Discovery of a Novel Seminal Fluid Microbiome and Influence of Estrogen Receptor Alpha Genetic Status

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Bacteria harbored in the male reproductive system may influence reproductive function and health of the male and result in developmental origins of adult health and disease (DOHaD) effects in his offspring. Such effects could be due to the seminal fluid, which is slightly basic and enriched with carbohydrates; thereby, creating an ideal habitat for microbes or a potential seminal fluid microbiome (SFM). Using wild-type (WT) and estrogen receptor-alpha (ESR1) knockout (KO) male mice, we describe a unique SFM whose inhabitants differ from gut microbes. The bacterial composition of the SFM is influenced according to whether mice have functional Esr1 genes. Propionibacterium acnes, causative agent of chronic prostatitis possibly culminating in prostate cancer, is reduced in SFM of ESR1 KO compared to WT mice ($P \leq 0.0007$). In certain genetic backgrounds, WT mice show a greater incidence of prostate cancer than ESR1 KO, which may be due to increased abundance of $P. \text{acnes}$. Additionally, select gut microbiome residents in ESR1 KO males, such as Lachnospiraceae and Christensenellaceae, might contribute to previously identified phenotypes, especially obesity, in these mutant mice. Understanding how genetics and environmental factors influence the SFM may provide the next frontier in male reproductive disorders and possibly paternal-based DOHaD diseases.

The future health of offspring can be influenced by the condition of the male parent1–9. For example, paternal obesity in mice has been linked to delayed development, altered carbohydrate utilization, and mitochondrial disturbances10 and various other health effects, such as obesity, cardiovascular and reproductive disorders in F1 pups1,2,10–16. Analogous effects have been noted in humans16–18, but in neither species has the mechanism of transmission to the offspring been defined.

Paternal condition has been proposed to give rise to F1 offspring developmental origins of health and disease (DOHaD) and trans-generational (transmission to future descendants) effects through three mechanisms. The first is direct effects on the epigenome of male germ cells, such as alterations in DNA methylation status19 and microRNAs (miRs), piRNAs, enhancer-derived RNAs (eRNAs), and tRNA fragments (tRFs)20–23. The latter may be produced and released by epithelial cells of the epididymis and then taken-up by the spermatozoa as they mature. Histone protein modifications in sperm may also lead to phenotypic alterations in F1 offspring and F2 descendants24.

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A second proposed mechanism for paternal transmission of information to the F1 generation is via seminal fluid content, which could impact female reproductive tract physiology and conceptus development. A third proposed possible mechanism is that the female responds to the perceived fitness of her partner by modulating her investment in his offspring. In a recent review, it has been speculated that fathers may transmit information via microbiota to their partners and progeny. The most likely source of such microbiota is the seminal vesicles.

Almost all mammalian males, except carnivores, possess seminal vesicles (also termed vesiculagnan glands). These glands are simple tubular and situated below the urinary bladder and vas deferens. In humans, approximately 50–70% of the seminal fluid is produced by the seminal vesicles. This fluid is slightly basic (pH < 7.2) and contains fructose, proteins, enzymes, mucus, vitamin C, flavins, phosphorylcholine, and prostaglandins. Fructose is thought to provide the primary source of metabolic energy for the spermatozoa, but this nutrient can also be utilized by microorganisms, such as fructophilic lactic acid bacteria (FLAB). Therefore, the seminal vesicles could provide a unique niche for such microorganisms to thrive. The seminal vesicles also express ESR1 and 2, suggesting that their secretory activity might be affected by endogenous and environmental estrogenic hormones. Experiments performed on mice null for ESR1 and ESR2 also implicate estrogens in regulating the function of the seminal glands.

Bacteria are present in semen samples of men and can be transmitted to their female sexual partners, but it is unclear whether these bacteria originate from colonies established in the seminal vesicles or elsewhere in the male reproductive and urinary tract systems. To address whether the seminal vesicles harbor a distinct microbiome and whether the composition of this population of microorganisms can be potentially influenced by estrogens, we collected the seminal fluid and tissue from ESR1 KO and wild-type (WT) male mice. Fecal samples from the same animals were collected to determine the extent to which the putative SFM resembled that of the large bowel.

Results

Comparison of the seminal fluid microbiome to the fecal microbiome. The first goal was to ascertain whether the seminal fluid harbored a unique microbiome. By sequencing the V4 region of the 16S rRNA gene on the Illumina MiSeq, several microbiota were identified in the seminal fluid of WT and ESR1 KO males. Collectively, they comprise a seminal fluid microbiome (SFM, Fig. 1). After identifying the presence of a SFM, we sought to determine whether the microbial community in this region differed from that present in the fecal samples of the same genotype mice. We chose this comparison as the gut or fecal microbiome is one of the best characterized to date.

When the 16S rRNA sequencing results were compared by using Greengenes Version 13.8 (which is available through QIIME, http://qiime.org/home_static/dataFiles.html ftp://greengenes.microbio.me/greengenes_release/gg_13_5/gg_13_8.otus.tar.gz), clear distinctions were evident in the various bacterial classes when comparing fecal to seminal fluid samples (Fig. 1A). A PcoA analysis and cladogram further confirmed that the classes of bacteria inhabiting these two biological samples clustered separately (Fig. 1B and C, p ≤ 0.0001 by PERMANOVA). The Venn diagram revealed that of 2690 operational taxonomic units (OTUs) only 285 were common to both sample types. Taken together, the data strongly support the finding that the seminal fluid harbors a unique microbiome relative to that present in the fecal samples.

Alpha-diversity was analyzed by plotting and comparing the Chao1 and Shannon-Weiner indices for each sample type (Supplementary Fig. 1). Additionally, rarefaction metrics were plotted and compared between the two sample types. Taken together, these data indicate that the seminal fluid microbial community is somewhat less species-rich and less diverse than the fecal community.

LEfSe comparison (Fig. 2) of microbiota in the seminal fluid or fecal samples also emphasizes the uniqueness of the seminal fluid microbiome. The genera listed in green, most consistently describe seminal fluid. In contrast, those listed in red are the signature of fecal samples. The SFM was characterized by a preponderance of Bacilli, Proteobacteria, Actinobacteria, Fusobacteria, Flavobacteria, and Acidobacteria (P ≤ 0.05, Fig. 2). In contrast, Deferribacteres, Enericutes, Verrucomicrobia, Deltaproteobacteria, Erysipelotrichi, Clostridia, and Bacteroidetes are more consistently present in the fecal samples compared to the seminal fluid samples (P ≤ 0.05, Fig. 2). Taken together, the data strongly support the finding that the seminal fluid harbors a unique microbiome relative to that present in the fecal samples.

KEGG metabolic pathways correlating with fecal and seminal fluid microbiome differences are listed in Supplementary Data 1, and select examples are illustrated in Fig. 3. Based on the classes that differed between the fecal and SFM and KEGG correlation analyses, it is likely that the disparities in microbial composition noted above, would also contribute to a distinct metabolic milieu in seminal fluid, including changes in amino acids, carbohydrates, energy, glycan and lipid metabolites, cofactors, vitamins, and terpenoids metabolites, that could in turn impact host cell functions.

Influence of age on the seminal fluid and fecal microbiomes. As part of these studies, we screened various ages of adult WT and ESR1 KO mice (Supplementary Fig. 2A, where the fecal and seminal fluid microbiomes are organized by age of the animals). This was done to increase the likelihood of detecting a SFM, should it exist. To determine if age altered the SFM and fecal microbiome, PCoA and PERMANOVA analysis were done based on age for both microbiomes. Age did not alter the SFM composition (Supplementary Fig. 2B, p = 0.4 by PERMANOVA). However, the fecal microbiome was altered by age (Supplementary Fig. 2C, p = 0.01 by PERMANOVA). To determine which OTUs differed by age, metagenomeSeq was performed. Thirty OTUs differed based on age. Top genera that were increased in older compared to young males include Lactobacillus, Peptostreptococcaceae, Bifidobacterium pseudolongum, Clostridiales, and Mollicutes (Supplementary Table 1). In contrast, Lachnospiraceae, Dehalobacterium, Bacteroides acidifaciens, Oscillospira, and another Peptococcaceae were more abundant in younger than older animals.
Influence of estrogen receptor 1 gene status on the seminal fluid microbiome. We next investigated whether ESR1 gene status had an impact on the microbiota present in the seminal fluid samples. While the majority of OTUs occurred in both the seminal fluid of WT and ESR1 KO males, each genotype also possessed genera not present in the other genotype (Fig. 4A), although these differences were insufficient to lead to statistically significant clustering of the samples, as illustrated in the PCoA analysis (Fig. 4B). To investigate the potentially subtle microbiome differences in the seminal fluid of these two genotypes, we used metagenomeSeq. This analysis identified several genera that characterized the SFM of WT or ESR1 KO males (Table 1). For example, *Propionibacterium acnes* was much more prevalent in the seminal fluid of WT than ESR1-/- mice, *p* = 0.0007. Other genera resulting in significant positive and negative metabolic pathway correlations in the seminal fluid of WT males included *Streptococcus*, *Anaerococcus*, *Lactococcus*, *Staphylococcus*, *Pseudomonas veronii*, *Finegoldia*, *Acinetobacter*, *Neisseriaceae*, and *Sphingomonas*. In ESR1 KO males, several metabolic pathway alterations correlated with microbiome changes in the seminal fluid. In this group, increased abundance of *P. acnes* positively correlated with several amino acid metabolic pathways but negatively correlated with signal transduction mechanisms. Other genera resulting in significant positive
and negative metabolic pathway correlations in the seminal fluid of ESR1 KO males included Streptococcus, Haemophilus parainfluenzae, Pseudomonas, Alcanivorax diselolei, Staphylococcus, Achromobacter, Gemellaceae, Nocardioidaceae, Corynebacterium, Peptoniphilus, Brevibacterium, Pseudomonas veronii, Actinomyces, Alloiococcus, Pseudomonas viridiflava, Acinetobacter, Neisseriaceae, Sphingomonas, and Xanthomonadaceae.

Influence of estrogen receptor 1 gene status on the fecal microbiome. As with the seminal fluid results, the fecal samples from WT and ESR1 KO males had more OTUs in common than were unique to each genotype (Fig. 6A). However, these differences did not lead to distinct clusters for each genotype in cluster analysis, as illustrated in Fig. 6B. Analysis with metagenomeSeq revealed several OTUs that differed in the fecal samples of WT compared to ESR1 KO males. The fecal samples of WT males were enriched with Ruminococcus, Dehalobacterium, Dorea, Sutterella, and Oscillospira; whereas, Parabacteroides, Coprococcus, and Clostridium were greater in the fecal samples of ESR1 KO males (Table 2).

The fecal microbiome changes in WT and ESR1 KO were correlated with alterations in several metabolic and other pathways (Fig. 7 and Supplementary Data 3). In WT, the genera that resulted in the most pronounced correlations included Coprococcus, Clostridiales, Bacteroidales, Bifidobacterium pseudolongum, Turicibacter, Allobaculum, Lachnospiraceae, Mogibacteriaceae, Sutterella, Oscillospira, Ruminococcaceae, Bacteroides acidifaciens and sp. Those that resulted in the greatest associations in ESR1 KO males were Clostridiales, Parabacteroides, Bacteroidales, Bifidobacterium pseudolongum, Turicibacter, Allobaculum, Lachnospiraceae, Sutterella, Oscillospira, Dehalobacterium, Ruminococcaceae, Bacteroides acidifaciens, and Clostriaceae.

Figure 2. LEfSe comparison of microbiota in seminal fluid or fecal samples. The genera listed in green most consistently describe seminal fluid. In contrast, those listed in red most consistently describe fecal samples. P values are listed by genera.
Discussion
Speculation exists as to whether the seminal vesicles harbor a unique microbiome. The current studies confirm that they do so, at least in mice. The occupants of the SFM are distinct from those residing in the fecal microbiome of these mice. This finding is not surprising as the microhabitat, including nutrient substrate availability, within each of these biological samples differs immensely. Therefore, the microbiomes present within each of these environments have seemingly evolved over time to the specific qualities of each niche. The bacterial phyla that dominate in the seminal fluid, including Proteobacteria, Actinobacteria, Fusobacteria, and Firmicutes may be able to utilize fructose and other carbohydrates produced by the seminal fluid glands as energy sources.
The microbiome alterations in the seminal fluid may also induce changes in virulence factors and the metabolic milieu in these secretions. As shown in Fig. 5, P. acnes, Streptophyta spp, Corynebacterium spp, Peptoniphilus spp, and Acinetobacter spp in WT males and P. acnes, H. parainfluenzae, Acinetobacter spp, and Sphingomonas are positively associated with significant amounts of metabolic pathway changes in the seminal fluid. Thus, some of the previously identified diet-induced metabolite changes in the seminal fluid may represent a combination of bacterial and host metabolites. The former may influence male reproductive function and overall health (including risk for prostate cancer in the case of P. acnes). The genome of P. acnes encodes all of the essential components of oxidative phosphorylation. In WT males, increased abundance of this bacterium is associated with greater oxidative phosphorylation metabolic pathways, which may increase the amount of reactive oxygen species, damage free radicals, and increase the risk for diseases, including prostate cancer. Additionally, transfer of the microbiome to a reproductive partner could influence her reproduction/health, as well as that of the resulting offspring, and even more distant descendants. Therefore, a better understanding of the SFM and how it might be influenced by intrinsic and extrinsic factors is essential.

The microbiomes in fecal samples from WT and ESR1 KO were analyzed in these studies primarily to determine how they compared to the SFM. However, our findings may also partially explain some of the prior phenotypic studies with these animals. For instance, ESR1 KO mice demonstrate obesity, adipocyte hyperplasia and hypertrophy, insulin resistance, and impaired glucose tolerance. At the time, these and other phenotypic disruptions identified in this transgenic mouse model were presumed to be mediated by direct ablation of the ESR1 gene. However, gut dysbiosis can result in metabolic, neurological, and other disease states. For instance, two Lachnospiraceae were increased in the fecal samples of ESR1 KO males (Table 2), and gut colonization by this bacterial family is associated with increased body weight and hyperglycemia in germ-free ob/ob mice. Christensenellaceae is another bacterium augmented in the stool of this group. Increased abundance of this
Bolded genera are greater in ESR1 KO males; whereas non-bolded genera are more abundant in metagenomeSeq.

Table 1. OTUs that differ in the seminal fluid between WT and ESR1 KO mice, as determined by metagenomeSeq. Bolded genera are greater in ESR1 KO males; whereas non-bolded genera are more abundant in WT males. *Bacterium associated with chronic prostatitis culminating in prostate cancer in men and rodent models.

| Bacterial Taxonomy | Log 2 Fold Change (WT vs. ESR1 KO) | Adjusted P Value |
|--------------------|------------------------------------|------------------|
| Proteobacteria; Gammaproteobacteria; Pseudomonadales; Pseudomonadaceae | −2.30 | 0.0141 |
| Firmicutes; Bacilli; Lactobacillales; Aerococcaceae; Alloprevotella | −1.90 | 0.0183 |
| Proteobacteria; Betaproteobacteria; Rhodocyclales; Rhodocyclaceae | −1.63 | 0.0171 |
| Actinobacteria; Actinomycetales; Nocardioidaceae | −1.62 | 0.0103 |
| Proteobacteria; Alphaproteobacteria; Sphingomonadales; Sphingomonadaceae; Sphingomonas | −1.67 | 0.0357 |
| Proteobacteria; Gammaproteobacteria; Pseudomonadales; Pseudomonadaceae; Pseudomonas; viridiflava | −0.913 | 0.0212 |
| Firmicutes; Bacilli; Lactobacillales; Streptococcaeae; Streptococcus | −0.750 | 0.0062 |
| Bacteroidetes; Sphingobacteria; Sphingobacteriales; Sphingobacteriaceae; Pedobacter | −0.698 | 0.0141 |
| Actinobacteria; Actinomycetales; Brevibacteriaceae; Brevibacterium | −0.491 | 0.0162 |
| Actinobacteria; Actinomycetales; Actinomycetaceae; Actinomyces | −0.465 | 0.0167 |
| Proteobacteria; Gammaproteobacteria; Pseudomonadales; Moraxellaceae; Acinetobacter | −0.407 | 0.0212 |
| Firmicutes; Bacilli; Gemellales; Gemellaceae | −0.383 | 0.0103 |
| Proteobacteria; Gammaproteobacteria; Xanthomonadales; Xanthomonadaceae | −0.359 | 0.0483 |
| Firmicutes; Clostridia; Clostridiales; [Tissierellaceae]; Peptoniphilus | −0.328 | 0.0112 |
| Actinobacteria; Actinomycetales; Corynebacteriaceae; Corynebacterium | −0.174 | 0.0106 |
| Cyanobacteria; Chloroplast; Streptophyta | −0.126 | 0.0103 |
| Proteobacteria; Betaproteobacteria; Burkholderiales; Alcaligenaceae; Achromobacter | −0.111 | 0.0099 |
| Firmicutes; Bacilli; Lactobacillales; Streptococcaeae; Lactococcus | 0.171 | 0.0095 |
| Proteobacteria; Gammaproteobacteria; Pseudomonadales; Pseudomonas | 0.185 | 0.0095 |
| Proteobacteria; Gammaproteobacteria; Pasteurellales; Pasteurellaceae; Haemophilus; parainfluenzae | 0.229 | 0.0063 |
| Proteobacteria; Gammaproteobacteria; Oceansporillales; Alcanivoracaceae; Alcanivorax; dieseloei | 0.243 | 0.0095 |
| Firmicutes; Clostridia; Clostridiales; [Tissierellaceae]; Anaerococcus | 0.243 | 0.0062 |
| Proteobacteria; Alphaproteobacteria; Rhizobiales; Methylobacteriaceae; Methylobacterium | 0.312 | 0.0162 |
| Actinobacteria; Actinomycetales; Propionibacteriaceae; Propionibacterium; acnes* | 0.337 | 0.0007 |
| Firmicutes; Bacilli; Bacillales; Staphylococcaceae; Staphylococcus; equorum | 0.409 | 0.0162 |
| Firmicutes; Bacilli; Lactobacillales | 0.503 | 0.0063 |
| Firmicutes; Bacilli; Bacillales; Staphylococcaceae; Staphylococcus | 0.641 | 0.0099 |
| Proteobacteria; Gammaproteobacteria; Pseudomonadales; Pseudomonadaceae; Pseudomonas; veronii | 0.680 | 0.0162 |
| Firmicutes; Bacilli; Lactobacillales; Lactobacillaceae; Lactobacillus; iners | 0.717 | 0.0063 |
| Proteobacteria; Betaproteobacteria; Neisseriales; Neisseriaceae; | 0.838 | 0.0316 |
| Proteobacteria; Betaproteobacteria; Burkholderiales; Comamonadaceae; | 0.971 | 0.0103 |
| Firmicutes; Clostridia; Clostridiales; [Tissierellaceae]; Finegoldia | 1.05 | 0.0171 |
| Proteobacteria; Betaproteobacteria; Burkholderiales; Comamonadaceae; Schlegelella; | 1.08 | 0.0104 |

In conclusion, we have made the novel discovery that the seminal vesicles harbor a unique microbiome. The microbial composition of the SFM can be affected by whether mice possess functional Esr1 genes. Microbiome shifts in the SFM and the potential changes in the abundance of concomitant metabolic pathways may impact male reproduction and health, his female reproductive partners, offspring, and future descendants. Thus, understanding the SFM and how the resident populations may be influenced by genetic background and environmental factors may spawn future research in biomedical sciences. Increased abundance of P. acnes and associated metabolic pathways in the seminal fluid of WT animals might render them at greater risk for prostate cancer under certain genetic and environmental conditions. Lastly, dysbiosis in the fecal microbiome of ESR1 KO mice might induce systemic effects.
**Methods**

**Animals.** The animal experiments were approved by the University of Missouri Animal Care and Use Committee (Protocol #7948) and performed in accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. Mice were ad libitum fed 5008 Purina Chow (Bourn Feed, Columbia, MO) and provided free access to water. They were maintained on a 12 hr light: 12 hr dark cycle with the lights on at 7:30 hrs and off at 19:30 hrs. To generate WT and ESR1 KO mice, males and females heterozygous for the ESR1 gene were bred. At weaning (3 weeks of age), approximately 1–2 mm section of tail was excised, DNA was isolated, and the genotype status for ESR1 determined as reported previously64.

**Collection of fecal and seminal fluid samples.** Prior to euthanasia, each animal was placed one per cage without any bedding. Adult males ranging in age from 3 to 13 months of age were used for these studies. Four to five fecal boli were collected from each animal. We collected the fecal samples ex vivo to replicate the approach used in similar studies65,66 and to ensure that the bacterial composition was reflective of the entire gastrointestinal system, including the rectum and anus. The animals were then intra-peritoneally injected with ~0.8 to 0.9 ml of 1.2% concentration of avertin (Sigma-Aldrich, Saint Louis, Missouri). After they were anesthetized, as evidenced by lack of corneal and leg withdrawal reflexes, the fur from the abdominal region extending from the xiphoid process to the pelvic region was shaved with a Wahl Clipper Corp, model 9962 (Wahl, Shelton, CT). The animals were then surgically scrubbed with a combination of 75% BD alcohol swabs (Becton, Dickinson and Company; Franklin Lakes, NJ) and betadine (Equate, Bentonville, Arkansas). The surgically prepped males were placed in

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**Figure 5.** Bacterial metabolic and other pathway differences in the seminal fluid samples of WT vs. ESR1 KO mice. As described in Fig. 7 of Ma et al.66, correlations between the PICRUSt-generated functional profile and QIIME-generated genus level bacterial abundance were calculated and plotted against genotype status for the seminal fluid samples in WT and ESR1 KO mice. Those genera that metagenomeSeq identified as being different between the two genotypes are depicted. Metabolic pathway designations are delineated at the bottom of the figure. Shading intensity and size of the circles indicates the Kendall rank correlation coefficient between matrices. Orange/red indicates a positive correlation; whereas blue designates a negative correlation. Red squares surrounding the circles are indicative of a P value ≤ 0.05. To aid in interpretation of the figure, the log2 values listed in Table 1 are included alongside the genera. Negative values indicate that the OTU is greater in the SFM of ESR1 KO males; whereas, positive values are greater in SFM of WT animals. The same number of replicates was analyzed for these data as listed in Fig. 1.
a laminar flow-hood (Nuaire NU 407–600, Plymouth, Minnesota) that had been previously cleaned with bleach and 75% EtOH followed by overnight UV radiation. The two experimenters surgically scrubbed their hands with E-Z scrub 205 betadine sponges (Becton, Dickinson and Company; Franklin Lakes, NJ) and donned a sterile surgical gown, gloves, and mask. After the animals were placed in the hood, the abdominal skin and muscles and linea alba were incised. The seminal vesicles were excised and placed into a petri dish. The seminal fluid was then gently extruded into a 2.0 ml Corning Cryogenic Vial (Corning Incorporated, Corning, NY). The fecal and seminal fluid samples were placed on dry ice and then stored at −80 °C until the samples were processed.

Isolation of seminal fluid and fecal microbial DNA. The microbial DNA from seminal fluid samples was isolated with either the Ultra-Deep Microbiome Prep kit (CaerusBio Inc., Downington, PA) or Qiamp DNA microbiome kit (Qiagen, Valencia, CA). Both of these kits destroy and remove any host cells in the process. The fecal microbial DNA was isolated using the PowerFecal DNA Isolation kit (Mo Bio Laboratories, Inc., Carlsbad, CA).

### Table 2. OTUs that differ in the fecal microbiome between WT and ESR1 KO males, as determined by metagenomeSeq.

| Bacterial Taxonomy | Log 2 Fold Change (WT vs. ESR1 KO) | Adjusted P Value |
|--------------------|------------------------------------|------------------|
| Bacteroidetes; Bacteroidia; Bacteroidales; Bacteroides | −0.488 | 0.0198 |
| Cyanobacteria; 4C0d-2; YS2 | −0.432 | 0.0054 |
| Bacteroidetes; Bacteroidia; Porphyromonadaceae; Parabacteroides | −0.191 | 0.0025 |
| Firmicutes; Clostridia; Clostridiales; Lachnospiraceae; Coprococcus | −0.147 | 0.0003 |
| Bacteroidetes; Bacteroidia; Rikenellaceae | −0.070 | 0.0038 |
| Firmicutes; Clostridia; Clostridiales; Lachnospiraceae | −0.049 | 0.0054 |
| Firmicutes; Clostridia; Clostridiales; Christensenellaceae | −0.030 | 0.0031 |
| Bacteroidetes; Bacteroidia; Bacteroidales; Bacteroides; acidifaciens | 0.108 | 0.0249 |
| TM7; TM7-3; CW040; F16 | 0.140 | 0.0071 |
| Firmicutes; Clostridia; Clostridiales; Ruminococcaceae | 0.145 | 0.0156 |
| Firmicutes; Clostridia; Clostridiales | 0.158 | 0.0005 |
| Firmicutes; Clostridia; Clostridiales; Dehalobacteriaceae; Dehalobacterium | 0.164 | 0.0087 |
| Firmicutes; Clostridia; Clostridiales; Lachnospiraceae; Dorea | 0.240 | 0.0002 |
| Bacteroidetes; Bacteroidia; Bacteroidales | 0.295 | 0.0038 |
| Proteobacteria; Betaproteobacteria; Burkholderiales; Alcaligenaceae; Sutterella | 0.342 | 0.0071 |
| Firmicutes; Clostridia; Clostridiales; [Mogibacteriaceae] | 0.372 | 0.0054 |
| Firmicutes; Clostridia; Clostridiales; Ruminococcaceae; Oscillospira | 0.478 | 0.0075 |
| Proteobacteria; Alphaproteobacteria; Rickettsiales; mitochondria | 0.551 | 0.0156 |
| Bacteroidetes; Bacteroidia; Bacteroidales; S24-7 | 0.617 | 0.0038 |
| Firmicutes; Clostridia; Clostridiales; Clostridiales | 0.747 | 0.0029 |
| Firmicutes; Bacilli; Turicibacteraceae; Turicibacteraceae; Turicibacter | 1.09 | 0.0038 |
| Firmicutes; Erysipelotrichi; Erysipelotrichales; Erysipelotrichaceae; Allobaculum | 1.52 | 0.0038 |
| Actinobacteria; Actinobacteria; Bifidobacteriales; Bifidobacteriaceae; Bifidobacterium; pseudolongum | 2.46 | 0.0038 |

**Figure 6.** Comparison of the fecal microbiome (SFM) in WT and ES1 KO males. (A) Venn diagram comparison of the OTUs that overlap in WT and ESR1 KO mice and those that unique to each genotype. (B) PCoA of the fecal microbiome in WT (blue circles) and ESR1 KO (red circles) mice (p = 0.83 by PERMANOVA). While the Venn diagram comparison reveals that there are differences in the SFM between WT and ESR1 KO animals, they did not result in distinct clustering between these two genotypes. Consequently, metagenomeSeq was used to determine which OTUs differed between WT and ESR1 KO males (Table 2).
CA). Respective protocols for each kit were followed. The quantity of DNA isolated was measured using Qubit 3.0 Fluorometer (Life Technologies, Grand Island, NY). The number of replicates is indicated in Supplementary Table 1.

16s rRNA sequencing. The University of Missouri DNA Core Facility prepared bacterial 16S ribosomal DNA amplicons from extracted fecal and seminal fluid DNA by amplification of the V4 hypervariable region of the 16s rDNA with universal primers (U515F/806R) flanked by Illumina standard adapter sequences. Universal primer sequences are available at proBase (http://www.microbial-ecology.net/probebase/) . A forward primer and reverse primer with a unique 12-base index were used in each PCR reaction. PCR reactions (50 ul) contained 100 ng of genomic DNA, forward and reverse primers (0.2 μM each), dNTPs (200 μM each), and Phusion High-Fidelity DNA Polymerase (1U). PCR amplification was performed as follows: 98°C (3:00) + [98°C(0:15) + 50°C(0:30) + 72°C(0:30)] × 25 cycles + 72°C(7:00). Maximum sample volume was added to each PCR reaction for seminal fluid samples which contained <100 ng of input DNA. Amplified product (5 ul) from each PCR reaction was combined and thoroughly mixed to prepare a single pool. Pooled amplicons were then purified by addition of Axygen AxyPrep MagPCR Clean-up beads (50 ul) to an equal volume of 50 ul of the amplicon library pool and incubated at room temperature for 15 minutes. Products were placed on a magnetic stand for 5 minutes and supernatant (95 μl) was removed and discarded. Each well was washed by addition of 200 ul of freshly prepared 80% EtOH, incubation at room temperature for 30 seconds, and removal of supernatant. Wash steps were repeated once and plate was allowed to dry on magnetic stand for 15 minutes. The dried pellet was resuspended in Qiangen EB Buffer (32.5 ul), incubated at room temperature for 2 minutes, and then placed on the magnetic stand for 5 minutes. Supernatant (30 ul) was transferred to low binding microcentrifuge tube for storage. The final amplicon pool was evaluated using the Advanced Analytical Fragment Analyzer automated electrophoresis system, quantified with the Qubit fluorometer using the quant-iT HS dsDNA reagent kit (Invitrogen), and diluted according to Illumina's standard protocol for sequencing on the MiSeq.
Bioinformatics and amplicon analysis. Paired-end Illumina MiSeq DNA reads were joined using FLASH\(^9\). Usearch\(^{71}\) was used to clean contigs and remove those with E > 0.5, as explained here: http://.drive5.com/usearch/manual/exp_errs.html. Contigs were clustered to 97% identity against DNA sequences in the GreenGenes database\(^{72}\), version 13_5, using the QIME\(^{73}\), version 1.10.0, script pick_closed_reference_otus.py, which obviates chimera and PCR error detection. For alpha-diversity, Chao1 (species richness) and Shannon diversity were calculated and plotted using the phyloseq R package\(^{74}\). Rarefaction metrics were calculated using the alpha_rarefaction.py script in the Qiime package\(^{75}\) and plotted using Microsoft Excel (Supplementary Fig. 1).

Measurements of beta-diversity were facilitated by the QIIME script beta_diversity_through_plots.py and visualized using Principal Coordinate Analysis, PCoA, as implemented in QIIME\(^{76}\). LEfSe\(^{77}\) was used to identify genera most consistently different between sample types. LEfSe results were visualized using taxonomy bar-chart and cladogram plots, as implemented on the LEfSe website, http://huttenhower.sph.harvard.edu/galaxy/. PCoA was used to determine whether age of WT and ESR1 KO mice affected the seminal fluid and fecal microbiome samples, where two age-ranges where compared: < 220 and > 220 days of age. For the fecal microbiome, metagenomeSeq\(^{78}\), version 1.10.0 was used to identify the OTUs in WT and ESR1 KO that varied based on these two age groups. To identify genera associated with the WT and ESR1 KO genotypes within fecal and seminal sample types, we used metagenomeSeq\(^{79}\), version 1.10.0. Bacterial metabolic characterization of sample types was facilitated with PICRUSt\(^{80}\), version 1.0.0. To correlate the genera changes with metabolic characteristics of sample types, we used a custom R script provided as a gift from Dr. Jun Ma and Kjersti Aagaard-Tillery, Baylor College of Medicine, Houston, TX.

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**Author Contributions**

A.B.J. and C.S.R. designed the study, A.B.J., A.M.M.A., D.B.L., N.J.B. and K.H.B. performed the experiments, A.B.J., W.G.S., S.A.J., M.R.E., S.A.G. and C.S.R. analyzed the data and A.B.J., W.G.S., N.J.B., S.A.G. and C.S.R. prepared the manuscript. All authors reviewed the manuscript.

**Additional Information**

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**Corrigendum:** Discovery of a Novel Seminal Fluid Microbiome and Influence of Estrogen Receptor Alpha Genetic Status

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In this Article, Figure 4 is a duplication of Figure 6. The correct Figure 4 appears below as Figure 1.

![Figure 1](https://example.com/figure1.png)

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