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Colitis-induced colorectal cancer and intestinal epithelial estrogen receptor beta impact gut microbiota diversity

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Chronic inflammation of the colon (colitis) is a risk factor for colorectal cancer (CRC). Hormone-replacement therapy reduces CRC incidences, and the estrogen receptor beta (ERβ/ESR2) has been implicated in this protection. Gut microbiota is altered in both colitis and CRC and may influence the severity of both. Here we test the hypothesis that intestinal ERβ impacts the gut microbiota. Mice with and without intestine-specific deletion of ERβ (ERβKO^{Vil}) were generated using the Cre-LoxP system. Colitis and CRC were induced with a single intraperitoneal injection of azoxymethane (AOM) followed by administration of three cycles of dextran sulfate sodium (DSS) in drinking water. The microbiota population were characterized by high-throughput 16S rRNA gene sequencing of DNA extracted from fecal samples ($N = 39$). Differences in the microbiota due to AOM/DSS and absence of ERβ were identified through bioinformatic analyses of the 16S-Seq data, and the distribution of bacterial species was corroborated using qPCR. We demonstrate that colitis-induced CRC reduced the gut microbiota diversity and that loss of ERβ enhanced this process. Further, the Bacteroidetes genus *Prevotellaceae*_UCG_001 was overrepresented in AOM/DSS mice compared to untreated controls (3.5-fold, $p = 0.004$), and this was enhanced in females and in ERβKO^{Vil} mice. Overall, AOM/DSS enriched for microbiota impacting immune system diseases and metabolic functions, and lack of ERβ in combination with AOM/DSS enriched for microbiota impacting carbohydrate metabolism and cell motility, while reducing those impacting the endocrine system. Our data support that intestinal ERβ contributes to a more favorable microbiome that could attenuate CRC development.

Introduction

Colorectal cancer (CRC) is the second leading cause of cancer death worldwide.[1] Inflammation of the gut (colitis) is considered an important risk factor.[2] Colitis is thought to contribute to cancer development through several mechanisms, including release of cytokines that activate pro-survival NFκB and STAT3 signaling[3] and alter the immune system,[4] and by increasing risk of gene mutations[5] and epigenetic changes.[6] Colitis can also change the dynamics of the gut microbiota which can lead to dysbiosis and contribute to cancer development (reviewed in

Key words: microbiota, colitis-associated colon cancer, AOM/DSS model, estrogen receptor beta

Abbreviations: AOM: azoxymethane; CRC: colorectal cancer; DSS: dextran sulfate sodium; ERβ: estrogen receptor beta; KO: knockout; LEfSe: Linear discriminant analysis effect size; NMDS: nonmetric dimensional scaling; OTU: operational taxonomic unit; PERMANOVA: permutational analysis of variance; PICRUSt: Phylogenetic investigation of communities by reconstruction of unobserved states; qPCR: quantitative polymerase chain reaction; WT: wild type

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What's new?

Chronic inflammation of the colon is a risk factor for colorectal cancer (CRC). Hormone-replacement therapy reduces CRC incidence, and the estrogen receptor beta (ERβ/ESR2) has been implicated in this protection. The microbiota of the gut is altered in both colitis and CRC, but whether intestinal ERβ affects gut microbiota remains to be investigated. Here, the authors demonstrate, in a mouse model, that colitis-induced CRC reduces the gut microbiota diversity and that loss of ERβ enhances this process. The findings could enable novel therapeutic or preventive approaches toward a more favorable microbiome in inflammatory bowel disease and/or colon cancer development.

[7]). Dysbiosis is characterized by a reduction of beneficial bacteria (anaerobic species) and an increase of facultative anaerobic bacteria, such as pathogenic *Enterobacteriaceae*.[8] In attempts to identify specific microbial species that may contribute to colorectal carcinogenesis, studies have characterized microbiota diversity in individuals with colorectal cancer.[9,10] These studies have, however, not yet reached consensus. A number of factors make these analyses challenging, including large variations regarding biological, environmental, dietary, inter-individual, and technical parameters such as sample origin (fecal or mucosal samples) and time of sample collection.[11–13] Genetically homogenous mice, housed in controlled environment and fed identical diet, provide a less variable setting. A mouse model that combines one azoxymethane (AOM, a pro-carcinogenic agent) injection with dextran sulfate sodium (DSS, a chemical colitogen) in drinking water, causes epithelial injury and induces wound-healing response, that mimic human colitis-induced CRC.[14] Some studies have found changes in the richness and diversity of the microbiota composition due to AOM and/or DSS exposure.[15–18]

Further, estrogen through estrogen receptor β (ERβ/ESR2) appears to reduce the risk of CRC development (reviewed in [19]). Our previous cell-based analyses have shown that re-introduction of ERβ into CRC cells exhibits anti-proliferative functions, represses oncogenes, and mediates anti-inflammatory signaling.[20–22] Studies in mice have generated support *in vivo* for such a preventive role.[23,24] Application of the AOM/DSS model on full-body ERβ knockout (BERKO) mice demonstrated an increase in number and size of polyps or tumors compared to wild type (WT) mice,[24] and similar results were found using the $APC^{min/+}$ model.[25] However, whether intestinal ERβ affects the microbiota of the gut has not been investigated. To explore the effect of ERβ in the intestines, we have generated intestine-specific knockout of ERβ (referred to as $ERβKO^{Vil}$ or simply KO in the remainder of the text).[20] Here, we characterize how the microbiota composition is affected by AOM/DSS-driven colitis and CRC development in mice with and without intestinal ERβ.

Materials and Methods

Animals

The animal study was performed at University of Houston and approved by the Institutional Animal Care and Use Committee (12–026 and 13–012). Mice with floxed ERβ (B6.129X1-Esr2tmGust) were crossed with mice expressing Cre-recombinase under effect of the Villin promoter (B6.SJL-Tg(Vilcre)997 Gum/J), generating intestine-specific ERβ knockout mice ($ERβKO^{Vil}$ or KO). $ERβKO^{Vil}$ (n = 21) and corresponding WT littermates (n = 18) were distributed into two major experiments, 9 weeks (n = 20) and 16 weeks (n = 19) after AOM injection, and respective controls (no AOM/DSS). The successful deletion of ERβ expression in the intestinal epithelium of these mice has been previously demonstrated,[20] and ERβ expression in its main organs (primarily the ovary [26]) is maintained (data not shown) and the mice are fully fertile (full KO of ERβ generate sub-fertile females with nearly no offspring). The layout of the experiment is illustrated in (Fig. 1). WT and $ERβKO^{Vil}$ littermates were housed in the same cages, separated only by sex and AOM/DSS treatment, thereby minimizing cage, strain, and age-dependent effects in terms of microbiota. At age 8–11 weeks and an average weight of 27 g for males and 21 g for females, mice were injected intraperitoneally (IP) with 10 mg azoxymethane (AOM, Sigma-Aldrich) per kg body-weight, followed by three 1-week cycles of 2.5% dextran sulfate sodium (DSS, MP Biomedical) in drinking water. The DSS cycles were separated by 2 weeks of regular drinking water. Stool pellets were collected 9 weeks [WT (n = 9) and $ERβKO^{Vil}$ (n = 11)], and 16 weeks [WT (n = 9) and $ERβKO^{Vil}$ (n = 10)] after AOM injection, and stored at −20 °C.

DNA extraction, library construction and 16S-sequencing

The microbiota composition was determined by 16S-Seq of the variable V3-V4 genomic regions of bacterial 16S rRNA. Libraries for sequencing were prepared from DNA extracted using QIAamp DNA stool mini kit (Qiagen, Sweden), according to the manufacturer's recommendations. Briefly, each fecal pellet was weighed, homogenized by pellet pestles in lysis buffer, vortexed and centrifuged. The supernatant was treated with InhibitEX tablets to adsorb PCR inhibitors, and with proteinase K to remove proteins. DNA was precipitated in ethanol (99%), purified using spin columns, and eluted in 200 μL elution buffer. Purity and concentration of DNA were measured using Nanodrop 2000 spectrophotometer (Thermo Scientific). Libraries were generated for all samples at the same time: Ten nanograms of DNA per sample was PCR amplified with Phusion master mix (20 cycles of 95 °C for 30 s, 55 °C for 30 s, 72 °C for 30 s) and degenerated forward (5'-TCG TCG GCA GCG TCA GAT GTG TAT AAG AGA CAG CCT ACG GGN GGC WGC AG-3′) and reverse (5'-GTC TCG

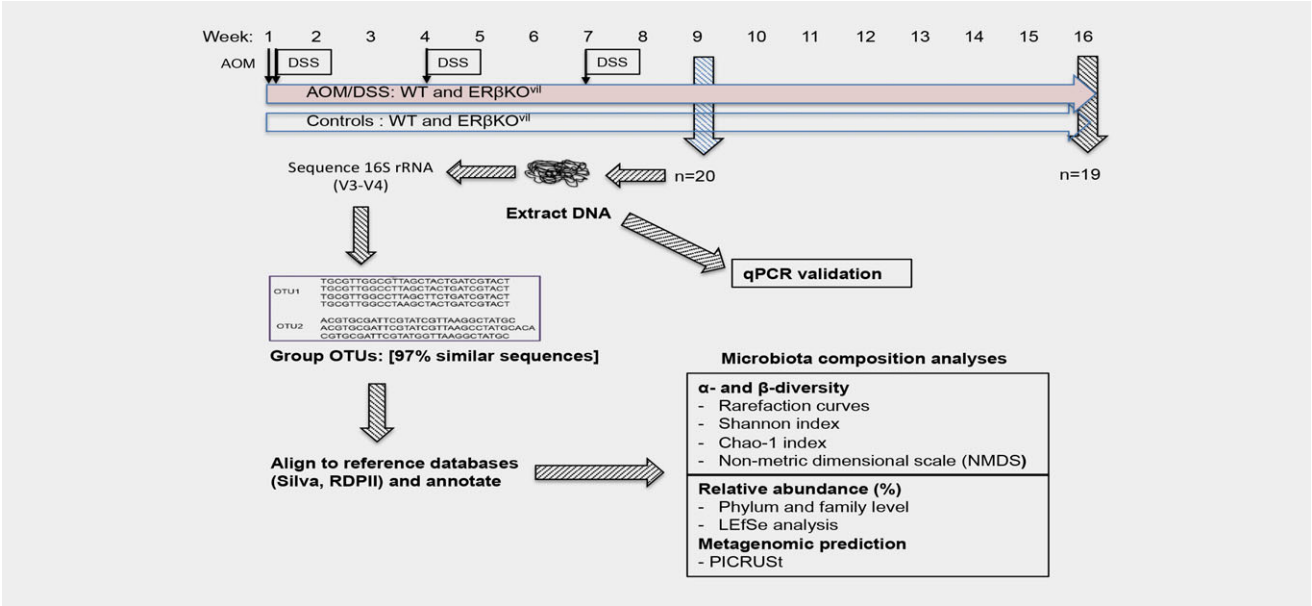

Figure 1. Schematic workflow of experimental design and analysis. Male and female mice on the same mixed C57BL/6 J background, with and without deletion of intestinal ERβ, were either injected with AOM (10 mg/kg bw, IP) at time point 0 and subjected to DSS (2.5%, drinking water) at weeks 1, 4 and 7, or maintained as parallel untreated controls. Stool pellets were collected from all mice at 9 weeks and 16 weeks. DNA was extracted, and genomic 16S rRNA gene was sequenced, OTUs were aligned to reference databases, annotated and analyzed for alpha and beta diversity, relative abundance of taxa at phyla and family level, differential abundance (LEfSe) and metagenomic prediction (PICRUSt). The sequencing results were validated with qPCR. [Color figure can be viewed at wileyonlinelibrary.com]

TGG GCT CGG AGA TGT GTA TAA GAG ACA GGA CTA CHV GGG TAT CTA ATC C-3′) primers. These primers target the variable V3-V4 genomic regions of bacterial 16S rRNA,[27] and the 5′ ends include Illumina adapter sequences. The expected amplicon size was approximately 550 bp, and unbound primers and primer-dimer fragments were removed using AMPure XP beads according to the manufacturer's protocol. Next, dual indices were attached to the Illumina sequencing adapters using the Nextera XT index kit and a second PCR was performed to complete the library (13 cycles of 95 °C for 30 s, 55 °C for 30 s, 72 °C for 30 s). The resulting product (expected size approximately 630 bp) was purified using AMPure XP beads, and the quality and size of end products was assessed with a DNA Bioanalyzer 1000 broad range kit. DNA concentration was assessed with the QuantIt kit, and adjusted to 4 nM. Ten microliters of each library was pooled, denatured with NaOH to generate single-stranded DNA, and sequenced in one run using Illumina MiSeq V3 kit with 5% PhiX library as spike-in internal control. In total, 3,397,153 reads were generated for these samples.

Bioinformatic pipeline and statistical analyses

A software designed to remove adapter sequences from reads, Cutadapt,[28] was used to trim away the primer sequences as well as 3′-bases with a Phred quality score below 15. Read pairs where one read was shorter than 120 bp were discarded. Remaining read pairs were merged using Uparse,[29] and pairs that did not overlap or were predicted to have more than 3 errors over the full sequence length were discarded. Overall, 640,965 reads were kept. They were dereplicated and denoised with Unoise2,[30] producing 2000 corrected sequences with abundance >1. All remaining reads were mapped back to these corrected sequences for quantification. We ensured that the samples of different groups were sequenced to approximately the same depths: The final 16S-Seq analysis generated 126,551 approved read pairs for the 9-week experiment (n = 20) with an average of 7723 reads per sample (range: 4140-10,837), and 178,519 read pairs for the 16-week experiment (n = 19) with an average of 6437 (range: 4236-9832) reads per sample. There was no significant difference of read counts between AOM/DSS and control mice, or between WT mice and KO mice within the experiments. This supports that the removal of potential PCR contaminants (such as DSS) with InhibitEX was successful during the preparation protocol. Taxonomic assignment was based on the SILVA database[31] using the strategy described by Hugerth *et al.*[32] All unique operational taxonomic units (OTUs) and their 16S rRNA sequences, as well as the codes and scripts used in our study, are attached as Additional file 1 and Additional file 2, respectively. Our data is submitted to the National Center for Biotechnology Information (NCBI) Sequence Read Archive (SRA) as BioProject [PRJNA475738] and BioSamples [SAMN09405040-SAMN09405078].

Bioinformatic microbiota analyses

Alterations of the microbiota were analyzed bioinformatically through several measures, including rarefaction curves, alpha-

diversity, beta diversity, Bray-Curtis divergence, and linear discriminant analysis (LDA) effect size (LEfSe). Rarefaction curves were used to confirm that sufficient sequencing coverage had been achieved, and to identify differences of species diversity between groups. Significant differences between rarefaction curves of different groups were calculated as described by Cayuela *et al.* [33] using the function EcoTest.sample from R package rareNMtests v1.1. Alpha diversity is a measure of microbiota abundance and how evenly these are distributed (evenness), and was estimated through Shannon's diversity index. Another estimate of alpha diversity is the richness of the microbiota, which was estimated through the Chao1 index. Beta diversity is the ratio of the diversity between groups *versus* individuals within each group, and we used the Bray-Curtis divergence to quantify this compositional dissimilarity between different groups. Statistical testing was performed using permutational multivariate analysis of variance (PERMANOVA). All these calculations were performed in R v. 3.3.1 using packages Vegan v. 2.4–5, Fossil v. 0.3.7,[34] and Vioplot v. 0.2.[35] LEfSe[36] was used to discover enriched features applying an LDA threshold of 2.0 and a significance cutoff less than 0.05. AOM/DSS was used as main category (Classes) while the genotype or sex were analyzed as subcategories and plotted individually. Other comparisons with different classes and subclasses as input parameters were also conducted, as specified in the text and figures. Finally, to predict the functionality of the metagenomic content in each sample, phylogenetic investigation of communities by reconstruction of unobserved states (PICRUSt) software was used, based on the abundance of corresponding OTUs. [37]

Quantitative PCR

qPCR was used for validation of 16S-Seq data. Specific primers were designed for *Prevotellaceae*_UCG_001 and *Parasutterella,* or adopted for *Bacteroidetes*, *Firmicutes*, *Gammaproteobacteria*, *Bifidobacteria*, *Cyanobacteria*, *Tenericutes*, *Verrucomicrobia,* and total bacteria (primer sequences in Supporting Information Table S1). The designed primer pairs were confirmed for specificity of the intended target group by alignment to the ribosomal database project (RDPII) using the probe match tool. Ten nanograms of fecal DNA was amplified in a total volume of 10 μL using iTaq Universal SYBR Green Supermix (Bio-Rad Laboratories) according to the manufacturer's recommendations (5 min at 95 °C, 40 cycles of 30 s at 95 °C, 30 s at 60 °C, 30 s at 72 °C), and followed by disassociation curve analysis to ensure amplification of a single amplicon. Each sample was analyzed in duplicates, and relative expression calculated using the $2^{-\Delta\Delta Ct}$ method normalized to total bacteria (total genomic 16S rRNA). The WT controls were used as calibrator samples in each experiment. Differences were tested with Welch's *t*-test, and a *p*-value equal or less than 0.05 was considered significant.

Results

To reduce the impact of normal variation of microbiota we compared co-housed (shared cages) and age-matched KO and WT siblings. All mice (males and females, WT and KO) were on the same mixed C57BL/6 J background. An overview of the experiment is presented in Figure 1.

Identification of baseline microbiota compositions in WT controls

We first compared the microbiota diversity of the two different WT control groups (the untreated 9-week and 16-week mice) in order to investigate the general variability of the microbiota and to establish a baseline microbiota composition and species diversity. We also corroborated the relative abundance of phyla in the WT controls of both time points using qPCR. We did not observe significant differences between the controls of the two different time points, except for two phyla (*Firmicutes* and *Tenericutes)* which increased at the later time point (Supporting Information Fig. S1). The ages differ by an average of 7 weeks between the two groups (19 *versus* 26 weeks old at end of experiments), but both experimental groups are categorized as young mice according to the frailty index scores of origin strains (C57BL/6).[38] We conclude that, overall, the microbiota does not differ significantly between the two different groups of WT control mice.

AOM/DSS reduces gut microbiota diversity in WT animals

In order to identify the general impact that colitis-induced CRC development has on the composition of microbiota, we compared the species diversity between AOM/DSS and untreated WT animals through several measures. First, we assessed species richness per sampling by plotting the number of detected species as a function of number of sequences analyzed. The resulting rarefaction curves leave the exponential phase around 2000 reads per sample, and signs of saturation are noted after around 5000 reads, confirming that sequencing coverage was sufficient (Fig. 2*a*, *b*). These curves also show that AOM/DSS exposure reduced the number detected species (diversity) with more than 100 OTUs (Fig. 2*a*, *b*) at both the 9-week ($p = 0.025$) and 16-week ($p = 0.035$) time point. Next, we investigated the abundance of the microbiota and how evenly these are distributed (evenness) using Shannon's alpha-diversity index, and their richness using the Chao-1 alpha-diversity index (Fig. 2*c*, *d*). We find that AOM/DSS in WT mice decreased the Shannon index from 4.9 to 4.2 (not significant) and the Chao-1 index from 940 to 630 ($p = 0.036$) at 9 weeks. A decrease was noted also at 16 weeks, where Shannon index was reduced from 5.15 to 4.85 ($p = 0.009$) and Chao-1 index from 910 to 750 ($p = 0.08$). We also explored the compositional dissimilarity of the species diversity between AOM/DSS and control groups by calculating beta diversity using Bray–Curtis dissimilarity. We found a significant dissimilarity between AOM/DSS-treated mice and their

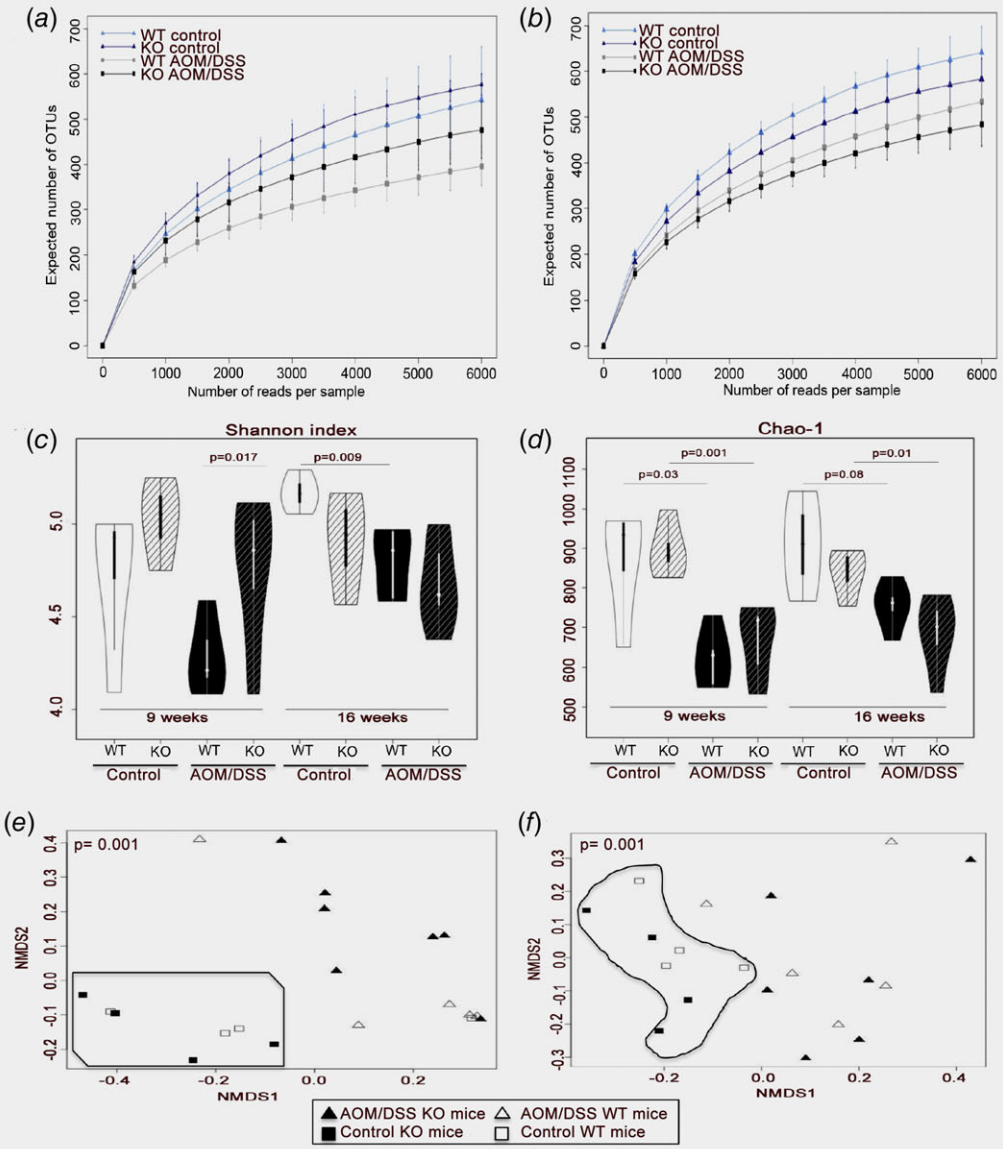

Figure 2. Microbiota diversity is reduced by treatment and lack of intestinal ERβ. (*a*, *b*) Rarefaction curves (mean +/− SD) show that sufficient sequencing depth was reached, and that a reduced number of species were detected in WT mice 9 weeks after treatment ($p = 0.025$, (*a*) and in both WT and KO mice after 16 weeks (WT: $p = 0.035$, KO: $p = 0.030$, B). (*c*, *d*) Diversity and richness measured with Shannon's and Chao1, respectively. AOM/DSS significantly reduces the diversity, and after 16 weeks, the lowest diversity was found in treated KO mice. (*e*, *f*) Beta diversity visualized with non-metric dimensional scattering plot (NMDS), is significantly separated by AOM/DSS treatment at both 9 weeks ($n = 20$, $p = 0.001$, panel e) and 16 weeks ($n = 19$, $p = 0.001$, panel f). Significance calculated by PERMANOVA test, statistical comparisons are summarized in Supplemental Table 2. [Color figure can be viewed at wileyonlinelibrary.com]

controls at both time points ($p = 0.001$, Fig. 2*e*, *f*, Supporting Information Table S2). Pooling of WT control mice from both time points ($n = 16$), maintained significant separation from the AOM/DSS cohorts ($p = 0.001$, Supporting Information Fig. S2A-B, Supporting Information Table S3). We conclude that AOM/DSS-mediated colitis and subsequent CRC significantly reduce the microbiota diversity in WT mice.

AOM/DSS in WT animals alters the abundance of species related to metabolism

Next, we plotted the relative abundance of microbiota at the phyla level as bar charts (Fig. 3*a*, *b*, with values of relative abundance at both time points summarized in Supporting Information Tables S4-S5). Relative abundance at the family levels is presented in Supporting Information Figure S2C-D

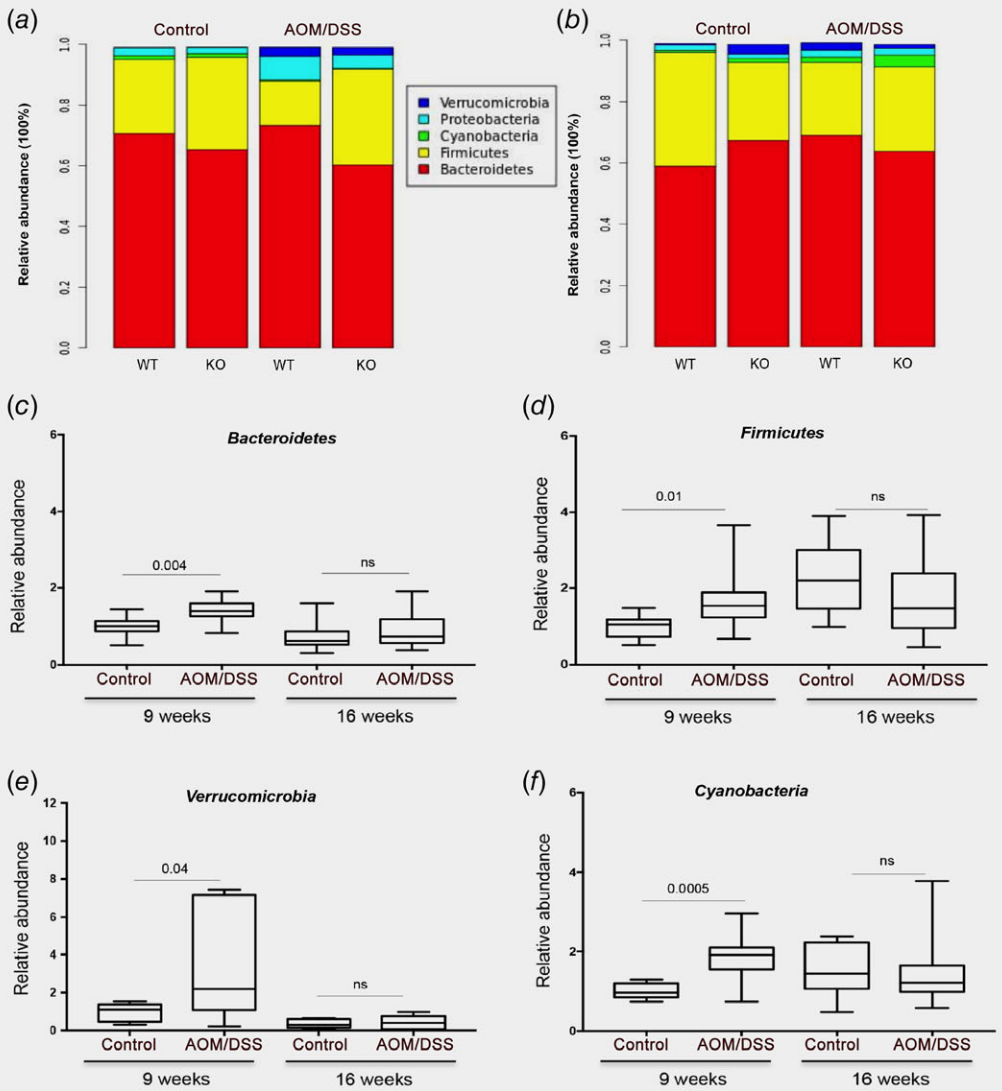

Figure 3. Relative abundance of microbiota. (*a*, *b*) Microbiota distribution at the phyla level at 9 weeks (a) and 16 weeks (b), with corresponding values of relative abundances summarized in Supplemental Tables 4–7. (*c*–*f*) Relative abundance measured with qPCR for *Bacteroidetes* (c), *Firmicutes* (d), *Verrucomicrobia* (e), and *Cyanobacteria* (f) are statistically significant (Welch's *t*-test, WT and KO combined) at 9 weeks. [Color figure can be viewed at wileyonlinelibrary.com]

with corresponding values in Supporting Information Tables S6-S7. The phyla *Bacteroidetes* and *Firmicutes* were the most prevalent taxa in all groups and both time points, followed by *Proteobacteria*, *Verrucomicrobia* and *Cyanobacteria*. A transient AOM/DSS-mediated increase of several phyla (*Bacteroidetes*, *Firmicutes*, *Verrucomicrobia*, and *Cyanobacteria*) after 9 weeks was corroborated using qPCR, but none of these changes remained after 16 weeks (Fig. 3*c*–*f*). To discover significant alteration of taxa abundance in response to AOM/DSS in a systematic manner, we conducted LEfSe. This approach revealed 18 OTUs as significantly affected 9 weeks after initiation of AOM/DSS treatment, and 12 OTUs after 16 weeks (Fig. 4*a*). *Parasutterella* was the most induced species (5.5-fold enrichment) by AOM/DSS at 9 weeks (Fig. 4*a*, left panel, and 4b) and *Prevotellaceae*_UCG_001 (phylum *Bacteroidetes*, family *Prevotellaceae*) the most reduced species (4.5-fold reduced, Fig. 4*a* and *c*) in WT mice. This was supported by qPCR (Fig. 4*d*, *e*). At 16 weeks, *Prevotellaceae*_UCG_001 was enriched by AOM/DSS treatment, along with the taxa *Odoribacter*, *Bacteroides* and *Parabacteroides* (Fig. 4*a*, right panel). We corroborated also this impact on *Prevotellaceae*_UCG_001 using qPCR (3.3-fold enrichment, $p = 0.004$, Fig. 4*e*). Finally, in order to make a prediction of functional effects resulting from the altered microbiota

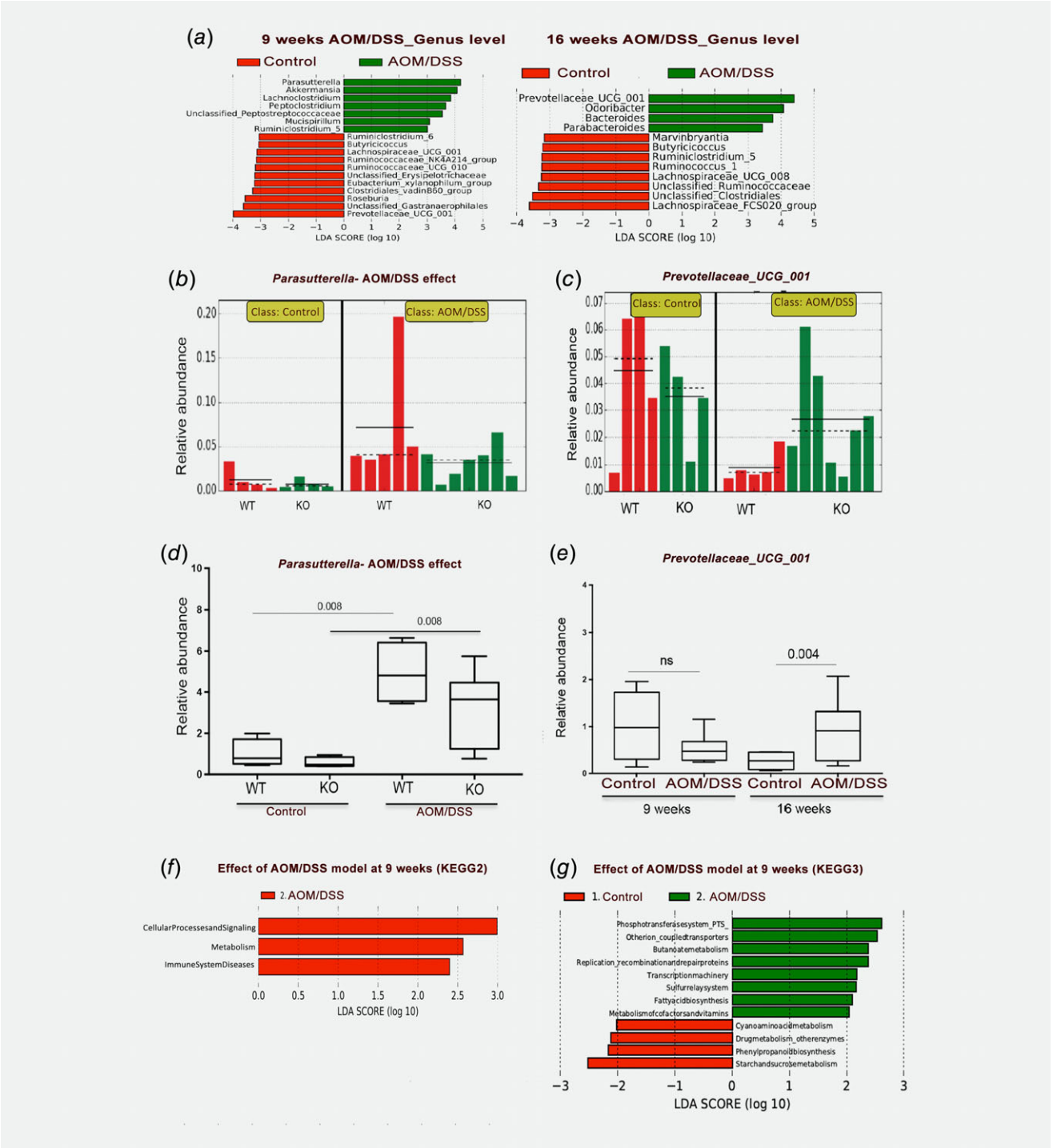

Figure 4. Differential abundance of microbiota demonstrated after AOM/DSS treatment. (*a*) LDA score indicate differentially abundant taxa (genus level) due to AOM/DSS at 9 weeks (left) and 16 weeks (right), per LEfSe analysis ($p < 0.05$ for all). (*b*) Histogram of the relative distribution of *Parasutterella* at 9 weeks, which shows an enrichment in WT mice after AOM/DSS treatment. (*c*) Histogram of the relative distribution of *Prevotellaceae_UCG_001* at 9 weeks, which shows the abundance in control WT and KO. (*d*, *e*) qPCR supports 16S-Seq identified alterations of *Parasutterella* (d) and *Prevotellaceae*_UCG_001 (e). (*f*, *g*) Functional classification of the predicted metagenome (PICRUSt) in response to AOM/DSS at 9 weeks using KEGG level 2 (f) and level 3 (g) pathways and visualized with LEfSe. [Color figure can be viewed at wileyonlinelibrary.com]

community, we used PICRUSt software. PICRUSt analyses can predict the functional KEGG pathways that are related to the composition of a metagenome, and has been demonstrated to provide a good representation of metagenomic prediction.[37] Applying LEfSe onto this analysis revealed that several predicted KEGG pathways were enriched due to AOM/DSS

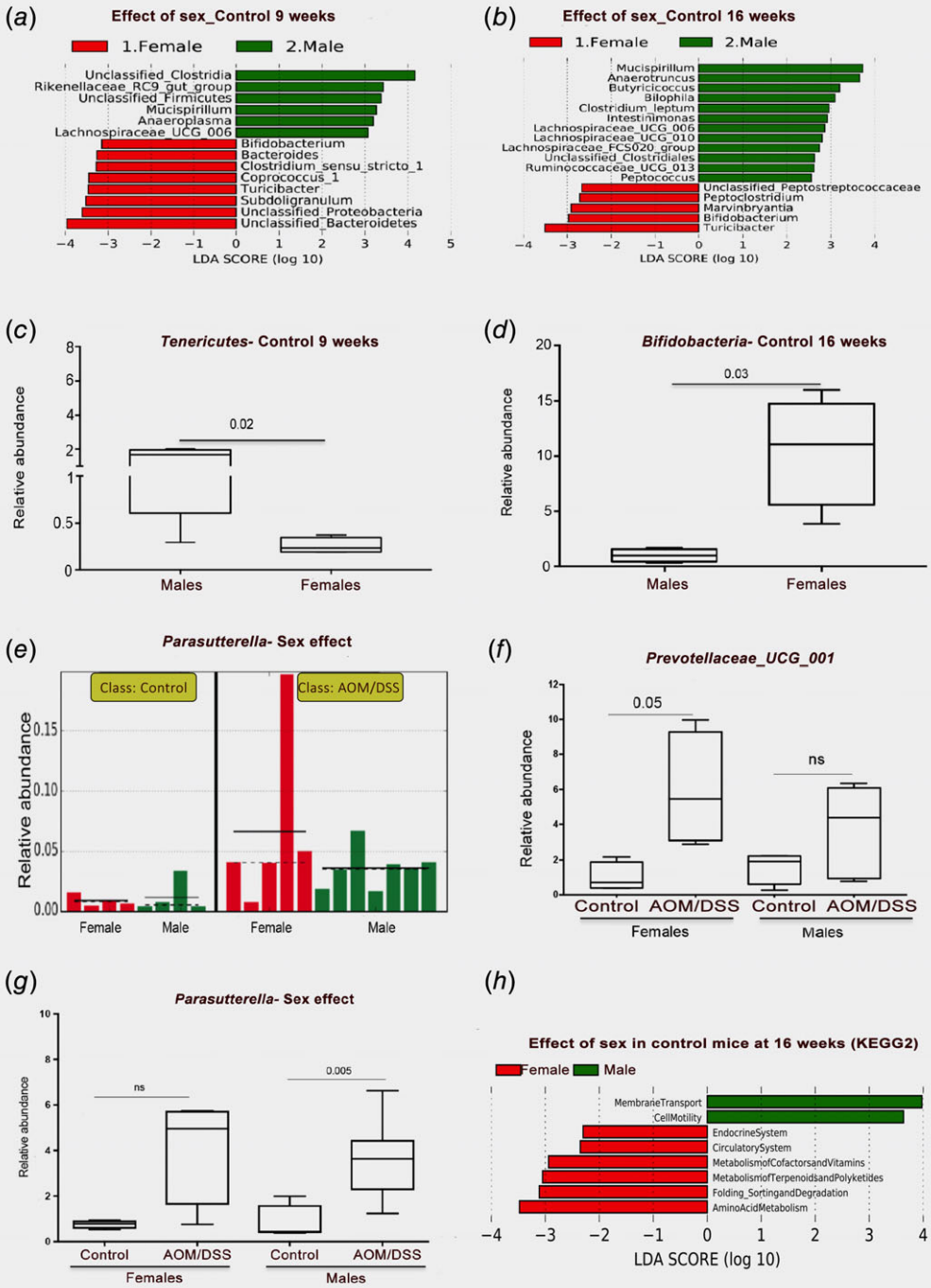

Figure 5. Microbiota abundance are sex-dependent. (*a*, *b*) LEfSe analysis of differentially abundant taxa (genus level) indicates sex differences in control mice at 9 weeks (a) and 16 weeks (b). (*c*, *d*) qPCR corroborated differences identified in a-b for phylum *Tenericutes* (genus *Anaeroplasma*) and phylum *Bifidobacteria* (genus *Bifidobacterium*). Welch's *t*-test was used for significance testing. (e) Relative distribution of *Parasutterella* in individual mice after 9 weeks, per LEfSe analysis with AOM/DSS model as class and sex as subclass (corroborated by qPCR in panel g). (*f*) Sex-dependent enrichment of *Prevotellaceae*_UCG_001 in AOM/DSS-treated females at 16 weeks. (*g*) *Parasutterella* is significantly enriched in AOM/DSS-treated males at 9 weeks. (*h*) Metagenomic prediction (PICRUSt) of sex differences using KEGG pathways and visualized with LEfSe, for untreated mice at 16 weeks. [Color figure can be viewed at wileyonlinelibrary.com]

treatment. After 9 weeks, AOM/DSS significantly increased bacteria associated with metabolism and immune system diseases (KEGG level 2, LDA >2, Fig. 4*f*). More specifically (KEGG level 3), we find that AOM/DSS enhanced bacteria that metabolize butanoate, cofactors and vitamins, while reducing those that metabolize cyanoaminoacid, starch, and sucrose (Fig. 4*g*). We also find that AOM/DSS increased bacteria associated with phosphotransferase system (PTS), fatty acid biosynthesis, and transcription machinery (Fig. 4*g*). We conclude that AOM/DSS treatment and subsequent colitis and CRC significantly alter specific microbial species in the colon of WT mice, and that this could impact metabolism, transcription, and the immune system of the gut.

Microbiota diversity and specific species are influenced by sex

To investigate whether the microbiota diversity is different between male and female mice, we explored the impact of this parameter on the beta diversity using Bray–Curtis dissimilarity and PERMANOVA testing. We found that sex alone impacted the diversity at both time points (9 W: $p = 0.048$ and 16 W: $p = 0.014$, Supporting Information Table S2). This separation remained also when controls from 9 weeks ($n = 8$) and 16 weeks ($n = 8$) were pooled (9 W: $p = 0.066$ and 16 W: $p = 0.007$, Supporting Information Table S3). LEfSe further revealed specific species differences between the sexes in the untreated controls (Fig. 5*a*, *b*). *Bifidobacterium* and *Turicibacter* were enriched in females at both time points, while males had higher levels of the genera *Mucispirillum* (both time points) and *Anaeroplasma* (at 9 weeks). qPCR analysis for the phyla *Tenericutes* (genus *Anaeroplasma*) and *Bifidobacteria* (genus *Bifidobacterium*) confirmed sex differences (Fig. 5*c*, *d*). Males (untreated) had 5-fold higher levels of *Tenericutes* ($p = 0.02$ at 9 weeks), and females (untreated) had 11-fold higher levels of *Bifidobacteria* ($p = 0.03$ at 16 weeks). Further, the phylum *Verrucomicrobia* was 2.4-fold higher in males ($p = 0.009$ at 9-weeks, Supporting Information Fig. S3A), and *Gammaproteobacteria* higher in females ($p = 0.05$ at 16-weeks, Supporting Information Fig. S3C). PICRUSt and LEfSe analysis further proposed that sex-related microbiota differences impacted metabolism and the endocrine system in females, and cell motility and membrane transport functions in males (LDA >3, Fig. 5*h*).

After treatment, the same sex differences did not remain or were not significant (exemplified in Supporting Information Fig. S3B and D). However, we note that the induction of *Prevotellaceae*_UCG_001 by AOM/DSS at 16 weeks (Fig. 4*e*), was 6-fold enriched in females ($p = 0.05$) while not significantly enriched in males, as corroborated by qPCR (Fig. 5*f*). Further, the AOM/DSS-induced *Parasutterella* in WT mice ($p = 0.008$, Fig. 4*d*) was significant only in males ($p = 0.005$, Fig. 5*g*). Thus, our analyses demonstrated clear sex differences in the microbiota, which affect specific clades associated with colitis and dysbiosis.

Intestinal ERβ deletion modulates colitis-induced microbiota diversity

Finally, we explored whether ERβ in the intestinal epithelium impacts the microbiota diversity. First, we investigated untreated animals, where rarefaction curves indicated a slight increase in the number of OTUs in KO mice compared to WT mice (about 50 OTUs) at the 9-week time point (Fig. 2*a*) and a slight decrease (85 OTUs) at the 16-week time point (Fig. 2*b*), but these changes were not significant. Similarly, both the Shannon and Chao-1 indices indicated a slight increased diversity at 9 weeks, and decreased diversity at 16 weeks compared to WT mice, but neither reached statistical significance (Fig. 2*c*, *d*). Treatment with AOM/DSS reduced the number of OTUs detected per rarefaction curves in KO animals with about 100 OTUs compared to untreated KO animals at both time points (Fig. 2*a*, *b*, $p = 0.01$ at 9 weeks, $p = 0.03$ at 16 weeks), but the difference between KO and WT after AOM/DSS was not significant. There was, however, a significant increase of diversity in KO mice compared to WT after treatment per Shannon index ($p = 0.017$) at 9 weeks. Further, at the 16-week time point, all three measurements (rarefaction curves, Shannon and Chao-1 indices) indicated that AOM/DSS ERβKO^{Vil} mice exhibited the lowest microbiota alpha diversity. The statistical significance of the AOM/DSS-induced reduction of microbiota richness (Chao-1 index) was also more robust in KO mice (9w: $p = 0.001$, 16w: $p = 0.01$) than in WT mice (9w: $p = 0.03$, 16w: $p = 0.08$) for both time points (Fig. 2*d*). Analysis of beta diversity using Bray–Curtis dissimilarity revealed a separation due to genotype (ERβ) ($p = 0.028$), and genotype in combination with sex ($p = 0.011$) at 9 weeks (Supporting Information Table S2), but no significant separation at 16 weeks. Overall, our analyses indicate that absence of intestinal ERβ reduces the microbiota diversity in conjunction with AOM/DSS treatment and CRC.

ERβ affects specific microbiota species during induced colitis

In order to explore whether intestinal ERβ KO impacts the abundance of specific species, we conducted further analysis with LEfSe. Comparing KO and WT control mice, we found that deletion of ERβ impacted few genera (Fig. 6*a*–*c*). A reduction of unclassified *Firmicutes* were indicated in control KO mice at 16 weeks (Fig. 6*c*, *d*). This was supported by qPCR with generic *Firmicutes* primers ($p = 0.07$, Fig. 6*e*). After AOM/DSS treatment (9 weeks), more OTUs were enriched in KO animals compared to WT (Fig. 6*f*). Interestingly, this included butyrate-producing bacteria members of the phylum *Firmicutes*, such as *Lachnospiraceae* and *Intestinimonas* (Fig. 6*f*–*h*). Further, the AOM/DSS-mediated reduction of *Prevotellaceae*_UCG_001 at 9 weeks (Fig. 4*a* and *e*), was almost exclusively resulting from reduction in the WT mice, while the decrease in KO mice were minimal (Fig. 4*c*). We also note that while *Parasutterella* was significantly induced in both WT and KO animals ($p = 0.008$, Fig. 4*d*), its levels

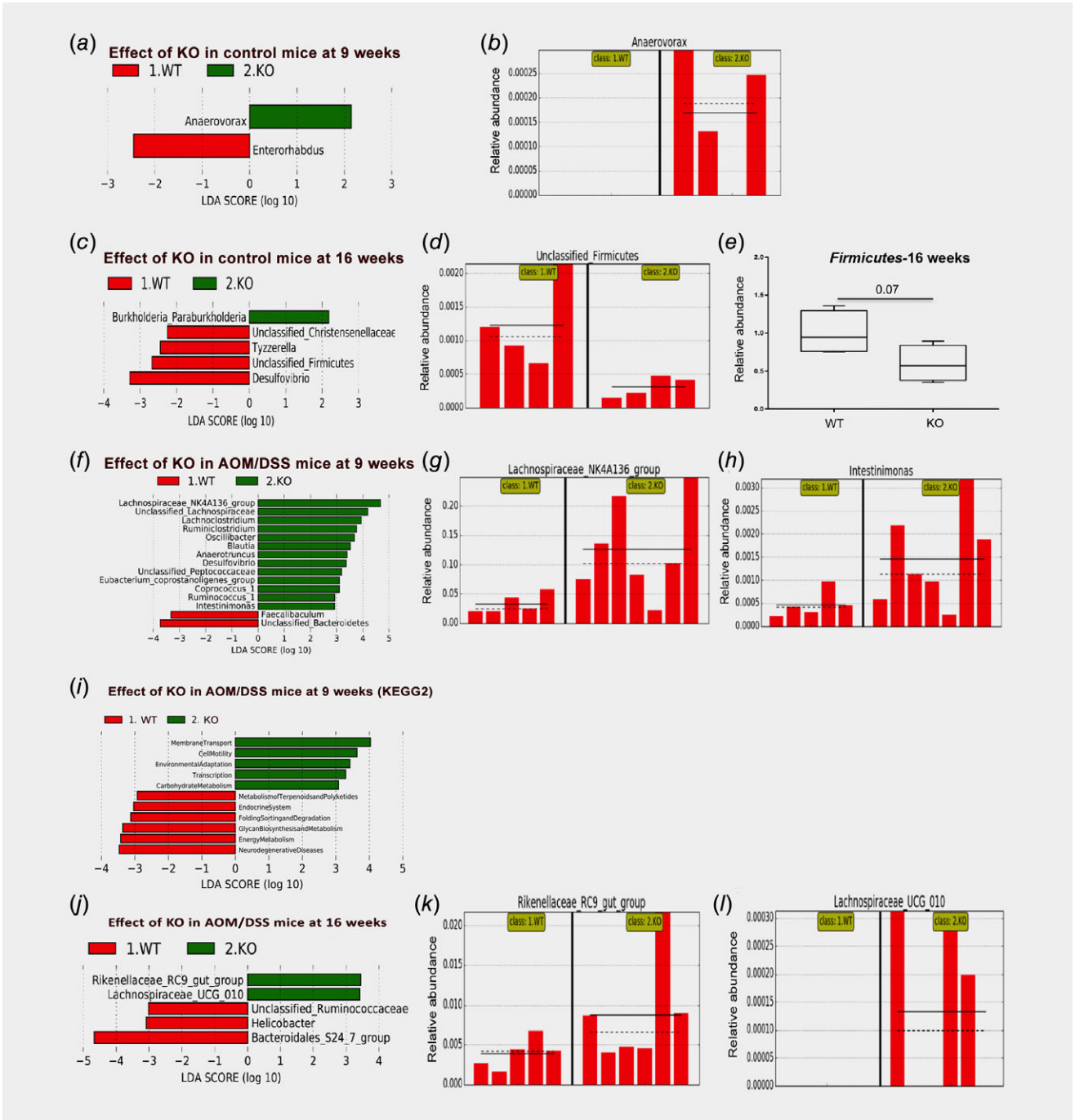

Figure 6. Specific taxa are differentially enriched in response to ERβ knockout. (*a*) LEFsE analysis indicates increase of the genus *Anaerovorax* (phylum *Firmicutes*) in KO untreated mice compared to WT, at 9 weeks, and (*b*) corresponding relative distribution of *Anaerovorax* in individual mice. (*c*) LEfSe analysis indicates decrease of unclassified *Firmicutes* in untreated KO mice at 16 weeks, and (*d*) corresponding relative distribution of unclassified *Firmicutes* in individual mice, which (*e*) is supported by qPCR of phylum *Firmicutes* (Welch's *t*-test for significance testing). (*f*) LEFsE analysis indicates differential abundance between AOM/DSS-treated WT and KO at 9 weeks, with (*g*, *h*) corresponding histograms illustrating increase in relative abundances of *Lachnospiraceae*_NK4A136 *group* and genus *Intestinimonas* in KO mice. (*i*, *j*) Metagenomic prediction (PICRUSt) of alterations in response to KO in AOM/DSS-treated mice at 9 weeks (panel i) and 16 weeks (panel j) using KEGG pathways and visualized with LEfSe. (*k*, *l*) The histogram of the increased abundances of *Rikenellaceae*_RC9_gut_group and genus *Lachnospiraceae*_UCG_010 in KO-treated mice at 16 weeks (corresponding to data in panel j). [Color figure can be viewed at wileyonlinelibrary.com]

appear lower in KO both before and after treatment (Fig. 4*b* and *d*). Applying PICRUSt followed by LEfSe analysis, indicated that lack of ERβ in combination with treatment (9 weeks) enriched for microbiota with functions in membrane transport, cell motility, and carbohydrate metabolism, and reduced functions related to endocrine systems and other types of energy metabolism (Fig. 6*i*). Some differences at the 16-week time point included enrichment of *Rikenellaceae*_RC9 and exclusive presence of *Lachnospiracae*_UCG_010 in AOM/DSS-treated KO (Fig. 6*j*–*l*). Overall, this may suggest that intestinal ERβ impacts the microbiota diversity and specific microbial species, especially during early onset of inflammatory events, which we hypothesize could contribute to progression of chronic inflammation and/or CRC development.

Discussion

In our study, we have characterized how colitis-associated CRC driven by AOM/DSS impacts the microbiota diversity in mice with and without intestinal ERβ. In particular, we have identified novel ERβ and sex-dependent effects.

Although our study was conducted in mice on mixed C57BL/6 J background, the identified AOM/DSS-induced alterations of the microbiota corroborates several previous reports using AOM and/or DSS in other mice strains. Such studies include analysis of the dynamic diversity of microbiota in fecal contents of BALB/c mice after AOM injection and 4 cycles of DSS (3%) for 7 days,[17] male A/J mice treated with AOM and DSS (2.5%) while on high-fat diet at 77 days[18], C57BL/6 mice after DSS only (5%) for 3 and 14 days, respectively[39], and C57Bl/10 treated with DSS only (3.5%) for 7 days.[15] Although different mice strains and different protocols of AOM and/or DSS treatment were used, all are in accordance with our study in terms of a decreased overall abundance and reduced beta diversity of the gut microbiota community after AOM and/or DSS treatment. However, there are also notable differences regarding the structural composition of the microbiota community, including the baseline composition of microbiota species.

The patterns we find are also in agreement with studies describing impact of colitis in the genetically modified IL10$^{-/-}$ colitis animal model,[8] and in patients diagnosed with inflammatory bowel disease (IBD).[40] For example, we found that the family *Lachnospiraceae*, was among the most decreased genera in AOM/DSS WT mice after 9 and 16 weeks (Fig. 4*a*, *b*). This family was also reduced in the IL10$^{-/-}$ colitis model[41] and in human IBD and CRC patients.[42,43] Other microbial species were transiently enriched by AOM/DSS (at 9 weeks), including *Verrucomicrobia*, *Firmicutes*, *Bacteroidetes,* and *Cyanobacteria*. Some members of those phyla are responsible for the degradation of host glycans and mucous layers which they use as nutritional and energy source, leading to disturbance of epithelial barriers.[44] Our data showed that pathogenic gram-negative bacteria *Parasutterella* (Fig. 4*a*, *b* and *d*) and host glycan-degradation bacteria *Akkermansia* (Fig. 4*a*) were enriched after AOM/DSS treatment. This can lead to gut barrier disruption, damaging the mucus layer and the epithelium and eventually induction of immune responses that may result in chronic inflammation.[4,45] This agrees with our PICRUSt metagenomic prediction, which indicated that AOM/DSS enriched for microbiota affecting functions involved in immune system diseases and metabolic functions. Moreover, phosphotransferase system (PTS) was highly enriched after AOM/DSS treatment. This function allows a selective uptake and phosphorylation of sugar molecules by the bacteria, which inhibits the efflux of sugar molecules back across the bacterial membrane.[46]

Our data are also in agreement with studies showing that DSS leads to a shift of the microbiota toward abundance of proinflammatory gram-negative bacteria. For example, we find enrichment of *Bacteroides* and *Odoribacter* in tumor-bearing mice which is in agreement with studies carried out by Zackular *et al.*[16] Further, *Parasutterella,* which we found increased by AOM/DSS (Fig. 4*d* and 5*g*), has been found to be higher in IBD patients with Crohn's disease,[47] and was one of only five genera significantly enriched in CRC patients compared to healthy volunteers.[43] Zackular *et al.* also found *Parasutterella* and *Akkermansia* to be enriched 7 days after DSS in C57BL/6 mice.[16] Several of the AOM/DSS affected species, including the enrichment of *Mucispirillum*, have the genetic arsenal to degrade the mucous layer,[48] or secrete pathogenic molecules such as lipopolysaccharides, that signal through Toll-like receptors (TLRs)[49], and could therefore contribute to colitis and cancer development. The agreement of our study with other reports of the impact of AOM or DSS on the microbiota, support the accuracy of observations and methodological considerations of our study. We also identify novel changes due to AOM/DSS-induced colitis, such as the emergence and enrichment of the genus *Prevotellaceae*_UCG_001 in AOM/DSS-treated mice at 16 weeks (Fig. 4*a* and *c*), especially in females (Fig. 5*f*). Members of the family *Prevotellaceae* have previously been shown to increase the severity of DSS-induced colitis in mice.[50]

Our study is the first study investigating the impact of intestinal epithelial ERβ on microbiota. A previous limited study used T-RFLP and Sanger's sequencing to characterize 16S rRNA gene variants in fecal pellets from body-wide ERβ knockout mice (BERKO).[12] That study found indications that the diet impacted microbiota differently in BERKO mice, especially the phyla *Bacteroidetes*, *Firmicutes* and *Proteobacteria.*[12] Our analysis, using 16S-Seq, found a lower abundance of *Firmicutes* in KO mice (Fig. 6*c*–*e*). Importantly, we showed that the reduced alpha diversity that resulted 16 weeks after AOM/DSS in WT animals was further reduced in KO mice. Additionally, the beta diversity was significantly impacted by loss of ERβ, and this was enhanced by sex (Supporting Information Tables S2 and S3), at 9 weeks after initiation of AOM/DSS treatment. A number of taxa were differently affected in AOM/DSS-treated KO mice compared to WT mice, including *Prevotellaceae*_UCG_001 which was

transiently reduced 5.6-fold in WT mice but barely reduced (1.2-fold) in KO mice at 9 weeks. This genus could be of particular interest to consider for future studies aimed at exploring how estrogen and ERβ impact gut inflammation and cancer *via* microbiota. Further, *Lachnospiraceae*_UCG_001 was enriched in treated KO mice at 16 weeks (Fig. 6*j* and *l*). This is important because it has been shown that *Lachnospiraceae*_UCG_001 serves as an energy source for epithelial cells *via* production of short chain fatty acids, which maintain the epithelial barrier integrity.[42,43] Fatty acid biosynthesis was induced by AOM/DSS, and membrane transport and carbohydrate metabolism functions were specifically enriched in KO mice exposed to AOM/DSS, per PICRUSt analysis. *Lachnospiraceae*_UCG_001 represents a source of energy for proliferation of the epithelial cells and tumor progression at 16 weeks, and it would be of interest to explore the role of this enrichment in future studies, and to explore whether knockout of intestinal ERβ also impacts the short chain fatty acids in the gut. ERβ in the colon has been proposed to maintain the integrity of epithelial barrier,[51] and our results support a role for ERβ in maintenance of the eubiosis status in the gut.

The effect of knockout was, however, not significant for all parameters analyzed. It is possible that more ERβ-related differences, including those that are specific for each gender, would be identified in a larger study. It is also possible that genotype-affected microbiota changes were diminished in our study due to our choice to co-host WT and KO littermates in the same cages. This was done in order to avoid cage effects, but may result in a partial sharing of microbiota. Finally, we did not explore the mucosal-associated microbiota profiles nor the impact of different diets on these mice. Further studies, exploring luminal microbiota, metabolic interactions with the host, a variety of diets including a purified diet, will yield further information.

In our study, we observed significant segregation of microbiota beta-diversity depending on sex after both 9 weeks and 16 weeks (Supporting Information Tables S2 and S3). We also found differences in the relative abundance of e.g. *Tenericutes, Bifidobacter Verrucomicrobia* and *Gammaproteobacteria* between males and females (Fig. 5*c* and Supporting Information Figure S3), although the difference was diminished after AOM/DSS treatment. Men have a higher incidence of CRC compared to women and it is possible that sex differences impact the microbiota diversity and hence susceptibility to microbiota-associated diseases. For example, ICR (Swiss albino) males have been reported to be more vulnerable to microbiota infections and development of inflammation than corresponding females.[52] A few animal studies have, indeed, demonstrated a correlation between microbiota diversity and sex[53], and in response to different nutrients.[54] Human studies have also indicated a difference in overall microbiota communities in the gut or of specific bacteria related to gender.[55] In addition, studies have shown that microbiota composition can influence the levels of sex hormones and also regulate autoimmune diseases.[56] However, other reports claim that there is no strong correlation between sex and microbiota diversity.[57] We have shown here that intestinal ERβ has an impact on the microbiota, and we have identified sex-dependent differences of the microbiota. It is possible that the sex-dependent differences are related to differential activation of ERβ in males and females. In order to investigate the combination of sex-differences, ERβ, and colitis and CRC, larger studies should be conducted in a time-dependent manner and in more mice strains, to assess the dynamic shifts of microbiota.

Conclusions

We identify that induced colitis and CRC reduce the microbiota diversity, enrich gram-negative bacteria, and strongly impact several phyla. The genus *Prevotellaceae*_UCG_001 is increased when tumors are established, especially in females, and *Parasutterella* is strongly increased in AOM/DSS-induced colitis but decreased once CRC is established. Furthermore, for the first time, we identify an effect of intestinal ERβ on the modulation of microbiota diversity.

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References

1. Ferlay J, Colombet M, Soerjomataram I, et al. Estimating the global cancer incidence and mortality in 2018: GLOBOCAN sources and methods. *Int J Cancer* 2018.
2. Eaden JA, Abrams KR, Mayberry JF. The risk of colorectal cancer in ulcerative colitis: a meta-analysis. *Gut* 2001;48:526–35.
3. Grivennikov SI, Karin M. Dangerous liaisons: STAT3 and NF-kappaB collaboration and crosstalk in cancer. *Cytokine Growth Factor Rev* 2010;21:11–9.
4. Shahanavaj K, Gil-Bazo I, Castiglia M, et al. Cancer and the microbiome: potential applications as new tumor biomarker. *Expert Rev Anticancer Ther* 2015;15:317–30.
5. Westbrook AM, Wei B, Braun J, et al. Intestinal mucosal inflammation leads to systemic genotoxicity in mice. *Cancer Res* 2009;69:4827–34.
6. Colotta F, Allavena P, Sica A, et al. Cancer-related inflammation, the seventh hallmark of cancer: links to genetic instability. *Carcinogenesis* 2009;30:1073–81.
7. Zitvogel L, Ma Y, Raoult D, et al. The microbiome in cancer immunotherapy: diagnostic tools and therapeutic strategies. *Science* 2018;359:1366–70.
8. Lupp C, Robertson ML, Wickham ME, et al. Host-mediated inflammation disrupts the

intestinal microbiota and promotes the overgrowth of Enterobacteriaceae. *Cell Host Microbe* 2007;2:119–29.
9. Zhu Q, Gao R, Wu W, et al. The role of gut microbiota in the pathogenesis of colorectal cancer. *Tumour Biol* 2013;34:1285–300.
10. Arthur JC, Jobin C. The complex interplay between inflammation, the microbiota and colorectal cancer. *Gut Microbes* 2013;4:253–8.
11. Benson AK, Kelly SA, Legge R, et al. Individuality in gut microbiota composition is a complex polygenic trait shaped by multiple environmental and host genetic factors. *Proc Natl Acad Sci U S A* 2010;107:18933–8.
12. Menon R, Watson SE, Thomas LN, et al. Diet complexity and estrogen receptor beta status affect the composition of the murine intestinal microbiota. *Appl Environ Microbiol* 2013;79:5763–73.
13. Hoy YE, Bik EM, Lawley TD, et al. Variation in taxonomic composition of the fecal microbiota in an inbred mouse strain across individuals and time. *PLoS One* 2015;10:e0142825.
14. Kuraishy A, Karin M, Grivennikov SI. Tumor promotion via injury- and death-induced inflammation. *Immunity* 2011;35:467–77.
15. Heimesaat MM, Fischer A, Siegmund B, et al. Shift towards pro-inflammatory intestinal bacteria aggravates acute murine colitis via toll-like receptors 2 and 4. *PLoS One* 2007;2:e662.
16. Zackular JP, Baxter NT, Iverson KD, et al. The gut microbiome modulates colon tumorigenesis. *MBio* 2013;4:e00692–13.
17. Klimesova K, Kverka M, Zakostelska Z, et al. Altered gut microbiota promotes colitis-associated cancer in IL-1 receptor-associated kinase M-deficient mice. *Inflamm Bowel Dis* 2013;19:1266–77.
18. Wang CZ, Huang WH, Zhang CF, et al. Role of intestinal microbiome in American ginseng-mediated colon cancer protection in high fat diet-fed AOM/DSS mice. *Clin Transl Oncol* 2017;20:302–12.
19. Williams C, Dileo A, Niv Y, et al. Estrogen receptor beta as target for colorectal cancer prevention. *Cancer Lett* 2016;372:48–56.
20. Nguyen-Vu T, Wang J, Mesmar F, et al. Estrogen receptor beta reduces colon cancer metastasis through a novel miR-205 - PROX1 mechanism. *Oncotarget* 2016;7:42159–71.
21. Edvardsson K, Nguyen-Vu T, Kalasekar SM, et al. Estrogen receptor beta expression induces changes in the microRNA pool in human colon cancer cells. *Carcinogenesis* 2013;34:1431–41.
22. Edvardsson K, Strom A, Jonsson P, et al. Estrogen receptor beta induces antiinflammatory and antitumorigenic networks in colon cancer cells. *Mol Endocrinol* 2011;25:969–79.
23. Hartman J, Edvardsson K, Lindberg K, et al. Tumor repressive functions of estrogen receptor beta in SW480 colon cancer cells. *Cancer Res* 2009;69:6100–6.
24. Saleiro D, Murillo G, Benya RV, et al. Estrogen receptor-beta protects against colitis-associated neoplasia in mice. *Int J Cancer* 2012;131:2553–61.
25. Cleveland AG, Oikarinen SI, Bynote KK, et al. Disruption of estrogen receptor signaling enhances intestinal neoplasia in Apc(min/+) mice. *Carcinogenesis* 2009;30:1581–90.
26. Andersson S, Sundberg M, Pristovsek N, et al. Insufficient antibody validation challenges oestrogen receptor beta research. *Nat Commun* 2017;8:15840.
27. Klindworth A, Pruesse E, Schweer T, et al. Evaluation of general 16S ribosomal RNA gene PCR primers for classical and next-generation sequencing-based diversity studies. *Nucleic Acids Res* 2013;41:e1.
28. Martin M. Cutadapt removes adapter sequences from high-throughput sequencing reads. *Bioinform Action* 2012;17:10–2.
29. Edgar RC. UPARSE: highly accurate OTU sequences from microbial amplicon reads. *Nat Methods* 2013;10:996–8.
30. Edgar RC. UNOISE2: improved error-correction for Illumina 16S and ITS amplicon sequencing. BioRxiv:081257 2016.
31. Quast C, Pruesse E, Yilmaz P, et al. The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. *Nucleic Acids Res* 2013;41:D590–6.
32. Hugerth LW SM, Pennhag AA, Du J, et al. A comprehensive automated pipeline for human microbiome sampling, 16S rRNA gene sequencing and bioinformatics processing. BioRxiv:286526 2018.
33. Cayuela L, Gotelli NJ, Colwell RK. Ecological and biogeographic null hypotheses for comparing rarefaction curves. *Ecol Monogr* 2015;85:437–55.
34. Vavrek MJ. *fossil*: Palaeoecological and palaeogeographical analysis tools. *Palaentol Electron* 2011;141T:16p.
35. D A. Vioplot: Violin Plot. R Package version 0.2. http://wsopuppenkiste.wiso.ni-goettingen.de/daldler 2005.
36. Segata N, Izard J, Waldron L, et al. Metagenomic biomarker discovery and explanation. *Genome Biol* 2011;12:R60.
37. Langille MG, Zaneveld J, Caporaso JG, et al. Predictive functional profiling of microbial communities using 16S rRNA marker gene sequences. *Nat Biotechnol* 2013;31:814–21.
38. Langille MG, Meehan CJ, Koenig JE, et al. Microbial shifts in the aging mouse gut. *Microbiome* 2014;2:50.
39. Nagalingam NA, Kao JY, Young VB. Microbial ecology of the murine gut associated with the development of dextran sodium sulfate-induced colitis. *Inflamm Bowel Dis* 2011;17:917–26.
40. Hansen J, Gulati A, Sartor RB. The role of mucosal immunity and host genetics in defining intestinal commensal bacteria. *Curr Opin Gastroenterol* 2010;26:564–71.
41. Ye J, Lee JW, Presley LL, et al. Bacteria and bacterial rRNA genes associated with the development of colitis in IL-10(−/−) mice. *Inflamm Bowel Dis* 2008;14:1041–50.
42. Frank DN, St Amand AL, Feldman RA, et al. Molecular-phylogenetic characterization of microbial community imbalances in human inflammatory bowel diseases. *Proc Natl Acad Sci U S A* 2007;104:13780–5.
43. Wang T, Cai G, Qiu Y, et al. Structural segregation of gut microbiota between colorectal cancer patients and healthy volunteers. *ISME J* 2012;6:320–9.
44. Derrien M, Vaughan EE, Plugge CM, et al. Akkermansia muciniphila gen. Nov., sp. nov., a human intestinal mucin-degrading bacterium. *Int J Syst Evol Microbiol* 2004;54:1469–76.
45. Davenport M, Poles J, Leung JM, et al. Metabolic alterations to the mucosal microbiota in inflammatory bowel disease. *Inflamm Bowel Dis* 2014;20:723–31.
46. McCoy JG, Levin EJ, Zhou M. Structural insight into the PTS sugar transporter EIIC. *Biochim Biophys Acta* 2015;1850:577–85.
47. Ricanek P, Lothe SM, Frye SA, et al. Gut bacterial profile in patients newly diagnosed with treatment-naive Crohn's disease. *Clin Exp Gastroenterol* 2012;5:173–86.
48. Derrien M, Van Baarlen P, Hooiveld G, et al. Modulation of mucosal immune response, tolerance, and proliferation in mice colonized by the Mucin-degrader Akkermansia muciniphila. *Front Microbiol* 2011;2:166.
49. Zarember KA, Godowski PJ. Tissue expression of human toll-like receptors and differential regulation of toll-like receptor mRNAs in leukocytes in response to microbes, their products, and cytokines. *J Immunol* 2002;168:554–61.
50. Elinav E, Strowig T, Kau AL, et al. NLRP6 inflammasome regulates colonic microbial ecology and risk for colitis. *Cell* 2011;145:745–57.
51. Wada-Hiraike O, Imamov O, Hiraike H, et al. Role of estrogen receptor beta in colonic epithelium. *Proc Natl Acad Sci U S A* 2006;103:2959–64.
52. Lee SM, Kim N, Son HJ, et al. The effect of sex on the Azoxymethane/dextran sulfate sodium-treated mice model of colon cancer. *J Cancer Prev* 2016;21:271–8.
53. Org E, Mehrabian M, Parks BW, et al. Sex differences and hormonal effects on gut microbiota composition in mice. *Gut Microbes* 2016;7:313–22.
54. Shastri P, McCarville J, Kalmokoff M, et al. Sex differences in gut fermentation and immune parameters in rats fed an oligofructose-supplemented diet. *Biol Sex Differ* 2015;6:13.
55. Dominianni C, Sinha R, Goedert JJ, et al. Sex, body mass index, and dietary fiber intake influence the human gut microbiome. *PLoS One* 2015;10:e0124599.
56. Markle JG, Frank DN, Mortin-Toth S, et al. Sex differences in the gut microbiome drive hormone-dependent regulation of autoimmunity. *Science* 2013;339:1084–8.
57. Kovacs A, Ben-Jacob N, Tayem H, et al. Genotype is a stronger determinant than sex of the mouse gut microbiota. *Microb Ecol* 2011;61:423–8.