of intact cell number and OD600 ($R^2$ = 0.9). (B) OD600 readings were recorded within the first 4 h. Two-way ANOVA was used followed by Tukey multiple comparisons. *$P$ < 0.05, ***$P$ < 0.001, and ****$P$ < 0.0001.

(Peters *et al.* 2022). Sex hormone changes during aging impact the gut microbiota and can play a pivotal role in identifying probiotics (An *et al.* 2018). Decreases in estradiol and *Akkermansia* are associated with metabolic syndrome in postmenopausal women and mice (Org *et al.* 2016, Peters *et al.* 2022). These studies suggested that *Akkermansia* is beneficial in regulating blood pressure, inflammation, and metabolic syndrome (Org *et al.* 2016, Ottman *et al.* 2017, Sun *et al.* 2019).

It is shown that prenatal testosterone exposure causes high blood pressure and gut microbiota dysbiosis (Sherman *et al.* 2018). We did not determine the growth of *Akkermansia* in the presence of testosterone because there was no significant difference in gut microbiome composition in castrated versus testes intact male mice. Therefore, we proceeded to evaluate the estradiol effect on the growth of *Akkermansia* because 17β-estradiol is shown to lower *Proteobacteria* and increase *Akkermansia* in ovariectomized mice with metabolic syndrome (Diviccaro *et al.* 2022). This concurs with our finding of downregulated *Akkermansia* in ovariectomized XXF and XYF mice, indicating a role for estrogen in regulating gut bacteria. While *Akkermansia*, *Anaerostipes*, and *Lachnoclostridium* were decreased with gonadectomy, multiple genera were increased in abundance in female mice (XXF and XYF), including *Eubacterium*, *Romboutsia*, and *Turicibacter*. Enrichment of *Blautia* in gonadal intact XYF mice suggested its association with the Y chromosome, given its abundance in control stool of male rats (Diviccaro *et al.* 2022). There was no overt change in Firmicutes and Bacteroides; however, our study shows a significant phylum change in Verrucomicrobia in female mice.

A decrease in 11-deoxycorticosterone was indicated in female mice independent of their sex chromosome complement. This is consistent with lower progesterone and corticosterone levels indicated in primary follicular depletion in rats (Carolino *et al.* 2019). In male mice, increased Tetrahydroxy 11-deoxycortisol suggested an underlying inflammatory response to upregulate glucocorticoids, adrenal, and metabolic dysfunction (Araujo-Castro *et al.* 2023, Suminska *et al.* 2023). Steroid analysis indicated that gonadectomy mediated dysregulation of mineralocorticoid synthesis and perhaps gut microbial composition. Nonetheless, based on the reported associations of *Akkermansia* with sex (Liu *et al.* 2017, Kaliannan *et al.* 2018, Moore & Pluznick 2023) we chose to focus on *Akkermansia* and demonstrate for the first time that the growth of *A. muciniphila* is indeed stimulated by estradiol. The enrichment of *Akkermansia* in gonadal intact female mice and the bacterium growth in the presence of estrogen points to the hormone–bacterium interdependence as demonstrated in our study. Intestinal enzyme β-glucuronidases can reactivate conjugated estrogens, including estrone and estradiol, through glucuronidation (Ervin *et al.* 2019). The estrogen-deconjugating β-glucuronidase enzyme known as estrobolome includes *Clostridium perfringens*, *B. fragilis*, and *R. gnavus* (Ervin *et al.* 2019). *Akkermansia* accounts for a more significant portion of the estrobolome phylum, indicating its correlation to the presence of estrogen in gonadal intact mice (Candeliere *et al.* 2022).

Our study provided a baseline to understand the contribution of sex hormones and sex chromosomes to the gut microbiome. However, there are drawbacks to

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our study design and the animal model used. Bedding transfer and maintained diet are essential to reproducible gut microbiota analysis (Miyoshi *et al.* 2018). However, our study did not transfer mice wood chips from old to new cages. Our study maintained a similar mice age; however, it is shown that aging and sex are independent nonmodifiable contributors to gut microbiome diversity (Miyoshi *et al.* 2018). It will be interesting to study FCG mice in separate cages since housing type, including isolator versus individually ventilated cages, impacts α diversity and β diversity of gut microbiome composition (Thurman *et al.* 2021). We used fecal matter in our study; however, it is shown that there is a significant difference when fecal vs gut mucosa microbiome are incorporated in analysis with *Oscillibacter*, *Alloprevotella*, *Colidextribacter*, and *Fournierella* taxa enriched in mucosa while *Fusicaenibacter*, *Lactobacillus*, and *Ruminococcus* reported being higher in the stool of rats (Diviccaro *et al.* 2022).

In the future, considering a standardized approach to studying gut microbiome will enhance the consistency, quality, and reproducibility of data acquisition and analysis. Moreover, time of day or night for fecal collection can impact gut microbiome composition shown to interact with circadian genes, including Bmal1 (Heddes *et al.* 2022). Our study provides a fundamental framework for the impact of sex hormones and sex chromosomes on gut microbiome content in addition to identifying *Akkermansia muciniphila* as an estradiol-responsive bacterium. It remains to be determined whether *Akkermansia muciniphila* may confer protection in ovariectomized female mice with hypertension and autoimmunity shown to be exacerbated in XXF than XYF mice (Ji *et al.* 2010, Itoh *et al.* 2019).

**Supplementary materials**

This is linked to the online version of the paper at https://doi.org/10.1530/MAH-23-0010.

**Declaration of interest**

Bina Joe is the editor-in-chief of *Microbiota and Host*. Bina Joe was not involved in the review or editorial process for this paper, on which she is listed as an author. The authors declare no conflict or competing interest.

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**Author contribution statement**

AK, PB, RT, FMJ, TY, BJ, BOO: Devised and proposed research topic; AK, PB, RT, TY, BJ, BOO: Executed experiments and collected data; AK, PB, RT, TY, BJ, BOO: Prepared figures, tables, and analyzed data; AK, RT, TY, BJ, BOO: Interpretation of results and drafted manuscript; AK, PB, RT, TY, BY, BOO: Edited and revised manuscript; AK, PB, RT, FMJ, TY, BJ, BOO: Approved the final version of the manuscript.

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