american physiological society
PHYSIOLOGICAL GENOMICS

Physiol Genomics 56: 417–425, 2024.
First published April 19, 2024; doi:10.1152/physiolgenomics.00003.2024

RESEARCH ARTICLE

The Microbiome in Health and Disease

Gonadal sex and chromosome complement influence the gut microbiome in a mouse model of allergic airway inflammation

Carolyn Damilola Ekpruke,[1]* Rachel Alford,[1]* Erik Parker,[2] and Patricia Silveyra[1,3]

[1]*Department of Environmental and Occupational Health, School of Public Health Bloomington, Indiana University, Bloomington, Indiana, United States;* [2]*Department of Epidemiology and Biostatistics, Biostatistics Consulting Center, School of Public Health, Indiana University, Bloomington, Indiana, United States; and* [3]*School of Medicine, Indiana University, Indianapolis, Indiana, United States*

Abstract

Evidence abounds that gut microbiome components are associated with sex disparities in the immune system. However, it remains unclear whether the observed sex disparity in asthma incidence is associated with sex-dependent differences in immune-modulating gut microbiota, and/or its influence on allergic airway inflammatory processes. Using a mouse model of house dust mite (HDM)-induced allergic inflammation and the four core genotypes (FCGs) model, we have previously reported sex differences in lung inflammatory phenotypes. Here, we investigated associations of gut microbiomes with these phenotypes by challenging FCG mice [mouse with female sex chromosome and male gonad (XXM), mouse with female sex chromosome and female gonad (XXF), mouse with male sex chromosome and male gonad (XYM), and mouse with male sex chromosome and female gonad (XYF); $n = 7$/group] with HDM (25 μg) or PBS intranasally for 5 wk and collecting fecal samples. We extracted fecal DNA and analyzed the 16S microbiome via Targeted Metagenomic Sequencing. We compared α and β diversity across genotypes and assessed the *Firmicutes/Bacteroidetes* (F/B) ratio. When comparing baseline and after exposure for the FCG, we found that the gut F/B ratio was only increased in the XXM genotype. We also found that α diversity was significantly increased in all FCG mice upon HDM challenge, with the highest increase in the XXF, and the lowest in the XXM genotypes. Similarly, β diversity of the microbial community was also affected by challenge in a gonad- and chromosome-dependent manner. In summary, our results indicated that HDM treatment, gonads, and sex chromosomes significantly influence the gut microbial community composition. We concluded that allergic lung inflammation may be affected by the gut microbiome in a sex-dependent manner involving both hormonal and genetic influences.

NEW & NOTEWORTHY Recently, the gut microbiome and its role in chronic respiratory disease have been the subject of extensive research and the establishment of its involvement in immune functions. Using the FCG mouse model, our findings revealed the influence of gonads and sex chromosomes on the microbial community structure before and after exposure to HDM. Our data provide a potential new avenue to better understand mediators of sex disparities associated with allergic airway inflammation.

allergic inflammation; four core genotypes; gonadal hormones; gut microbiome; sex chromosomes

INTRODUCTION

Most of the microorganisms that exist in the human body are in the gut. Over a thousand species of bacteria are located in the human intestine and each of them has at least hundreds of subspecies (1). The gut microbiota is being investigated in multiple diseases and conditions and has been implicated as one of the factors that contribute to the risk of developing asthma (2, 3). *Firmicutes* and *Bacteroidetes* are some of the most abundant phyla present in the human gut, and their ratio has been associated with asthma outcomes (4). Within the first 100 days of life, the relative abundance of some bacteria including the *Lachnospira* and *Veillonella* has been found to decrease in infants who are at risk of developing asthma (2). In addition, early life exposure to allergens disrupts the gut microbiome and is associated with an increased risk of developing asthma in childhood (5, 6). Moreover, the relationship between *Lachnospira* and immune cell counts in the bronchoalveolar lavage fluid was negatively correlated, showing that this group of bacteria may reduce lung inflammation

*C. D. Ekpruke and R. Alford contributed equally to this work.
Correspondence: P. Silveyra (psilveyr@iu.edu).
Submitted 16 January 2024 / Revised 10 April 2024 / Accepted 17 April 2024

(2). Importantly, distinct bacteria are associated with the different phenotypes of allergic asthma (7), indicating that bacterial taxa present in the gut may be associated with susceptibility and risk of developing asthma (2, 8). Other common phyla include *Proteobacteria*, *Verrucomicrobia*, *Actinobacteria*, *Cyanobacteria*, and *Fusobacteria* (9, 10).

Researchers have suggested that gut microbiota plays a vital role in the pathophysiology of inflammatory diseases (11). The gut microbiome contributes to the development of the immune system and the maintenance of health, but can also be disrupted by external factors (12) including exposure to environmental stressors such as allergens (13, 14), administration of antibiotics (15), temperature (16), and diet (17). These factors contribute to microbial imbalance which could be loss or gain in microbial communities or relative abundance. An increase in diversity and richness of gut microbial components is of great health benefit to the host and reflects microbial community stability (18–20). On the other hand, disruption of the microbial community can lead to the depletion or deletion of the normal beneficial roles it plays. The microbiota effect has been seen both in innate and adaptive immunity (21) and has been implicated in inflammatory diseases (22, 23).

Sex is an essential player when studying the relationship between gut microbiome and environmental factors (24–26). Biological sex has been identified to play a role in the treatments that disrupt the microbial community (22, 23, 27–30). However, scientists have found it difficult to establish the influence of sex in gut microbial composition. A few researchers have reported sex-based differences in microbiota composition (31, 32). Recent studies using mouse models have also demonstrated that sex hormones influence individual gut microbial composition by mediating sex-dependent communication within the microbiota (31, 33–35). For example, Org et al. (36) observed that the host genotype and sex had an impact on microbial composition and that the sex hormone effect was greatly felt in the gut microbiome of male gonadectomized rats than in the females. At the same time, some investigators have reported that gender has little or no effect on gut microbiota (9, 37), whereas others reported associations (32).

Asthma is a chronic respiratory disease with a complex etiology and sex disparities in its outcomes (38). Chronic exposure to environmental allergens such as house dust mites (HDM) is known to result in allergic responses (39). Evidence abounds that gut microbiome components drive sex disparities in the immune system and an individual's susceptibility to diseases (40). However, it remains unclear if the sex disparity in asthma results from sex-dependent differences in the immune-modulating gut microbiota in allergic airway inflammation disease. Therefore, to study the phenotypic effect of sex chromosomes versus gonads on the gut microbiome following HDM exposure, we used the four core genotype (FCG) mouse model (41). This model comprises four groups of mice that are genetically modified to have both male and female gonads irrespective of their biological sex producing "XX" or "XY" mice with male or female gonads (hence, the four cores are XXM, XXF, XYM, and XYF). Using this model, we evaluated changes in the gut microbiome in response to the HDM allergen challenge to determine the influence of sex chromosomes and gonads in the previously observed sex differences in gut microbial changes triggered by the allergen challenge. We hypothesize that gonads and chromosome complement influence gut microbiome disruption following allergen exposure, and thus may contribute to the sex disparity observed in asthma incidence.

MATERIALS AND METHODS

Animal Model

The animal protocol (21-012) for this study was approved by Bloomington's Institutional Animal Care and Use Committee before the commencement of the study. This study used an inbred strain from the FCG mouse model (Strain No. 010905) originally obtained from Jackson Laboratories (Bar Harbor, ME). Mice were bred according to protocol and maintained in Indiana University's Laboratory Animal Resources facility. The wild-type females (C57BL/6J) were bred with the XYM (ArnoJ) male mice to produce pups belonging to one of the FCG (XXM, XXF, XYM, and XYF). Tissues from these mice were collected for genotyping to ascertain genotype. FCG mice of 8 to 10 wk old of age were randomly assigned ($n = 7$ animals/group) to the control and experimental groups. The mice were treated following the guidelines of the National Institutes of Health (NIH) for the care and use of laboratory animals.

Intranasal Instillation of PBS or HDM Challenge

FCG mice assigned to the HDM-treated group were intranasally administered 50 μL of HDM solution (25 μg of the allergen extract from HDM, *D. pteronyssinus*, and *D. farinae* mix) (Citeq biologics, Groningen, Netherlands) and suspended in 50 μL of phosphate-buffered saline (PBS) daily (5 days/wk) for 5 total weeks to induce phenotypic airway inflammation. Those assigned to the PBS-treated group had 50 μL of PBS daily through the same route, throughout the study. The 50 μL HDM or PBS was intranasally administered to the FCG mice using 20–200 μL pipette tips after light anesthesia with 5% isoflurane using the SomnoSuite device (Kent Scientific). The fecal samples of these mice were collected at baseline before the commencement of the treatment and at *week 5* at the end of the study.

Fecal Pellet Collection and Processing

Fecal pellet samples were collected from individual animals by placing an animal in an empty autoclaved cage with no bedding and allowing the mouse to defecate normally. Fecal pellets from individual animals were collected into 1.5 mL Eppendorf tubes with sterile forceps and placed at −80°C until analyzed. Samples were collected from each mouse 1 to 3 days before the commencement of treatment or control to measure preexposure bacterial composition. Once treatment began, fecal pellet samples were collected from each mouse immediately at the end of the 5-wk treatment period. To account for cage variance, all animals of the same genotype or in the same treatment group were kept in different cages (two to three mice of the same litters per cage) and arranged in different columns on the same rack for the 5 wk of study. Researchers have demonstrated that 2–5 wk of HDM exposure induced allergic airway

inflammation in female mice evidenced by an increase in eosinophils, the presence of perivascular and peribronchial inflammation, and increased airway hyperresponsiveness (42, 43). These features have also been observed in our previous study (44). Fecal pellet samples collected during *week 0* (pre-exposure) and *week 5* (final exposure) were processed with ZymoBIOMICS Service: Targeted Metagenomic Sequencing (Zymo Research, Irvine, CA). DNA was extracted to a 50 μL elution volume using ZymoBIOMICS-96 MagBead DNA kit with ZymoBIOMICS Microbial Community Standard as a positive control. Afterward, the sample was prepared for targeted sequencing with Quick-16STM NGS Library Prep kit using customized primers from the Quick-16STM Primer Set V3–V4. Final products from polymerase chain reactions (PCRs) performed in real-time PCR machines were quantified with qPCR fluorescence readings and pooled together based on equal molarity to prepare a sequencing library. ZymoBIOMICS Microbial Community DNA Standard was used as a positive control for each target and negative controls were included. The final library was cleaned up using the Select a-Size DNA Clean & Concentrator and then quantified with TapeStation (Agilent Technologies, Santa Clara, CA) and Qubit (Thermo Fisher Scientific, Waltham, WA). The library was sequenced on Illumina MiSeq with a v3 reagent kit (600 cycles). The sequencing was performed with a 10% PhiX spike-in.

Statistical Analysis and Plots

All analyses were conducted at the Indiana University Bloomington Biostatistics Consulting Center. Taxonomy assignment was performed using Uclust from Qiime v.1.9.1 with the internally designed and curated Zymo Research Database used as a reference. Sequencing output from the Illumina MiSeq PE250 platform was converted to fastq format and demultiplexed using Illumina Bcl2Fastq 2.18.0.12. The resulting reads were processed using Qiime v. 1.9.1. *Firmicutes/Bacteroidetes* ratio (F: B) was calculated. The raw amplicon sequence variant (ASV) output from QIIME 2 was used to generate stacked taxa bar plots, permutational ANOVA (PERMANOVA), Bray–Curtis Nonmetric Multidimensional Scaling (NMDS) plots, and ANOVA-like differential expression (ALDEx) analyses in *R*. ALDEx was used to evaluate the specific taxa that were differentially abundant among samples based on the different variables. Linear discriminant analysis effect size (LEfSe) was used to find ASVs differentially expressed at the threshold of 1.2-logFC among sample groups (45). Chao1 and Shannon's indices were used to analyze the α diversity among the groups and significant differences were based on Tukey-adjusted pairwise *t* tests between estimated mean obtained from a linear mode. Alpha diversity values were expressed in means ± standard deviation and only Chao1 index was reported. Data are represented as the mean and standard deviation of both α diversity metrics for each of the levels of the grouping variables. For each α diversity metric, we determined the significant level using the Tukey-adjusted pairwise *t* tests between estimated means at P value $<$ 0.05. NMDS plots were made using the Bray–Curtis distance among the samples based on the counts of different microbial species called the ASVs (46). A P value of 0.05 was considered statistically significant for all analyses.

RESULTS

Chronic HDM Exposure Alters the Gut Microbiome of FCG Mice

Among the 17 common microbial amplicon sequence variants (ASVs) that were selected as meeting a threshold of 5% abundance in at least one fecal pellet sample, phyla *Bacteroidetes* occupied 47%, *Firmicutes* 35%, *Actinobacteria* 12%, *and Verrucomicrobia* 6% of the microbial population in the gut as shown in Fig. 1. Interestingly, the microbial community at baseline in all the genotypes had the lowest percentage of microbial amplicon sequence variants that do not meet up to at least 5% threshold, whereas the HDM-treated mice had the most. *Actinobacteria*: *Actinobacteria*; *Bifidobacterium Pseudolongum* increased in the animals treated with HDM or PBS, but it was absent in the animals at baseline and PBS-treated XYM genotype mice only. *Firmicutes*, *Clostridia*, *and Ruminococcaceae sp35380* appeared only in the guts of FCG mice with male chromosomes, XYF and XYM, at baseline. *Firmicutes*; *Bacilli lactobacillus johnsonii* was abundant at baseline but declined in PBS-treated and HDM-treated mice in all the genotypes. The relative abundance of *Verrucomicrobia* was high in the gut of HDM-treated XXF genotype compared with the baseline value.

The LEfSe analysis revealed ASVs that were differentially expressed at the threshold of 1.2-logFC among the groups at baseline and after treatment, as shown in Figure 2, *A* and *B*. The PBS-treated XYF genotype had the highest number of ASVs (11) that were significantly increased in relative abundance at a threshold of 1.2 logFC. Interestingly, at baseline, XXF, XYF, and XYM genotypes had significantly increased relative abundance of ASVs in the gut microbiome, but not XXM. In the PBS-treated group, all genotypes had a significant relative abundance of more than one ASVs, except the XYM genotype which had only one, *g _Anaerosporobacter*. However, all the HDM-treated FCG except the XYM genotype did not have any ASVs that were significantly increased in relative abundance in their gut microbiome (Fig. 2*B*).

Firmicutes/Bacteroidetes Ratio in the Gut of PBS- and HDM-Challenged FCG Mice

The pretreatment (baseline) and after-treatment (HDM or PBS) *Firmicutes/Bacteroidetes*(F/B) ratio in the gut of the FCG mice are shown in Fig. 3, *A–C*. At baseline, the gut F/B ratio was higher in the XYF and XYM genotypes (2.769 and 2.633, respectively) than in the other genotypes (XXM and XXF which were 1.529 and 2.112, respectively) (Fig. 3*A*). In HDM-treated mice, the basal values decreased drastically in all the genotypes except in the XXM, in which the ratio increased from 1.529 to 1.747. The XYF mice, which had the highest F/B ratio at baseline (2.769), had the lowest value after HDM treatment (1.282). Interestingly, in the PBS-treated group, all the genotypes had a decline in their F/B ratio compared with baseline, except the XXM mice, which increased to 1.759, almost the same value as that of the HDM-treated mice with the same genotype. The PBS-treated XYF and XYM genotypes showed more than two times decrease when compared with the basal values of the gut F/B ratio.

When comparing the gut F/B ratio of FCG mice carrying the same gonads (irrespective of the chromosomal complement)

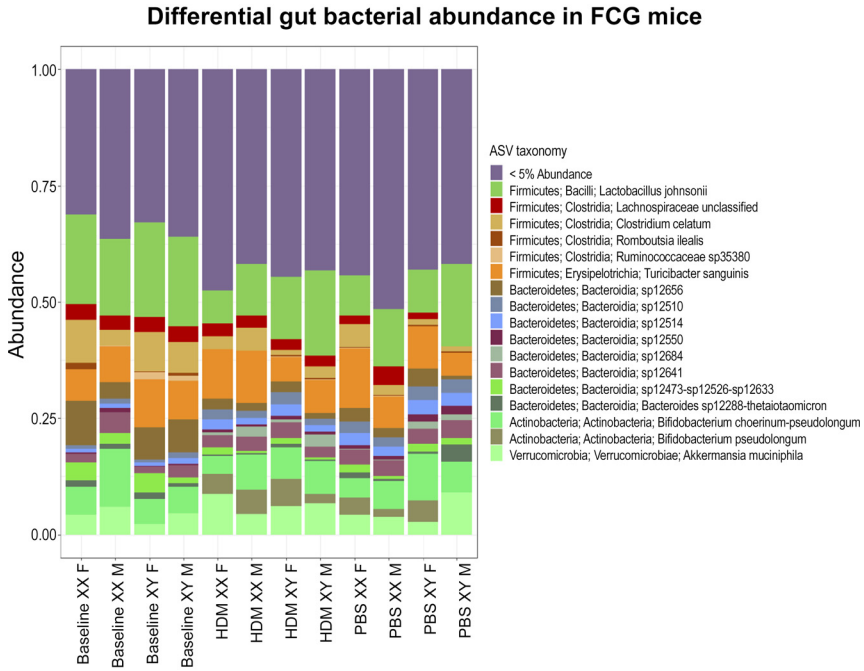

Figure 1. Stacked taxa bar plot of FCG mice at baseline and after 5 wk of PBS or HDM exposure. This plot shows all the samples on the *x*-axis, grouped by "Identity," which are defined as unique combinations of treatment, week, gonads, and sex chromosomes. Each of these bars then shows the abundance of named taxa (phylum; class; genus; species) found to be present at, at least 5% relative abundance in at least one sample. All other taxa falling below this threshold were then aggregated into a single "rare" category and placed at the top. $n = 7$–10/group. FCG, four core genotype; HDM, house dust mite; PBS, phosphate-buffered saline.

(Fig. 3*B*), we found that the average gut F/B ratio was 2.037 and 2.396 for mice with male or female gonads, respectively. In mice with male gonads, the F/B ratio also decreased to 1.4 and 1.903, respectively, for HDM- and PBS-treated groups. In females, the average gut F/B ratio was 1.232 in the PBS-treated group and 1.425 in the HDM-treated group. Interestingly, the F/B ratio was lower in the PBS-treated group than in the HDM-treated one with either male or female gonads. Overall, the F/B ratio was higher in males in the PBS-treated and HDM-treated groups compared with the females in the same group.

When comparing the F/B ratio in the gut of the FCG mice with the same sex chromosomes (Fig. 3*C*), we found that for animals with male chromosomes, the gut F/B ratio was 2.698 at baseline and decreased to 1.099 in the PBS-treated group and 1.628 in the HDM-treated group, respectively. Mice with female chromosomes had a gut F/B ratio of 1.826 at baseline, which was decreased to 1.551 in the PBS-treated group and 1.656 in the HDM-treated one. Interestingly, the average value of the F/B ratio in the animals with either male or female chromosomes was almost the same in the HDM-treated groups.

α Diversity in the Gut Microbiome of PBS- and HDM-Challenged FCG Mice

The α diversity for the different groupings of samples is shown using both the Chao1 and Shannon indices in

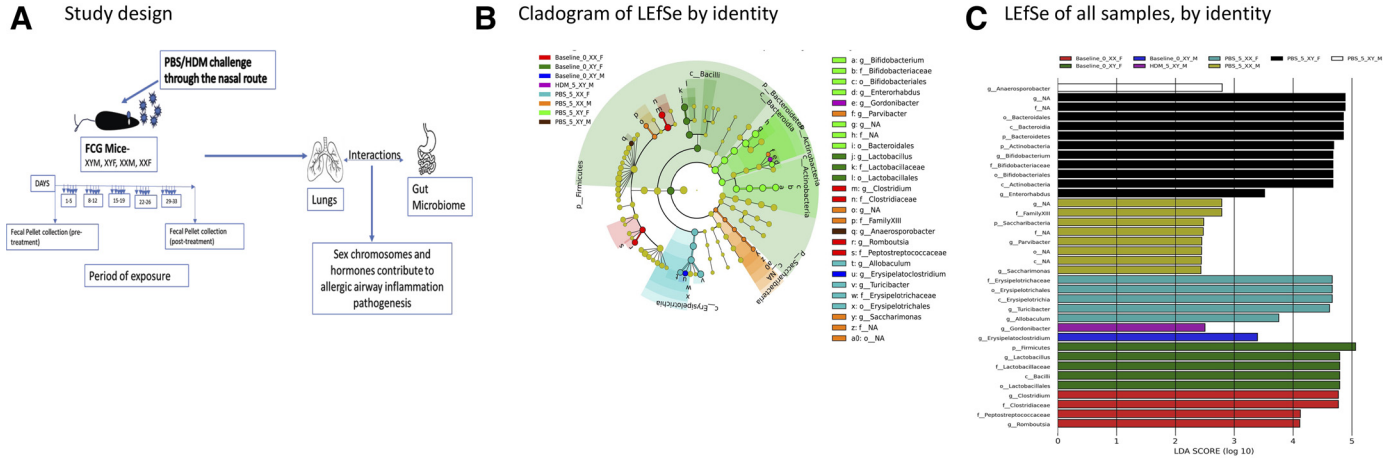

Figure 2. Cladogram and linear discriminant analysis effect size (LEfSe) results in the FCG mouse model treated with HDM or PBS (control). *A*: diagram showing the summary of treatment and duration of study in the FCG mouse model. *B*: cladogram of the FCG mouse model. The classification from the phylum to the genus is represented by the circle radiating inside out. Each small circle at different classifications represented a taxon, and the circle diameter was proportional to the relative abundance. Red, green, and blue dots represent the core bacterial populations in each respective group. *C*: LEfSe diagram. The graph shows the linear discriminant analysis (LDA) scores obtained from linear regression analysis of the significant microbial community in the FCG mouse model, and the result corresponds to a differential species. These diagrams only showed the groups that were differentially expressed at the threshold of 1.2 logFC, with NA representing samples that were unclassified or unassigned at a particular level of taxonomy in both diagrams. $n = 7$–10/group. FCG, four core genotype; HDM, house dust mite; PBS, phosphate-buffered saline.

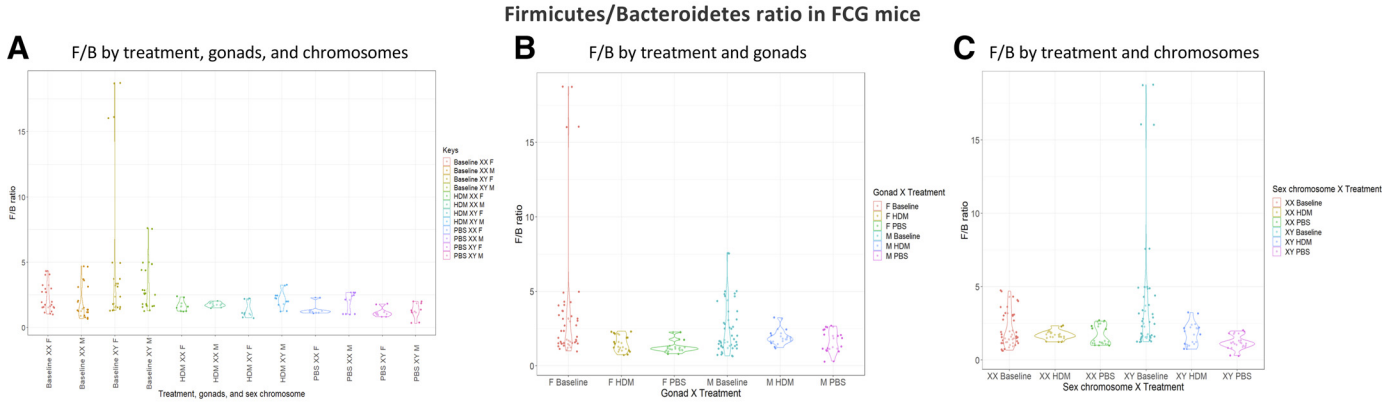

Figure 3. *Firmicutes/Bacteroidetes* (F/B) ratio among the FCG mouse model. *A*: F/B ratio in the gut microbiome of all the FCG at baseline and after treatment. *B*: effect of gonads and treatment on the F/B ratio in the gut microbiome among the groups. The ratio was greater than 1 in all the groups with the female (baseline) having the highest value and the female (PBS-treated) having the lowest value. *C*: effect of sex chromosomes and treatment on the F/B ratio in the gut microbiome among the groups. $n = 7$–10/group. FCG, four core genotype; PBS, phosphate-buffered saline.

Fig. 4, *A*–*C*. We compared the effects of *1*) sex chromosomes, *2*) gonads, and *3*) four core genotypes by treatment, as indicated below.

Effect of sex chromosomes and treatment.

At baseline, the average α diversity value was 159.021 ± 0.326 and 152.001 ± 0.460 in mice with male and female chromosomes, respectively, and increased in the HDM-treated group to 185.360 ± 0.369 and 192.893 ± 0.346 (Fig. 4*A*). These values were also increased in the PBS-treated group reaching 155.480 ± 0.250 and 184.829 ± 0.333 in males and females, respectively. The average value of α diversity increased after treatment in both groups of mice with either the male or female chromosome. Mice with the female chromosomes (XX) irrespective of their gonads in the HDM-treated group had a significant increase in the α diversity of their gut microbiome compared with that of those with female chromosomes at baseline (Chao1 index: P value = 0.0078). Also, the baseline average α diversity of the gut microbiome of mice with female chromosomes was significantly lower than those in the PBS-treated group (Chao1 index: P value = 0.00019) and that of the male chromosomes HDM-treated group (Shannon index: P value = 0.00464) (Fig. 4*A*).

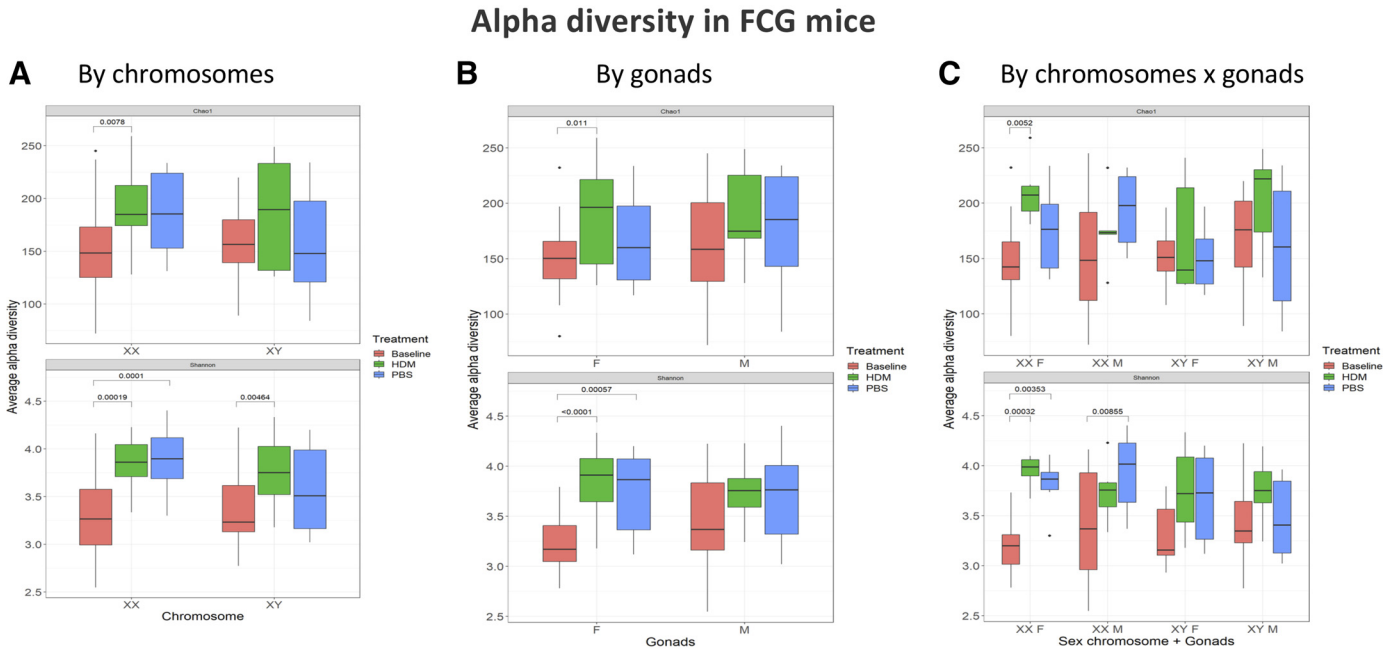

Figure 4. Boxplots of the α diversity in the FCG mouse model before and after exposure to PBS or HDM using the Chao1 and Shannon indices Fig. 4, *A*–*C*. Data were represented in the mean and standard deviation of both α diversity metrics for each of the levels of the grouping variables. For each α diversity metric, we determined the significant level using the Tukey-adjusted pairwise *t* tests between estimated means at P value < 0.05. *A*: sex chromosome has no effect (Chao1: P value =0.3751) on the α diversity in gut microbiome if considered only except when considered with treatment (baseline, HDM, and PBS). *B*: the value of α diversity of gut microbiome was significantly increased in the PBS-treated (Shannon index: 0.00057) or HDM-treated (Chao index: P value =0.011; Shannon index: $s < 0.0001$) mice with female gonads compared with the baseline. *C*: effect of genotypes on the average value of α diversity on the gut microbiome. $n = 7$–10/group. FCG, four core genotype; HDM, house dust mite; PBS, phosphate-buffered saline.

Effect of male and female gonads by treatment.

The average α diversity value in mice with male gonads (161.485 ± 0.383) was higher than in the females (149.537 ± 0.414) at baseline. These values increased both in the HDM-treated and PBS-treated groups. In the HDM-treated group with the male gonads, the average α diversity value was 189.046 ± 0.371 and 189.206 ± 0.342 in the females. In the PBS-treated group, those with the male gonads had an average α diversity value of 177.168 ± 0.305 and the females had 163.14 ± 0.0304 values. Interestingly, the value was not different in the mice with male or female gonads in the HDM-treated group (Fig. 4*B*). The α diversity value in mice with female gonads was increased in the HDM-treated group (Chao1 index: *P* value = 0.011) and PBS-treated group (Shannon index *P* value = 000057), as it occurred in mice with male gonads when compared with baseline (Fig. 4*B*).

Effect of genotypes.

The average baseline α diversity values in the XXF, XXM, XYF, and XYM genotype mice were 149.165 ± 0.511, 154.837 ± 0.393, 149.909 ± 0.263, and 168.134 ± 0.375, respectively (Fig. 4*C*). These values increased after treatment in both HDM and PBS groups. In the HDM-treated group, the values increased to 210.144 ± 0.42 for XXF; 175.641 ± 0.207 for XXM; 167.948 ± 0.353 for XYF; and 202.771 ± 0.417 for XYM. On the other hand, in the PBS-treated group, values were 175.631 ± 0.370 for XXF; 194.027 ± 0.318 for XXM; 150.651 ± 0.083 for XYF; and 160.309 ± 0.298 for XYM. The α diversity values were lowest in the XYF genotype in both the HDM-treated and PBS-treated groups. Among the genotypes, the differences between XXF (baseline) and XXF (HDM-treated) were significant at *P* value = 0.0052; XXF (baseline) and XYM (HDM-treated) were significant at *P* value = 0.023; XXM (baseline) and XXF (HDM-treated) was significant at *P* value = 0.016; XYF (baseline) and XXF (HDM-treated) were significant at *P* value = 0.0051; XYF (baseline) and XYM (HDM-treated) was significant at *P* value = 0.03; XXF (HDM-treated) and XYF (PBS-treated) was significant at *P* value = 0.036. However, comparing the baseline and PBS or HDM-treated groups, within each genotype, α diversity values increased significantly in the XXF (HDM-treated and PBS-treatment) and XXM PBS-treatment only (Fig. 4*C*).

Diversity in the Gut Microbiome of PBS- and HDM-Challenged FCG Mice

Figure 5 shows the β diversity in the FCG mice as nonmetric multidimensional caling plots, using the Bray–Curtis distance sampling among groups. When comparing the effect of treatment, gonads, and sex chromosomes using PERMANOVA (Supplemental Table S1), we found that this interaction was significant (*P* value = 0.001), indicating a significant explanatory variable of differences in microbial community structure among the groups. Interestingly, the three-way interaction among treatment, gonads, and treatment versus sex chromosomes was not significant (*P* value = 0.653). However, when we considered two-way interactions between treatment versus gonads and treatment versus sex chromosomes, the interaction was significant (Treatment and Gona,- *P* value = 0.009; Treatment and Sex Chromosomes, *P* value = 0.046), indicating that the β diversity of the microbial community in mice with male or female gonads and those with male or female chromosomes responded differently to the treatment of either PBS or HDM (Fig. 5).

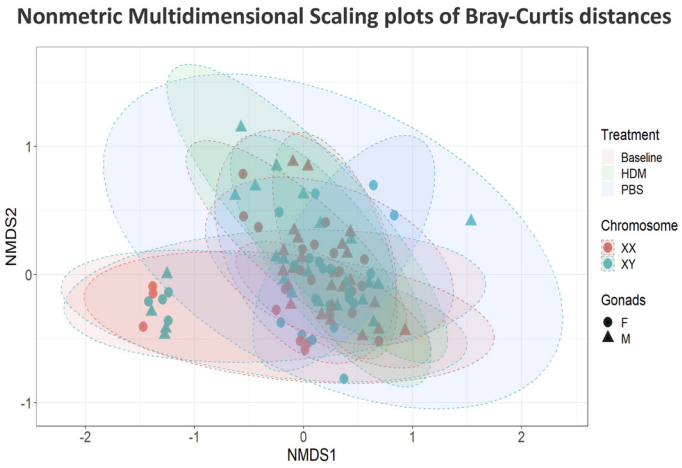

Figure 5. Nonmetric multidimensional scaling (NMDS) plots were made using Bray–Curtis distances between samples. This was used to evaluate the β diversity of the gut microbiome in the FCG mouse model of allergic airway. The diagram is showing the ordination of all samples using the Bray–Curtis distance and NMDS. *n* = 7–10/group. FCG, four core genotype.

DISCUSSION

In our previous study, we identified genes that were differentially expressed in the FCG mouse model of allergic airway inflammation (44), and the alterations of microRNA by female gonadal hormones in the same group of mice (47). We observed that sex chromosomes and gonadal hormones contribute to the changes in gene expression and inflammation phenotypes. In the present study, we used the FCG mouse model to show for the first time that there is an effect of sex chromosomes and gonads on the gut microbiome following HDM exposure. All the genotypes (XXM, XXF, XYM, and XYF) were treated intranasally to either HDM or PBS challenge daily for over 5 wk (*n* = 7 animals/group). To our knowledge, this study is the first to evaluate the role of sex chromosomes and gonads in the disruption of gut microbiome in allergic airway inflammation.

Diversity of the gut microbial community is important for the development of the immune system (48) and early exposure aids the maturation of the immune system response (49–51). Specifically, we found that the phyla *Bacteroidetes and Firmicutes* predominate in the gut microbial community, which was similar to what others have reported (52–54). However, we found that the relative abundance of *Verrucomicrobia* was increased in all the HDM-induced groups, except those with XXF genotypes. Interestingly, *Verrucomicrobia* phylum species such as *Akkermansia muciniphila* have been identified as advantageous microbes that are prominent in the gut of apparently healthy individuals (55) and play a role in controlling inflammatory processes (56). Reduction in the relative abundance of *Verrucomicrobia* has suggested a disruption of microbial community in the gut (57). In addition, its relative abundance was reduced in mice from the XXF genotype, which was associated with severe allergic airway inflammation compared with mice from the other genotypes in our study. This

microbe has also been investigated in other diseases such as type 2 diabetes mellitus and was found to be reduced in individuals diagnosed with the disease (58). Therefore, the *Verrucomicrobia* phylum could represent a target for the development of therapeutics for the management of allergic airway inflammation, especially in women known to have severe asthma symptoms. The Phylum *Firmicutes* was lower in relative abundance in the HDM and PBS groups after exposure, whereas a group reported an increase in male asthmatic rats (59). We observed that the relative abundance of *Bifidobacterium* species, specifically *pseudolongum*, was absent in the gut microbiome of the FCG mouse model at baseline and in the XYM genotype (after PBS exposure). In this regard, Hevia et al. previously reported that this phylum was lower in patients with asthma than in healthy individuals (60). Waligora–Dupriet et al. (61) also suggested that this species was not related to allergic status, whereas other researchers reported otherwise (62–64).

In our study, we observed that the α diversity was increased in HDM and PBS-treated mice and was higher in males than in females' chromosomes or gonads. This was partially in agreement with the work of Xiong et al. (59), which also observed an increase in gut microbiome α diversity in rats with asthma, although they used only male rats and did not evaluate sex as a biological variable (59). It is important to note that low levels of α diversity have been linked to chronic diseases in humans (65, 66) and used as an indicator to describe the gut microbial community. Thus, it is possible that α diversity represents a potential biomarker for human studies. Interestingly, recent clinical studies also reported that α diversity was increased in biofluids from patients with asthma, but without considering sex as a biological variable (67, 68). However, this finding was inconsistent with another report that showed that α diversity was lower in individuals with asthma than in controls (69) and that this decrease was associated with the development of asthma (66). Similarly, Hua et al. (70) reported non-sex-specific similarities between the diversity of α and β diversity in individuals with asthma and healthy individuals. Beta diversity analysis showed that the microbial community in the gut microbiome of the mice with male or female gonads and those with male or female chromosomes responded differently to the treatment of PBS or HDM at different times (baseline and after exposure). Both α and β diversity were influenced by the sex chromosomes and gonads in the different groups.

Together, all the abovementioned analysis indicates that there are consistently significant effects of gonads and sex chromosomes on microbial community similarity, whereas the interaction of treatments (HDM and PBS) with gonads is significant at *week 5* (posttreatment). Therefore, to our knowledge, the current study demonstrates for the first time that both the sex chromosomes and gonadal hormones influence alterations of the gut microbiome after allergen exposure.

Perspectives and Significance

This current study investigated the contributions of sex chromosomes and gonads in the microbial community similarities in the gut microbiome of the FCG mouse model following HDM exposure. We identified the effect of treatment (HDM and PBS), gonads (male and female), sex chromosomes (male and female), and genotypes (XXF, XXM, XYF, and XYM) on the gut microbiome. Most importantly, we revealed alterations in the α and β diversity, *Firmicutes/Bacteroidetes ratio*, and alterations in the relative abundance of ASVs of the gut microbiome among the FCGs. These results revealed the importance of the gut microbiome in the pathogenesis of chronic respiratory disease and the consideration of sex as a biological variable in asthma research.

DATA AVAILABILITY

All sources of data can be found here: https://doi.org/10.17605/OSF.IO/X2KR3.

SUPPLEMENTAL DATA

Supplemental Table S1: https://doi.org/10.6084/m9.figshare.24986535.

GRANTS

This work was supported by National Heart, Lung, and Blood Institute Grants R01HL159764 and R03HL141618 (to P.S.).

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

P.S. conceived and designed research; C.D.E. and R.A. performed experiments; C.D.E., R.A., and E.P. analyzed data; C.D.E. and P.S. interpreted results of experiments; C.D.E. and E.P. prepared figures; C.D.E. drafted manuscript; C.D.E., E.P., and P.S. edited and revised manuscript; C.D.E., R.A., E.P., and P.S. approved final version of manuscript.

REFERENCES

1. **Sommer F**, **Bäckhed F.** The gut microbiota—masters of host development and physiology. *Nat Rev Microbiol* 11: 227–238, 2013. doi:10.1038/nrmicro2974.
2. **Arrieta M-C**, **Stiemsma LT**, **Dimitriu PA**, **Thorson L**, **Russell S**, **Yurist-Doutsch S**, **Kuzeljevic B**, **Gold MJ**, **Britton HM**, **Lefebvre DL**, **Subbarao P**, **Mandhane P**, **Becker A**, **McNagny KM**, **Sears MR**, **Kollmann T**, **Mohn WW**, **Turvey SE**, **Finlay BB**; **CHILD Study Investigators.** Early infancy microbial and metabolic alterations affect risk of childhood asthma. *Sci Transl Med* 7: 307ra152, 2015. doi:10.1126/scitranslmed.aab2271.
3. **Trompette A**, **Gollwitzer ES**, **Yadava K**, **Sichelstiel AK**, **Sprenger N**, **Ngom-Bru C**, **Blanchard C**, **Junt T**, **Nicod LP**, **Harris NL**, **Marsland BJ.** Gut microbiota metabolism of dietary fiber influences allergic airway disease and hematopoiesis. *Nat Med* 20: 159–166, 2014. doi:10.1038/nm.3444.
4. **Zhao X**, **Hu M**, **Zhou H**, **Yang Y**, **Shen S**, **You Y**, **Xue Z.** The role of gut microbiome in the complex relationship between respiratory tract infection and asthma. *Front Microbiol* 14: 1219942, 2023. doi:10.3389/fmicb.2023.1219942.
5. **Lynch SV**, **Wood RA**, **Boushey H**, **Bacharier LB**, **Bloomberg GR**, **Kattan M**, **O'Connor GT**, **Sandel MT**, **Calatroni A**, **Matsui E**, **Johnson CC**, **Lynn H**, **Visness CM**, **Jaffee KF**, **Gergen PJ**, **Gold DR**, **Wright RJ**, **Fujimura K**, **Rauch M**, **Busse WW**, **Gern JE.** Effects of early-life exposure to allergens and bacteria on recurrent wheeze and atopy in urban children. *J Allergy Clin Immunol* 134: 593–601. e12, 2014. doi:10.1016/j.jaci.2014.04.018.

6. **Ludka-Gaulke T**, **Ghera P**, **Waring SC**, **Keifer M**, **Seroogy C**, **Gern JE**, **Kirkhorn S.** Farm exposure in early childhood is associated with a lower risk of severe respiratory illnesses. *J Allergy Clin Immunol* 141: 454–456.e4, 2018. doi:10.1016/j.jaci.2017.07.032.
7. **Wang Z**, **Lai Z**, **Zhang X**, **Huang P**, **Xie J**, **Jiang Q**, **Zhang Q**, **Chung KF.** Altered gut microbiome compositions are associated with the severity of asthma. *J Thorac Dis* 13: 4322–4338, 2021. doi:10.21037/jtd-20-2189.
8. **Abrahamsson TR**, **Jakobsson HE**, **Andersson AF**, **Björkstén B**, **Engstrand L**, **Jenmalm MC.** Low gut microbiota diversity in early infancy precedes asthma at school age. *Clin Exp Allergy* 44: 842–850, 2014. doi:10.1111/cea.12253.
9. **Human Microbiome Project Consortium.** Structure, function and diversity of the healthy human microbiome. *Nature* 486: 207–214, 2012. doi:10.1038/nature11234.
10. **Qin J**, **Li R**, **Raes J**, **Arumugam M**, **Burgdorf KS**, **Manichanh C, et al.** A human gut microbial gene catalogue established by metagenomic sequencing. *Nature* 464: 59–65, 2010. doi:10.1038/nature08821.
11. **Al Bander Z**, **Nitert MD**, **Mousa A**, **Naderpoor N.** The gut microbiota and inflammation: an overview. *Int J Environ Res Public Health* 17: 761, 2020. doi:10.3390/ijerph17207618.
12. **Vangay P**, **Johnson AJ**, **Ward TL**, **Al-Ghalith GA**, **Shields-Cutler RR**, **Hillmann BM**, **Lucas SK**, **Beura LK**, **Thompson EA**, **Till LM**, **Batres R**, **Paw B**, **Pergament SL**, **Saenyakul P**, **Xiong M**, **Kim AD**, **Kim G**, **Masopust D**, **Martens EC**, **Angkurawaranon C**, **McGready R**, **Kashyap PC**, **Culhane-Pera KA**, **Knights D.** US immigration westernizes the human gut microbiome. *Cell* 175: 962–972.e10, 2018. doi:10.1016/j.cell.2018.10.029.
13. **Sommer F**, **Anderson JM**, **Bharti R**, **Raes J**, **Rosenstiel P.** The resilience of the intestinal microbiota influences health and disease. *Nat Rev Microbiol* 15: 630–638, 2017. doi:10.1038/nrmicro.2017.58.
14. **Nascimento CM**, **Casaro MC**, **Perez ER**, **Ribeiro WR**, **Mayer MPA**, **Ishikawa KH**, **Lino-Dos-Santos-Franco A**, **Pereira JNB**, **Ferreira CM.** Experimental allergic airway inflammation impacts gut homeostasis in mice. *Heliyon* 9: e16429, 2023. doi:10.1016/j.heliyon.2023.e16429.
15. **Boscaini S**, **Cabrera-Rubio R**, **Golubeva A**, **Nychyk O**, **Fülling C**, **Speakman JR**, **Cotter PD**, **Cryan JF**, **Nilaweera KN.** Depletion of the gut microbiota differentially affects the impact of whey protein on high-fat diet-induced obesity and intestinal permeability. *Physiol Rep* 9: e14867, 2021. doi:10.14814/phy2.14867.
16. **Li B**, **Li L**, **Li M**, **Lam SM**, **Wang G**, **Wu Y**, **Zhang H**, **Niu C**, **Zhang X**, **Liu X**, **Hambly C**, **Jin W**, **Shui G**, **Speakman JR.** Microbiota depletion impairs thermogenesis of brown adipose tissue and browning of white adipose tissue. *Cell Rep* 26: 2720–2737.e5, 2019. doi:10.1016/j.celrep.2019.02.015.
17. **Bellenger J**, **Bellenger S**, **Bourragat A**, **Escoula Q**, **Weill P**, **Narce M.** Intestinal microbiota mediates the beneficial effects of *n*-3 polyunsaturated fatty acids during dietary obesity. *OCL* 28: 21, 2021. doi:10.1051/ocl/2021006.
18. **Rinninella E**, **Raoul P**, **Cintoni M**, **Franceschi F**, **Miggiano GAD**, **Gasbarrini A**, **Mele MC.** What is the healthy gut microbiota composition? A changing ecosystem across age, environment, diet, and diseases. *Microorganisms* 7: 14, 2019. doi:10.3390/microorganisms7010014.
19. **Marchesi JR**, **Adams DH**, **Fava F**, **Hermes GD**, **Hirschfield GM**, **Hold G**, **Quraishi MN**, **Kinross J**, **Smidt H**, **Tuohy KM**, **Thomas LV**, **Zoetendal EG**, **Hart A.** The gut microbiota and host health: a new clinical frontier. *Gut* 65: 330–339, 2016. doi:10.1136/gutjnl-2015-309990.
20. **Tang Q**, **Jin G**, **Wang G**, **Liu T**, **Liu X**, **Wang B**, **Cao H.** Current sampling methods for gut microbiota: a call for more precise devices. *Front Cell Infect Microbiol* 10: 151, 2020. doi:10.3389/fcimb.2020.00151.
21. **Mao K**, **Baptista AP**, **Tamoutounour S**, **Zhuang L**, **Bouladoux N**, **Martins AJ**, **Huang Y**, **Gerner MY**, **Belkaid Y**, **Germain RN.** Innate and adaptive lymphocytes sequentially shape the gut microbiota and lipid metabolism. *Nature* 554: 255–259, 2018. doi:10.1038/nature25437.
22. **Hiippala K**, **Jouhten H**, **Ronkainen A**, **Hartikainen A**, **Kainulainen V**, **Jalanka J**, **Satokari R.** The potential of gut commensals in reinforcing intestinal barrier function and alleviating inflammation. *Nutrients* 10: 988, 2018. doi:10.3390/nu10080988.
23. **Sankaran-Walters S**, **Macal M**, **Grishina I**, **Nagy L**, **Goulart L**, **Coolidge K**, **Li J**, **Fenton A**, **Williams T**, **Miller MK**, **Flamm J**, **Prindiville T**, **George M**, **Dandekar S.** Sex differences matter in the gut: effect on mucosal immune activation and inflammation. *Biol Sex Differ* 4: 10–12, 2013. doi:10.1186/2042-6410-4-10.
24. **Bridgewater LC**, **Zhang C**, **Wu Y**, **Hu W**, **Zhang Q**, **Wang J**, **Li S**, **Zhao L.** Gender-based differences in host behavior and gut microbiota composition in response to high fat diet and stress in a mouse model. *Sci Rep* 7: 10776, 2017. doi:10.1038/s41598-017-11069-4.
25. **Xie G**, **Wang X**, **Zhao A**, **Yan J**, **Chen W**, **Jiang R**, **Ji J**, **Huang F**, **Zhang Y**, **Lei S**, **Ge K**, **Zheng X**, **Rajani C**, **Alegado RA**, **Liu J**, **Liu P**, **Nicholson J**, **Jia W.** Sex-dependent effects on gut microbiota regulate hepatic carcinogenic outcomes. *Sci Rep* 7: 45232, 2017. doi:10.1038/srep45232.
26. **Sheng L**, **Jena PK**, **Liu H-X**, **Kalanetra KM**, **Gonzalez FJ**, **French SW**, **Krishnan VV**, **Mills DA**, **Wan Y-JY.** Gender differences in bile acids and microbiota in relationship with gender dissimilarity in steatosis induced by diet and FXR inactivation. *Sci Rep* 7: 1748, 2017. doi:10.1038/s41598-017-01576-9.
27. **Park H-J**, **Choi J-M.** Sex-specific regulation of immune responses by PPARs. *Exp Mol Med* 49: e364, 2017. doi:10.1038/emm.2017.102.
28. **Weng M**, **Walker W.** The role of gut microbiota in programming the immune phenotype. *J Dev Orig Health Dis* 4: 203–214, 2013. doi:10.1017/S2040174412000712.
29. **Schuijt TJ**, **Lankelma JM**, **Scicluna BP**, **de Sousa e Melo F**, **Roelofs JJ**, **de Boer JD**, **Hoogendijk AJ**, **de Beer R**, **de Vos A**, **Belzer C**, **de Vos WM**, **van der Poll T**, **Wiersinga WJ.** The gut microbiota plays a protective role in the host defence against pneumococcal pneumonia. *Gut* 65: 575–583, 2016. doi:10.1136/gutjnl-2015-309728.
30. **Fawkner-Corbett D**, **Simmons A**, **Parikh K.** Microbiome, pattern recognition receptor function in health and inflammation. *Best Pract Res Clin Gastroenterol* 31: 683–691, 2017. doi:10.1016/j.bpg.2017.11.001.
31. **Yurkovetskiy L**, **Burrows M**, **Khan AA**, **Graham L**, **Volchkov P**, **Becker L**, **Antonopoulos D**, **Umesaki Y**, **Chervonsky AV.** Gender bias in autoimmunity is influenced by microbiota. *Immunity* 39: 400–412, 2013. doi:10.1016/j.immuni.2013.08.013.
32. **Bolnick DI**, **Snowberg LK**, **Hirsch PE**, **Lauber CL**, **Org E**, **Parks B**, **Lusis AJ**, **Knight R**, **Caporaso JG**, **Svanbäck R.** Individual diet has sex-dependent effects on vertebrate gut microbiota. *Nat Commun* 5: 4500, 2014. doi:10.1038/ncomms5500.
33. **Park JY**, **Choi J**, **Lee Y**, **Lee JE**, **Lee EH**, **Kwon HJ**, **Yang J**, **Jeong BR**, **Kim YK**, **Han PL.** Metagenome analysis of bodily microbiota in a mouse model of Alzheimer disease using bacteria-derived membrane vesicles in blood. *Exp Neurobiol* 26: 369–379, 2017. doi:10.5607/en.2017.26.6.369.
34. **Gomez A**, **Luckey D**, **Taneja V.** The gut microbiome in autoimmunity: sex matters. *Clin Immunol* 159: 154–162, 2015. doi:10.1016/j.clim.2015.04.016.
35. **Markle JG**, **Frank DN**, **Mortin-Toth S**, **Robertson CE**, **Feazel LM**, **Rolle-Kampczyk U**, **Von Bergen M**, **McCoy KD**, **Macpherson AJ**, **Danska JS.** Sex differences in the gut microbiome drive hormone-dependent regulation of autoimmunity. *Science* 339: 1084–1088, 2013. doi:10.1126/science.1233521.
36. **Org E**, **Mehrabian M**, **Parks BW**, **Shipkova P**, **Liu X**, **Drake TA**, **Lusis AJ.** Sex differences and hormonal effects on gut microbiota composition in mice. *Gut Microbes* 7: 313–322, 2016. doi:10.1080/19490976.2016.1203502.
37. **Shastri P**, **McCarville J**, **Kalmokoff M**, **Brooks SP**, **Green-Johnson JM.** Sex differences in gut fermentation and immune parameters in rats fed an oligofructose-supplemented diet. *Biol Sex Differ* 6: 13, 2015. doi:10.1186/s13293-015-0031-0.
38. **Ekpruke CD**, **Silveyra P.** Sex differences in airway remodeling and inflammation: clinical and biological factors. *Front Allergy* 3: 875295, 2022. doi:10.3389/falgy.2022.875295.
39. **von Mutius E.** Gene-environment interactions in asthma. *J Allergy Clin Immunol* 123: 3–11, 2009. quiz 12–13 doi:10.1016/j.jaci.2008.10.046.
40. **Vemuri R**, **Sylvia KE**, **Klein SL**, **Forster SC**, **Plebanski M**, **Eri R**, **Flanagan KL.** The microgenderome revealed: sex differences in bidirectional interactions between the microbiota, hormones, immunity and disease susceptibility. *Semin Immunopathol* 41: 265–275, 2019. doi:10.1007/s00281-018-0716-7.
41. **Arnold AP.** Four core genotypes and XY* mouse models: update on impact on SABV research. *Neurosci Biobehav Rev* 119: 1–8, 2020. doi:10.1016/j.neubiorev.2020.09.021.
42. **Bracken SJ**, **Adami AJ**, **Szczepanek SM**, **Ehsan M**, **Natarajan P**, **Guernsey LA**, **Shahriari N**, **Rafti E**, **Matson AP**, **Schramm CM**, **Thrall**

RS. Long-term exposure to house dust mite leads to the suppression of allergic airway disease despite persistent lung inflammation. *Int Arch Allergy Immunol* 166: 243–258, 2015. doi:10.1159/000381058.

43. **Mostafa DH**, **Hemshekhar M**, **Piyadasa H**, **Altieri A**, **Halayko AJ**, **Pascoe CD**, **Mookherjee N.** Characterization of sex-related differences in allergen house dust mite-challenged airway inflammation, in two different strains of mice. *Sci Rep* 12: 20837, 2022. doi:10.1038/s41598-022-25327-7.
44. **Ekpruke CD**, **Alford R**, **Rousselle D**, **Babayev M**, **Sharma S**, **Commodore S**, **Buechlein A**, **Rusch DB**, **Silveyra P.** Transcriptomics analysis of allergen-induced inflammatory gene expression in the four-core genotype mouse model. *Physiol Genomics* 56: 235–245, 2023. doi:10.1152/physiolgenomics.00112.2023.
45. **Segata N**, **Izard J**, **Waldron L**, **Gevers D**, **Miropolsky L**, **Garrett WS**, **Huttenhower C.** Metagenomic biomarker discovery and explanation. *Genome Biol* 12: R60, 2011. doi:10.1186/gb-2011-12-6-r60.
46. **Palmer MW.** *Ordination Methods – An Overview*. Stillwater, OK: Oklahoma State University, 2004, p. 74078.
47. **Commodore S**, **Ekpruke CD**, **Rousselle D**, **Alford R**, **Babayev M**, **Sharma S**, **Buechlein A**, **Rusch DB**, **Silveyra P.** Lung pro-inflammatory microRNA and cytokine expression in a mouse model of allergic inflammation: role of sex chromosome complement and gonadal hormones. *Physiol Genomics* 56: 179–193, 2023. doi:10.1152/physiolgenomics.00049.2023.
48. **Cahenzli J**, **Köller Y**, **Wyss M**, **Geuking MB**, **McCoy KD.** Intestinal microbial diversity during early-life colonization shapes long-term IgE levels. *Cell host Microbe* 14: 559–570, 2013. doi:10.1016/j.chom.2013.10.004.
49. **Sjögren YM**, **Tomicic S**, **Lundberg A**, **Böttcher MF**, **Björkstén B**, **Sverremark-Ekström E**, **Jenmalm MC.** Influence of early gut microbiota on the maturation of childhood mucosal and systemic immune responses: gut microbiota and immune responses. *Clin Exp Allergy* 39: 1842–1851, 2009. doi:10.1111/j.1365-2222.2009.03326.x.
50. **Wang S**, **Hibberd ML**, **Pettersson S**, **Lee YK.** Enterococcus faecalis from healthy infants modulates inflammation through MAPK signaling pathways. *PLoS One* 9: e97523, 2014. doi:10.1371/journal.pone.0097523.
51. **West CE**, **Jenmalm M**, **Prescott S.** The gut microbiota and its role in the development of allergic disease: a wider perspective. *Clin Exp Allergy* 45: 43–53, 2015. doi:10.1111/cea.12332.
52. **Hufnagl K**, **Pali-Schöll I**, **Roth-Walter F**, **Jensen-Jarolim E.** Dysbiosis of the gut and lung microbiome has a role in asthma. *Semin Immunopathol* 42: 75–93, 2020. doi:10.1007/s00281-019-00775-y.
53. **Lal CV**, **Travers C**, **Aghai ZH**, **Eipers P**, **Jilling T**, **Halloran B**, **Carlo WA**, **Keeley J**, **Rezonzew G**, **Kumar R**, **Morrow C**, **Bhandari V**, **Ambalavanan N.** The airway microbiome at birth. *Sci Rep* 6: 31023, 2016. doi:10.1038/srep31023.
54. **Zhang D**, **Li S**, **Wang N**, **Tan HY**, **Zhang Z**, **Feng Y.** The cross-talk between gut microbiota and lungs in common lung diseases. *Front Microbiol* 11: 301, 2020. doi:10.3389/fmicb.2020.00301.
55. **Fujio-Vejar S**, **Vasquez Y**, **Morales P**, **Magne F**, **Vera-Wolf P**, **Ugalde JA**, **Navarrete P**, **Gotteland M.** The gut microbiota of healthy chilean subjects reveals a high abundance of the phylum *Verrucomicrobia*. *Front Microbiol* 8: 1221, 2017. doi:10.3389/fmicb.2017.01221.
56. **Zhang L**, **Qin Q**, **Liu M**, **Zhang X**, **He F**, **Wang G.** *Akkermansia muciniphila* can reduce the damage of gluco/lipotoxicity, oxidative stress and inflammation, and normalize intestine microbiota in streptozotocin-induced diabetic rats. *Pathog Dis* 76: fty028, 2018. doi:10.1093/femspd/fty028.
57. **de Vos WM.** Microbe profile: *Akkermansia muciniphila*: a conserved intestinal symbiont that acts as the gatekeeper of our mucosa. *Microbiology (Reading)* 163: 646–648, 2017. doi:10.1099/mic.0.000444.
58. **Le Chatelier E**, **Nielsen T**, **Qin J**, **Prifti E**, **Hildebrand F**, **Falony G**, **et al.** Richness of human gut microbiome correlates with metabolic markers. *Nature* 500: 541–546, 2013. doi:10.1038/nature12506.
59. **Xiong Y**, **Hu S**, **Zhou H**, **Zeng H**, **He X**, **Huang D**, **Li X.** High-throughput 16S rDNA sequencing of the pulmonary microbiome of rats with allergic asthma. *Genes Dis* 7: 272–282, 2020. doi:10.1016/j.gendis.2019.03.006.
60. **Hevia A**, **Milani C**, **López P**, **Donado CD**, **Cuervo A**, **González S**, **Suárez A**, **Turroni F**, **Gueimonde M**, **Ventura M**, **Sánchez B**, **Margolles A.** Allergic patients with long-term asthma display low levels of *Bifidobacterium adolescentis*. *PLoS One* 11: e0147809, 2016. doi:10.1371/journal.pone.0147809.
61. **Waligora-Dupriet AJ**, **Campeotto F**, **Romero K**, **Mangin I**, **Rouzaud G**, **Ménard O**, **Suau A**, **Soulaines P**, **Nicolis I**, **Kapel N**, **Dupont C**, **Butel MJ.** Diversity of gut *Bifidobacterium* species is not altered between allergic and non-allergic French infants. *Anaerobe* 17: 91–96, 2011. doi:10.1016/j.anaerobe.2011.04.003.
62. **Ferraris L**, **Aires J**, **Waligora-Dupriet A-J**, **Butel M-J.** New selective medium for selection of bifidobacteria from human feces. *Anaerobe* 16: 469–471, 2010. doi:10.1016/j.anaerobe.2010.03.008.
63. **Mullié C**, **Odou M-F**, **Singer E**, **Romond M-B**, **Izard D.** Multiplex PCR using 16S rRNA gene-targeted primers for the identification of bifidobacteria from human origin. *FEMS Microbiol Lett* 222: 129–136, 2003. doi:10.1016/S0378-1097(03)00245-3.
64. **Fallani M**, **Rigottier-Gois L**, **Aguilera M**, **Bridonneau C**, **Collignon A**, **Edwards CA**, **Corthier G**, **Doré J.** *Clostridium difficile* and *Clostridium perfringens* species detected in infant faecal microbiota using 16S rRNA targeted probes. *J Microbiol Methods* 67: 150–161, 2006. doi:10.1016/j.mimet.2006.03.010.
65. **Pickard JM**, **Zeng MY**, **Caruso R**, **Núñez G.** Gut microbiota: role in pathogen colonization, immune responses, and inflammatory disease. *Immunol Rev* 279: 70–89, 2017. doi:10.1111/imr.12567.
66. **Valverde-Molina J**, **García-Marcos L.** Microbiome and asthma: microbial dysbiosis and the origins, phenotypes, persistence, and severity of asthma. *Nutrients* 15: 486, 2023. doi:10.3390/nu15030486.
67. **Sohn KH**, **Choi S**, **Jung JW**, **Choi JH**, **Cho SH**, **Yi H**, **Kang HR.** Different inflammatory features of asthma according to gut microbiome enterotype. *Allergy* 78: 2997–3001, 2023. doi:10.1111/all.15768.
68. **Lee JH**, **Choi JP**, **Yang J**, **Won HK**, **Park CS**, **Song WJ**, **Kwon HS**, **Kim TB**, **Kim YK**, **Park HS**, **Cho YS.** Metagenome analysis using serum extracellular vesicles identified distinct microbiota in asthmatics. *Sci Rep* 10: 15125, 2020. doi:10.1038/s41598-020-72242-w.
69. **Zou XL**, **Wu JJ**, **Ye HX**, **Feng DY**, **Meng P**, **Yang HL**, **Wu WB**, **Li HT**, **He Z**, **Zhang TT.** Associations between gut microbiota and asthma endotypes: a cross-sectional study in South China based on patients with newly diagnosed asthma. *J Asthma Allergy* 14: 981–992, 2021. doi:10.2147/JAA.S320088.
70. **Hua X**, **Goedert JJ**, **Pu A**, **Yu G**, **Shi J.** Allergy associations with the adult fecal microbiota: analysis of the American Gut Project. *EBioMedicine* 3: 172–179, 2016. doi:10.1016/j.ebiom.2015.11.038.