ORIGINAL RESEARCH—BASIC

Chronic Intestinal Inflammation and Microbial Dysbiosis Are Associated With Female Reproductive Outcomes in a Mouse Model of Inflammatory Bowel Disease

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BACKGROUND AND AIMS: The mechanism for increased infertility and adverse pregnancy outcomes in women with active inflammatory bowel disease is unknown. We aimed to create a murine model of chronic gut inflammation to study the pathogenesis of reproductive outcomes in inflammatory bowel disease. **METHODS:** Chronic intestinal inflammation was induced with dextran sodium sulfate (DSS) in specific-pathogen–free (SPF) female mice. SPF mice not treated with DSS served as controls. Daily estrous cycle monitoring was performed. Age-matched groups were cohabitated with SPF males for mating purposes. Pup weights, litter sizes, reproductive hormone serologies, peripheral and mucosal immune changes, and 16S rRNA gene taxonomic profiling of the fecal microbiome were measured and characterized. **RESULTS:** DSS treatment led to weight loss, increased disease activity index scores, and reduced colon lengths. Compared to SPF controls, DSS mice spent less time in the estrus phase of the reproductive cycle ($P < .05$) and had decreased litter sizes and pup weights ($P < .05$). DSS-treated mice had lower anti-müllerian hormone and luteinizing hormone ($P < .05$) concentrations and higher estradiol ($P < .05$) concentrations. Among DSS mice, *Turicibacter* abundance correlated positively with the proportion of circulating neutrophils and proinflammatory cytokines and serum estradiol (Spearman $\rho = 0.538–0.650$, $P < .001–.002$). *Lactobacillus* and *Prevotellaceae* positively correlated with pup weights, litter size, estrus phase duration, luteinizing hormone, and immune cell changes from the colon and peripheral blood ($\rho = 0.475–0.695$, $P < .01$). **CONCLUSION:** Chronic bowel inflammation induces gut dysbiosis and likely contributes to adverse reproductive outcomes through endocrine imbalances. Further investigation with human studies is needed.

Keywords: Inflammatory Bowel Disease; Female Infertility; Intestinal Inflammation; Gut Microbiome

Introduction

The impact of inflammatory bowel disease (IBD) on fertility and pregnancy outcomes is poorly understood and stands at the forefront of concerns expressed by women, who are usually diagnosed before the age of 35.[1] Women with active IBD may have a 33% reduction in fertility[2] and are at increased risk for spontaneous miscarriage, small for gestational age infants and preterm birth.[3,4] Although avoiding active disease at the time of conception is associated with reduced risk of disease relapse during pregnancy, this is not always possible, especially for women with ulcerative colitis, who face an increased risk of disease flares during pregnancy regardless of disease control at the time of conception.[3,5]

Women with IBD may experience alterations in menstrual function and a decrease in ovarian reserve as measured by anti-müllerian hormone (AMH).[6–8] Additionally, peripheral immunologic changes and gut microbiota changes in the form of altered bacterial diversity and composition have been shown to occur during pregnancy in IBD.[9,10] Experiments linking alterations in reproductive cycling, endocrine functioning, immunity, and the gut microbiome to fertility and pregnancy outcomes in women with IBD have not been performed. Moreover, these experiments are particularly challenging given fertility and pregnancy outcomes in women are largely limited to retrospective research designs and high rates of voluntary childlessness. In this study, we aimed to establish a novel murine model of active IBD to gain insights into the etiopathophysiology of the effects of chronic gut inflammation on female fertility and pregnancy outcomes.

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Abbreviations used in this paper: **AMH, anti-müllerian hormone; ANOSIM, analysis of similarity; CCA, canonical correspondence analysis; DC, dendritic cells; DSS, dextran sodium sulfate; DAI, disease activity index; FRT, female reproductive tract; IBD, inflammatory bowel disease; PCoA, principal coordinate analysis; SPF, specific-pathogen–free; UC, ulcerative colitis.**

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Methods

Mice

Female specific–pathogen–free (SPF) C57BL/6 (B6) mice were purchased from Charles River Laboratories at 6 weeks of age (Wilmington, MA, USA). All mice were housed in American Association for Accreditation of Laboratory Animal Care approved animal facilities at the University of Minnesota. Littermates were randomly assigned to experimental groups (Figure A1). All mice were fed standard 18% protein chow throughout the study period, except during breeding, where mice were fed breeding chow. Mice were housed under a 12-hour light-dark cycle at 23 °C. All mice were used in accordance with the guidelines of the Institutional Animal Care and Use Committee at the University of Minnesota.

Dextran Sodium Sulfate (DSS) Model

Following baseline fecal and serum collections, as well as baseline estrous cycle assessment by vaginal smear (days 0–19), one cohort of SPF B6 mice was administered 2%–2.5% wt/vol dextran sodium sulfate (DSS; MP Biomedicals) in drinking water, intermittently substituting with normal drinking water in the event of severe colitis symptoms, to establish a murine model of chronic colonic inflammation while minimizing mortality (days 19–48).[11] During a subsequent 5-day breeding period (days 48–53), female DSS mice were not treated with DSS. Following the breeding period, DSS mice were placed back on 1.5% wt/vol DSS throughout the gestation period (days 54–75). Following the gestation period, DSS mice were no longer treated with DSS (day 75-sacrifice). Throughout DSS treatment, female DSS mice were monitored for evidence of loose stools, anal lesions, weight loss, rectal bleeding, and the presence of blood in cages to ensure successful implementation of the DSS model (Figure A1).[11] Disease activity index (DAI) scores were calculated according to stool consistency, the presence or absence of hematochezia, and percent weight loss from baseline, as previously described.[12]

Reproductive Cycle Assessment

Female mice estrus cycle duration was measured by vaginal cytology, as previously described.[13] Briefly, 20–40 uL of phosphate-buffered saline was deposited 1–2 mm into the vaginal vault and flushed 3 times. Vaginal smears were placed on glass slides and imaged on a Leica DM6000 B microscope. Cycle stage was identified by characterization of cell type and number in vaginal smears.

Leukocyte Isolation and Phenotyping

Leukocytes from blood, colon, and female reproductive tract (FRT) were isolated from female mice on the last day of diestrus at the study endpoint (day 75), as previously described.[14] Briefly, blood was treated twice with ACK Lysis Buffer then placed in Roswell Park Memorial Institute (RPMI) medium (Cytiva) supplemented with 5% heat inactivated fetal bovine serum (FBS) (Atlas Bio). Colon length was measured and cecal content was subsequently removed. The colon was cut transversely and minced before being incubated with RPMI + 5% FBS, 1 mM $MgCl_2$, 1 mM $CaCl_2$ and 100 U/mL Collagenase Type I (Worthington) for 45 minutes at 37 °C with shaking. The FRT, including vagina, cervix, uterine horns, and ovaries, were minced and placed in RPMI+ 5% FBS, 1 mM $MgCl_2$, 1 mM $CaCl_2$ and 0.5 mg/mL Collagenase Type IV (Sigma) for 45 minutes at 37 °C with shaking. Tissue was then digested with a GentleMacs Dissociator (Miltenyi) on setting m_spleen_01.01, digesting once for colon tissue and twice for FRT. Single cell suspensions were strained through a 70-micron filter, washed with RPMI + 5% FBS, and placed on ice until staining or incubation. A portion of single-cell suspensions were stimulated with 1x Cell Stimulation Cocktail (CytekBio) or left unstimulated in 200 microliters of RPMI plus 10% FBS, 1% non-essential amino acids, 1% Sodium Pyruvate, 1% L. glutamine and 1% Penicillin and Streptomycin, containing 0.5 uL of GolgiPlug (BD) at 37 °C for 3–4 hours.

Single cell suspensions were stained with antibodies against Ghost Dye (Tonbo), CD45, CD8a, CD8b, Ly6C, CD11c, Ly6G, CD44 (Biolegend) CD19, and CD4 (BD) for 30 minutes at 4 °C. Stimulated and unstimulated cells were treated with Fixation and Permeabilization (BD) then stained intracellularly with interferon-gamma (IFNg), tumor necrosis factor-alpha (TNFa), and interleukin-17A (IL17a) (Biolegend). Cell counts were acquired using PKH26 Reference Microbeads (Sigma Aldrich). All cells were fixed with 0.5% paraformaldehyde overnight and run on an LSR II or Fortessa cytometers (BD). Samples were analyzed using FlowJo Software (Treestar).

Hormone Measurement

Peripheral blood was collected from all mice during the diestrus phase on days 0, 19, 34, 48, and 75 (Figure A1). Serum was isolated following the University of Virginia Center for Research in Reproduction Ligand Assay and Analysis Core (Charlottesville, VA, USA) protocol. Briefly, blood was allowed to clot at room temperature for 90 minutes, then centrifuged at 200×g for 15 minutes. Serum was placed in a polypropylene centrifuge tube and stored at −20 °C until analysis. Serum estradiol, LH, and AMH levels were measured using mouse enzyme immunoassays (ELISA; ALPCO; Salem, NH, USA) by the University of Virginia Center for Research in Reproduction Ligand Assay and Analysis Core (Charlottesville, VA, USA).

Mating and Determination of Fertility Outcomes

Female SPF B6 and DSS mice were mated with C57BL/6-SPF male mice in a ratio of 1 female per male for 5 days. Female mice were checked daily for evidence of copulation plugs. At the first instance of an observable copulation plug, female mice were separated from male mice. Following delivery and before sacrifice, mouse pups were weighed, and the number of pups per litter was recorded (Figure A1).

Fecal Sample Collection, DNA Extraction, and Sequencing

Fresh fecal pellets were collected at 5 different time points on the last day of the diestrus phase (days 0, 19, 34, 61, 75) from mouse cages and immediately frozen and stored in sterile microfuge tubes at −80 °C. Bacterial DNA was extracted from individual mouse fecal pellets (approximately 0.1 g) using the DNeasy PowerSoil Pro DNA Isolation Kit (QIAGEN, Hilden, Germany). The V4 hypervariable region of the 16S rRNA gene was amplified and paired-end sequenced using the 515F/806R primer set[15] on the Illumina MiSeq Platform (Illumina, Inc, San Diego, CA, USA) at read length of 301 nucleotides (nt) by the

University of Minnesota Genomics Center (Minneapolis, MN, USA).[16,17] Raw data are deposited in the Sequence Read Archive under BioProject accession SRP337962.[18]

16S rRNA Processing and Analysis

All sequence processing was performed using mothur software (version 1.41.1), as previously described.[19,20] Sequences were pair-end-joined, trimmed for quality, and aligned against the SILVA database (version 138.1) for clustering. A 2% precluster was used to remove sequences likely to contain errors.[21] Chimeras were identified and removed using UCHIME (version 4.2.40).[22] Amplicon sequence variants were identified at 99% similarity using furthest-neighbor clustering and classified against the Ribosomal Database Project release (version 18). Alpha diversity, measured as Good's coverage and the Shannon and Chao1 indices, was calculated in mothur to determine within-sample diversity. Differences in beta diversity were calculated using Bray-Curtis similarities to determine between-sample diversity.[23]

Quantification and Statistical Analysis

Data are presented as means ± standard deviation. DSS colitis severity, immunological, reproductive cycling and outcomes, and hormone serological data were analyzed using student's T tests for parametric data and Mann–Whitney U and Kruskal–Wallis tests for nonparametric data in GraphPad Prism (Graphpad Software; Boston, MA). To analyze the number of dendritic cells (DCs) in the reproductive tract, outliers were removed based on the robust regression and outlier removal method in Prism. For microbiome data, statistically significant differences in alpha diversity were determined using analysis of variance (ANOVA) with Tukey's Honestly significant difference procedure for pairwise comparisons. Correlative analyses were carried out using canonical correspondence analyses (CCAs) and Spearman correlation tests. ANOVA, Kruskal–Wallis, CCAs, and Spearman correlation tests were completed using XLSTAT software (version 2022.5.1; Addinsoft, Belmont, MA, USA). Longitudinal analysis of predominant genera was performed using the *permuspliner* function of SplinectomeR, a permutation-based package in R that uses weighted local polynomials to test for group differences in longitudinal microbiome data.[24] Analysis of similarity (ANOSIM) was used to evaluate differences in community composition, and ordination was done using principal coordinate analysis (PCoA).[25,26] Correlation of genera abundances with axes positions was performed using Spearman correlation tests. ANOSIM, PCoA, and correlation analyses were performed using mothur. All statistical analyses were performed at $\alpha = 0.05$, and ANOSIM analyses were Bonferroni-corrected for pairwise comparisons.

Results

DSS Induces Chronic Inflammation in Key Tissues

DSS was shown to induce chronic colitis as indicated by weight loss from baseline and increased DAI score (Figure 1A and B). Compared to SPF mice, mice treated with DSS had significantly decreased colon lengths (110.3 ± 31.2 vs 57.4 ± 28.2, Mann–Whitney U test $P < .002$; Figure 1C), significantly increased colon weights (0.2 ± 0.1 vs 0.5 ± 0.5, $P = .021$; Figure 1D), and significantly increased ratios of colon lengths to weight (492.2 ± 149.8 vs 141.4 ± 87.3, $P < .0001$; Figure 1E), all indicators of chronic colitis in this model. There were several changes in immunologic markers of local and systemic inflammation in the colon, reproductive tract, and peripheral blood of DSS mice compared to SPF controls. In DSS-treated mice, the number of CD4+ T cells and IFNg, TNFa, and IL17a expressing CD4 T cells was increased in the colon (Figure 2C and E). There were no significant changes in the frequency of IFNg, TNFa, and IL17a expressing CD4 T cells in the colon among DSS-treated mice (Figure A2A). Interestingly, the frequency of CD8+ T cells and DCs (Figure 2A and B) was reduced, while the number of CD8+ T cells remained unchanged (Figure A2B). The frequency and number of neutrophils increased in DSS treated mice colons (Figure 2D, Figure A2C), suggesting that chronic DSS administration causes a proinflammatory immune phenotype in the colon.

In the FRT, DSS colitis induced changes in the innate and adaptive immune systems: increased frequency and number of neutrophils, number of DCs, and number of IL17+CD4+ cells were noted (Figure 2F–I). However, there were no significant changes in the frequencies of DCs and IL17a expressing CD4 T cells in the FRT (Figure A2D and E). Additionally, DSS mice had altered peripheral blood leukocyte populations compared to SPF mice. Specifically, DSS-treated mice had a higher percent of circulating B cells, neutrophils and monocytes and a lower percent of circulating DCs compared to SPF mice (Figure 2J–M). Taken together, these data indicate that chronic DSS administration results in changes to the immune composition of various compartments and suggests that chronic colitis causes a proinflammatory environment in the colon and FRT.

DSS Colitis Results in Changes to the Reproductive Cycle and Outcomes

There were no differences observed in the appearance of copulation plugs between SPF and DSS-treated mice. The number of days DSS-treated mice spent in the estrus phase of the estrous cycle, also known as "heat", or when females are receptive to mating with males,[24] significantly decreased following DSS initiation (Figure 3A). Among the 28 mated SPF mice across 2 breeding periods, 6 became pregnant, producing 5 viable litters and one nonviable litter. Similarly, of the 21 mated DSS mice, 5 became pregnant, resulting in 2 viable litters and 3 nonviable litters. Furthermore, litter sizes and pup weights were significantly decreased among pups from DSS-treated mothers (Figure 3B and C). These results indicate that DSS-induced colitis interferes with normal reproductive cycling and outcomes.

Reproductive Hormones are Altered in DSS-Treated Mice

Compared to SPF mice and mice before DSS treatment, the concentrations of AMH and LH significantly decreased

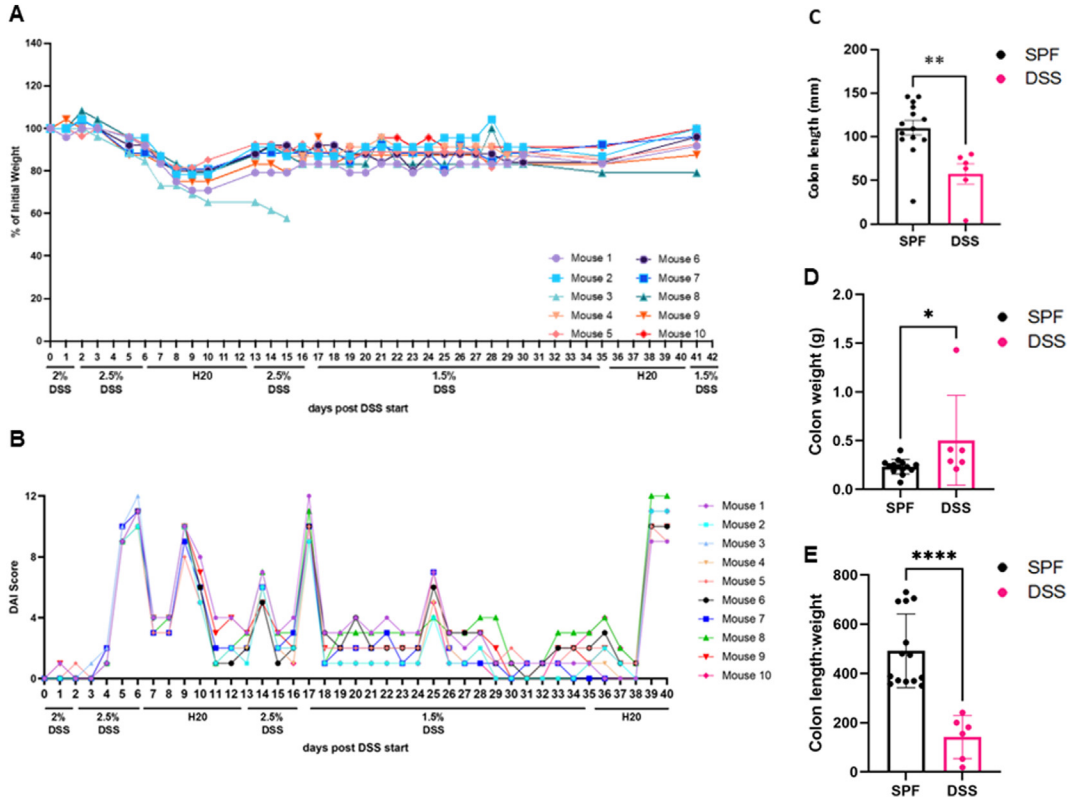

Figure 1. DSS leads to chronic disease at parturition in a mouse model of colitis. (A) Change in weight over time in individual mice receiving DSS are shown as the percent change from baseline weight. Mice were enrolled in breeding on days 36 and 72 (not shown, cohort 1 only) post-DSS initiation. Data shown include mice from one round of experimentation (n = 10). Of note, Mouse 3 died due to DSS treatment in the early phase of the experiment. Mouse 7 was euthanized at a later point in the experiment due to reasons unrelated to the study and per the animal care team. (B) DAI scores are shown over time in individual mice receiving DSS. Data shown include mice from one round of experimentation (n = 10). Mouse 3 died due to DSS treatment in the early phase of the experiment. Mouse 7 was euthanized at a later point in the experiment due to reasons unrelated to the study and per the animal care team. (C–E) Colon lengths, colon weights, and the ratios of colon lengths to colon weights are shown for SPF and DSS-treated mice. Error bars reflect the mean ± standard deviation. Asterisks reflect statistical significance (* = $P \leq .05$, ** = $P \leq .01$, **** = $P \leq .0001$). Each symbol reflects an individual mouse. Experiments were repeated twice; however, some mice could not be included in the final analysis due to demise during the experimental period (SPF n = 14, DSS n = 6).

after DSS treatment. (Figure 4A and B). Conversely, the concentration of circulating estradiol was significantly greater after DSS treatment (Figure 4C). Combining these results, we show that DSS-induced colitis is associated with changes in serum concentrations of reproductive hormones.

DSS is Associated with Changes in Fecal Bacterial Community Composition and Diversity

Among fecal samples, overall community composition and diversity were significantly altered based on treatment group and experimental time point. A mean Good's coverage of 98.4 ± 1.0% (mean ± standard deviation) was achieved from all fecal samples following rarefaction to 4600 sequence reads per sample. Significant changes in alpha diversity were observed among treatment groups and across time points, according to both the Shannon and Chao1 indices (Kruskal–Wallis $P = .0022$ and .0028, respectively), with a significant decrease in alpha diversity in the DSS group before pregnancy, (Dunn's *post-hoc* $P = .0023$ and .0009, respectively; Figure 5A and B). Taxonomic analysis revealed increases in the relative abundances of the bacterial genera *Bacteroides, Turicibacter*, and *Faecalibaculum* and a reduction in *Muribaculaceae, Lactobacillus, Alistipes*, and *Prevotellaceae* following DSS administration (Figure 5C). SplinectomeR longitudinal analysis confirmed significant differences in the relative abundances of *Lactobacillus* ($P = .005$), *Turicibacter* ($P = .002$), *Alistipes* ($P = .007$), and *Muribaculaceae* ($P = .001$) between SPF and DSS mice over time (Figure 5D–G). Ordination of Bray-Curtis dissimilarity matrices by PCoA revealed a significant separation of fecal bacterial communities based on treatment group and experimental time point (ANOSIM R = 0.667, $P < .001$; Figure 5H). Pairwise comparisons revealed a significant separation of samples between SPF and DSS mice

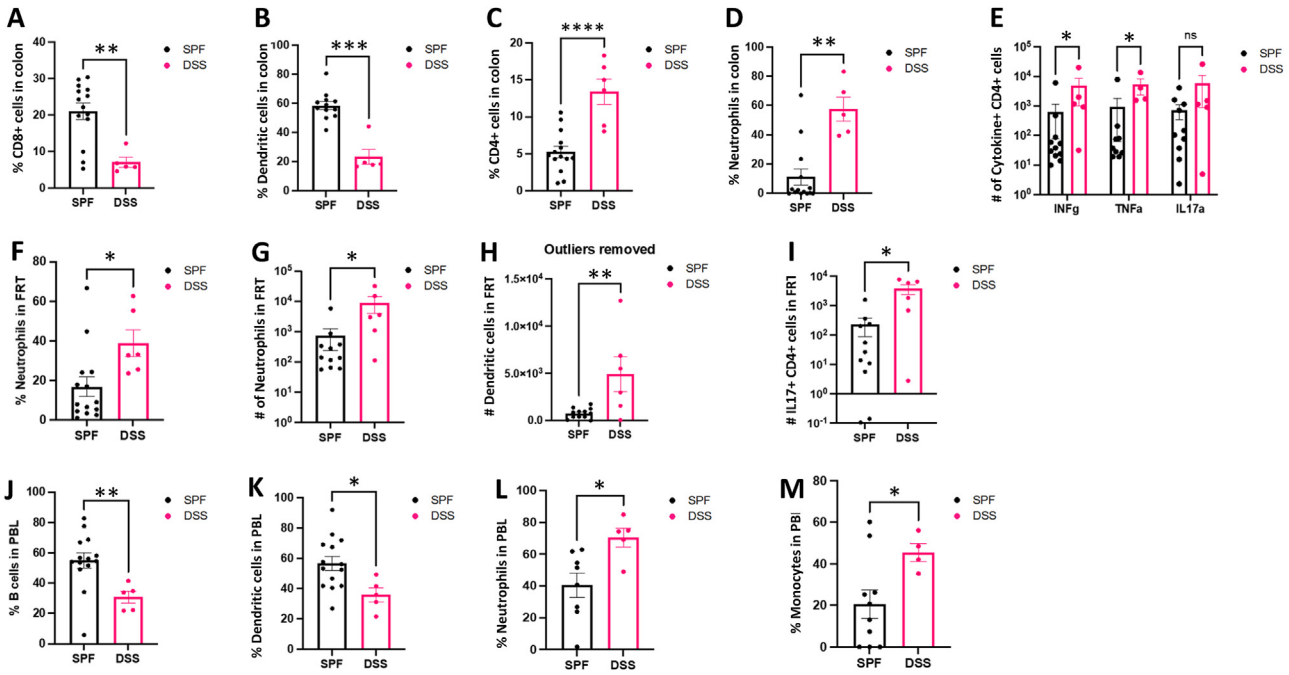

Figure 2. DSS lead to inflammation in the blood, colon, and FRT at parturition. (A–E) DSS-associated changes in immune cells of the colon. (F–I) DSS-associated changes in immune cells of the FRT. To analyze the number of DCs in the reproductive tract, outliers were removed based on ROUT in Prism. (J–M) DSS-associated changes in immune cells in the peripheral blood. Error bars reflect the mean ± standard deviation. Asterisks reflect statistical significance (ns = $P > .05$, * = $P \leq .05$, ** = $P \leq .01$, *** = $P \leq .001$, **** = $P \leq .0001$). Each symbol reflects an individual mouse. Experiments were repeated twice; however, some mice could not be included in the final analysis due to demise during the experimental period (SPF n = 14, DSS n = 6).

before pregnancy (pairwise R = 0.870, $P < .001$, Bonferroni corrected $\alpha = 0.002$). Spearman correlations of the relative abundances of predominant genera and ordination position revealed the DSS group was associated with significantly greater relative abundances of *Bacteroides, Turicibacter, Faecalibaculum*, and *Bifidobacterium* and significantly reduced relative abundances of *Muribaculaceae, Alistipes, Lactobacillus*, and *Lachnospiraceae* (Spearman $P < .05$, Figure 5H). Overall, these results suggest a significant effect of DSS-induced colitis on the composition and diversity of fecal (luminal) microbiota.

Temporal Variation Among Fecal Communities is Significantly Lower in SPF Relative to DSS-Treated Mice

Temporal analysis of fecal samples from SPF mice showed minimal variation in bacterial community composition and diversity over time. Alpha diversity did not vary significantly over time based on both the Shannon and Chao1 indices (ANOVA $P = .262$ and .192, respectively; Table 1). Similarly, taxonomic analysis revealed that the community compositions of fecal samples from SPF mice were relatively unchanged throughout the experimental period (Figure 5C). Furthermore, there were no bacterial genera that varied significantly in their relative abundances over time (Kruskal–Wallis $P > .05$). Significant and directional temporal changes in beta diversity were observed (ANOSIM R = 0.342, $P = .020$; Figure A3A). Further analysis of temporal trends in the SPF group revealed significant pairwise differences between the prepregnancy and postpregnancy time points (pairwise R = 0.621, $P = .002$; Bonferroni-corrected $\alpha = 0.008$; Figure A3A). Notably, there were no significant pairwise differences between the prepregnancy and midpregnancy time points among SPF mice (pairwise R = 0.117, $P = .261$).

Among DSS-treated mice, overall community composition and diversity were significantly altered temporally. Significant changes in alpha diversity were observed among DSS-treated mice, according to both the Shannon and Chao1 indices, with a significant decrease in alpha diversity following DSS treatment (ANOVA $P < .001$ and .009, respectively, Tukey's *post-hoc* $P < .05$; Table 1). Taxonomic analysis revealed increases in the relative abundances of the bacterial genera *Bacteroides, Turicibacter*, and *Faecalibaculum* and a reduction in *Muribaculaceae, Lactobacillus* and *Prevotellaceae* following the initial DSS administration (Figure 5C). Between the prepregnancy and midpregnancy time points, there were notable reductions in the relative abundances of *Lactobacillus* and *Bacteroides* (Figure 5C). Among communities characterized from DSS-treated mice, significant temporal changes in beta diversity were observed (ANOSIM R = 0.476, $P = .021$; Figure A3B). However, pairwise comparisons for specific time points

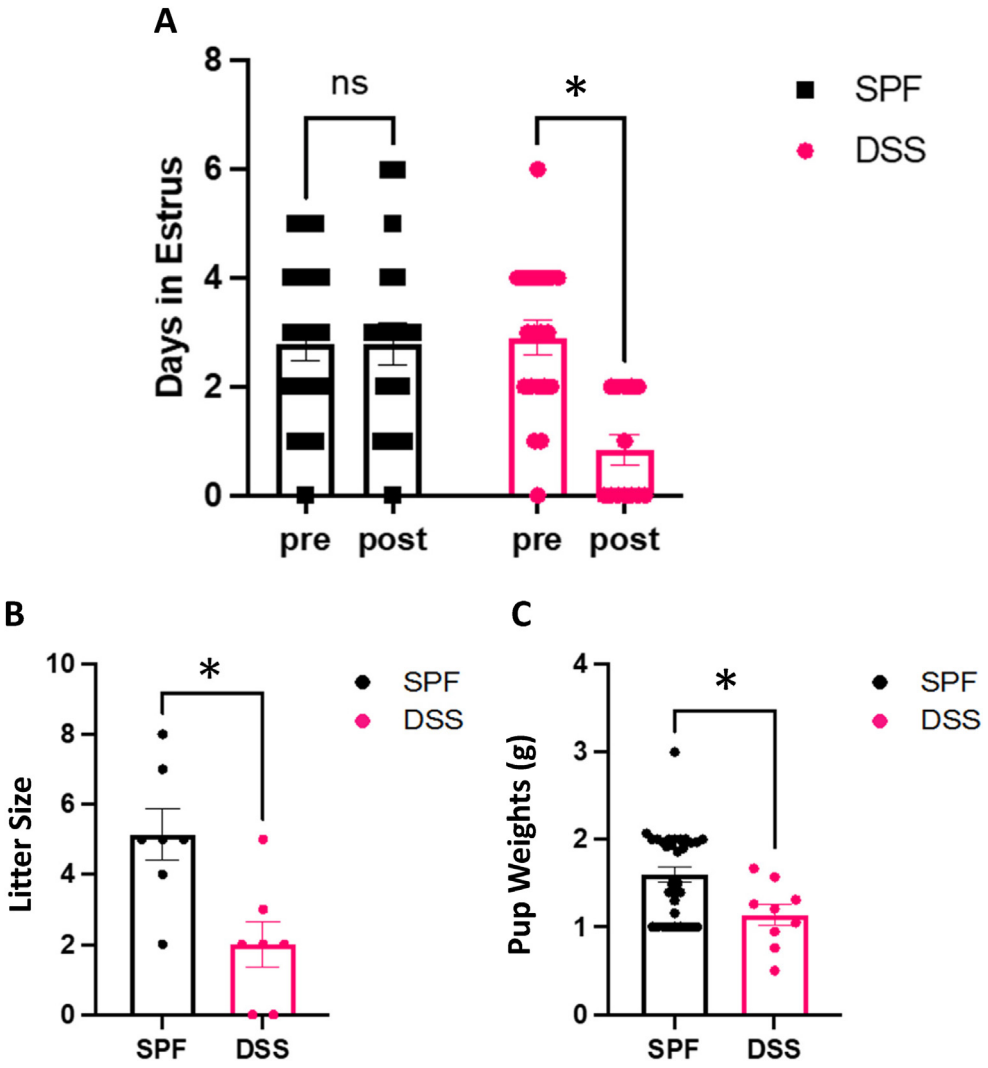

Figure 3. DSS is associated with changes in reproductive cycle and fertility outcomes. (A) Change in the number of days spent in the estrus phase before and after the initiation of DSS treatment. Experiments were repeated twice (n = 20 per treatment group). (B) Litter sizes of SPF and DSS-treated dams, as measured following delivery and before sacrifice (day 75). (C) Weights of pups born to SPF and DSS-treated dams, as measured following delivery and before sacrifice (day 75). Error bars reflect the mean ± standard deviation. Asterisks reflect statistical significance (ns = $P > .05$, * = $P \leq .05$). Each symbol reflects an individual mouse. Experiments were repeated twice; however, some mice could not be included in the final analysis due to demise during the experimental period or failure to become pregnant (SPF n = 7, DSS n = 7).

were insignificant in the DSS group (pairwise $P \geq .019$, Bonferroni-corrected $\alpha = 0.008$; Figure A3B). Spearman correlations based on predominant genera and ordination position revealed *Lactobacillus* was significantly correlated with fecal communities before DSS administration, while *Turicibacter, Bifidobacterium,* and *Bacteroides* were

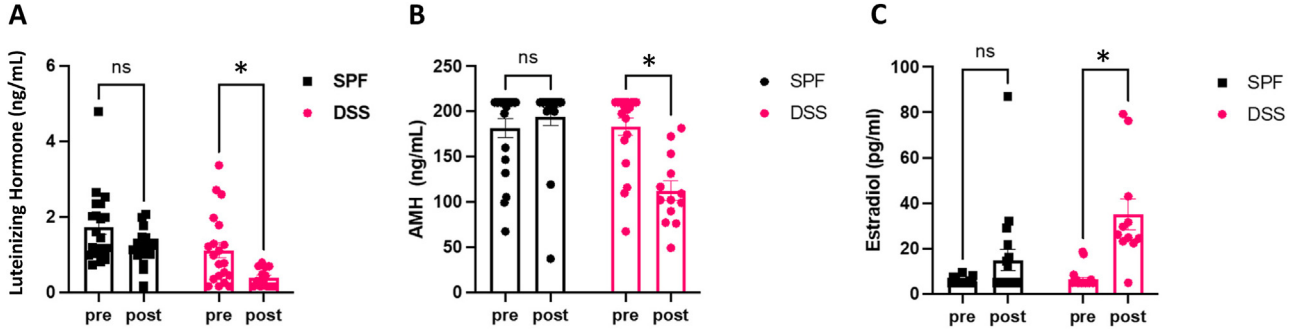

Figure 4. DSS is associated with changes in serum reproductive hormones at parturition. (A) Change in the concentration of LH before (day 0) and after DSS initiation (days 19, 34, 48, 75) on the last day of the diestrus phase. (B) Change in the concentration of AMH before (day 0) and after DSS initiation (days 19, 34, 48, 75) on the last day of the diestrus phase. (C) Change in the concentration of estradiol before (day 0) and after DSS initiation (days 19, 34, 48, 75) on the last day of the diestrus phase. Error bars reflect the mean ± standard deviation. Asterisks reflect statistical significance (ns = $P > .05$, * = $P \leq .05$). Each symbol reflects an individual mouse. Experiments were repeated twice (n = 20 per treatment group).

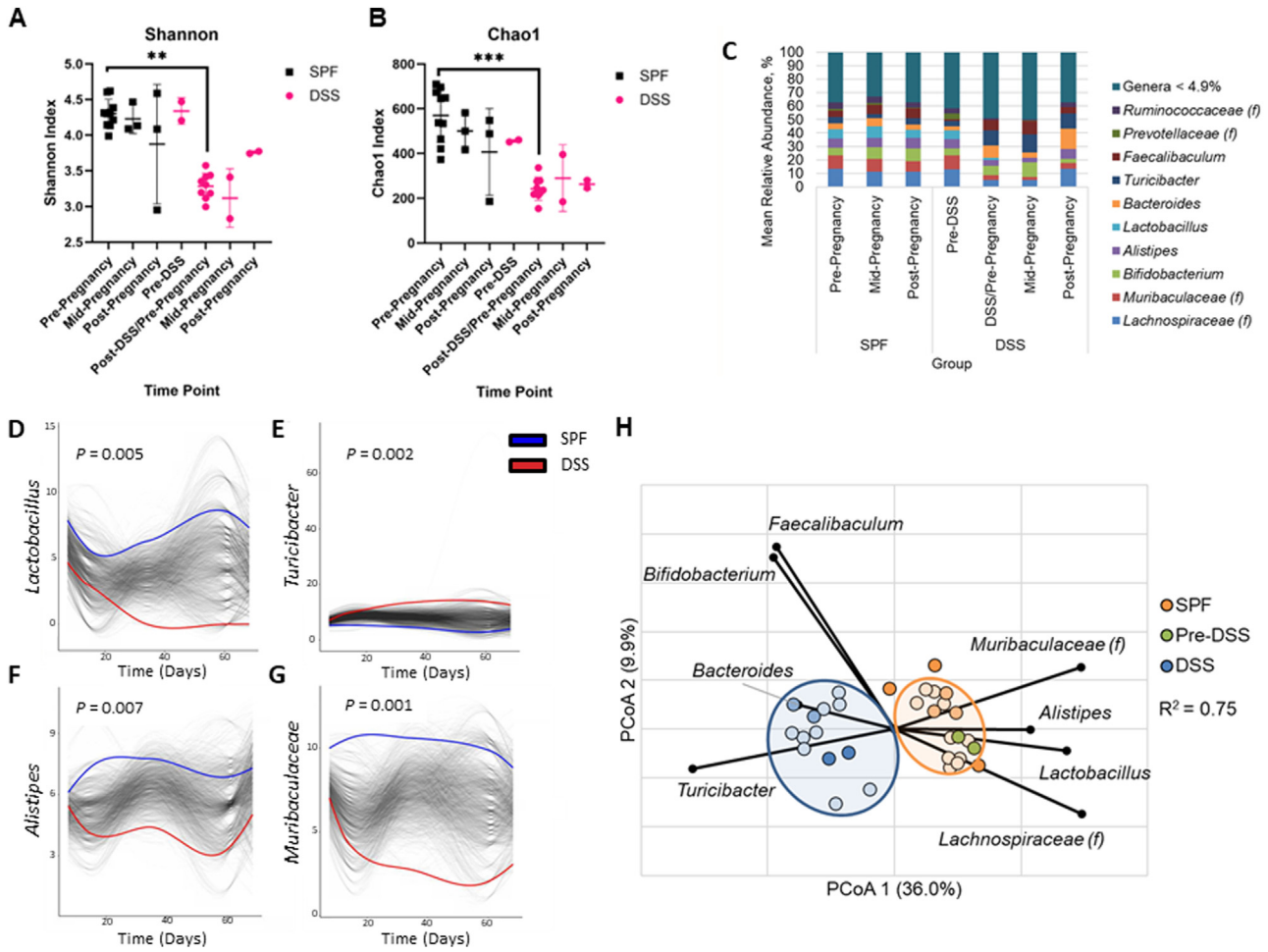

Figure 5. DSS is associated with changes in fecal bacterial community composition and diversity at parturition. (A–B) Average Shannon and Chao1 indices of bacterial taxa in fecal samples collected pre-DSS (day 0) and post-DSS (days 19, 34, 61, 75) on the last day of the diestrus phase. Error bars reflect the mean ± standard deviation. Asterisks reflect statistical significance (** = $P \leq .01$, *** = $P \leq .001$). Each symbol reflects an individual sample. (C) Taxonomic distribution and overall community composition of bacterial genera in fecal samples. Less abundant bacteria make up a mean <4.9% of sequence reads among all samples and were consolidated for clarity. (D–G) Longitudinal analysis of relative abundances of predominant fecal bacteria between SPF (blue) and DSS (red) mice, as determined by SplinectomeR. (H) PCoA of Bray–Curtis distances among bacterial communities in fecal samples. Sample shading becomes darker as time progresses. Each symbol reflects an individual sample. Experiments were repeated twice; however, only 1 cohort of samples was analyzed due to batch effects. Additionally, some mice could not be included in the final analysis due to demise during the experimental period or exclusion of samples due to rarefaction (SPF prepregnancy n = 10, SPF midpregnancy n = 3, SPF postpregnancy n = 3, pre-DSS n = 2, post-DSS/prepregnancy n = 9, DSS midpregnancy = 2, DSS postpregnancy = 2).

associated with fecal samples post-DSS treatment (Spearman $P < .05$).

Correlation Analysis of Fecal Microbiota, DSS-Induced Colitis Severity and Reproductive Cycling, Reproductive Hormones and Outcomes

The CCA comparing all variables revealed a significant relationship between the immunological, reproductive, and endocrine variables and intestinal microbiome data ($P < .0001$, Figure 6). Among fecal samples from DSS and SPF mice, serum estradiol concentration and the proportion of neutrophils, monocytes, and proinflammatory cytokines from the colon, reproductive tract, and peripheral blood had significant positive correlations with *Turicibacter* (Spearman $\rho = 0.538–0.650$, $P < .001–.002$). *Bacteroides* was also positively correlated with these variables, although this comparison did not reach statistical significance ($\rho = 0.235–0.343$, $P = .060–.203$). Additionally, DSS-treated mice exhibited significant positive correlations among pup weights, litter size, the number of days spent in each phase of the estrous cycle, LH concentration, and immune cells from the colon and peripheral blood and the bacterial taxa *Lactobacillus, Prevotellaceae*, and *Muribaculaceae* ($\rho = 0.475–0.695$, $P < .01$). Moreover, there was a moderate negative correlation between estradiol concentration and

Table 1. Average Shannon and Chao1 Indices of Bacteria in Fecal Samples

Group	Time point	Mean Shannon index	Mean Chao index	*P* value (Shannon)	*P*-value (Chao1)
DSS	Pre-DSS (2)	4.34 ± 0.19^{A}	456 ± 7^{A}	<0.001*	0.009*
	DSS/Premating (9)	3.29 ± 0.18^{BC}	243 ± 51^{B}		
	Midpregnancy (2)	3.12 ± 0.41^{C}	290 ± 149^{AB}		
	Postpregnancy (2)	3.76 ± 0.02^{AB}	263 ± 23^{B}		
SPF	Premating (10)	4.30 ± 0.20	571 ± 121	0.262	0.192
	Midpregnancy (3)	4.23 ± 0.21	500 ± 82		
	Postpregnancy (3)	3.87 ± 0.84	408 ± 194		

Alpha diversities according to the Shannon and Chao1 indices across treatment groups and time points. Samples were collected pre-DSS (day 0) and post-DSS (days 19, 34, 61, 75) on the last day of the diestrus phase. Values reflect the mean ± standard deviation. Asterisks (*) indicate a significant result ($P < .05$) using an ANOVA test. Samples sharing the same letter did not differ significantly in pairwise comparisons using the Tukey's Honestly significant difference procedure ($P > .05$). The number of samples (N) is indicated in parentheses.

Lactobacillus abundance in DSS mice ($\rho = -0.599$, $P < .001$). Estradiol concentration was also negatively correlated with time spent in the estrus phase, pup weights, and litter sizes ($\rho = -0.362$ to 0.768, $P < .0001$–$.046$).

Discussion

Infertility and adverse reproductive outcomes are increased in women with active IBD compared to the general population, but the pathogenesis remains unclear. We

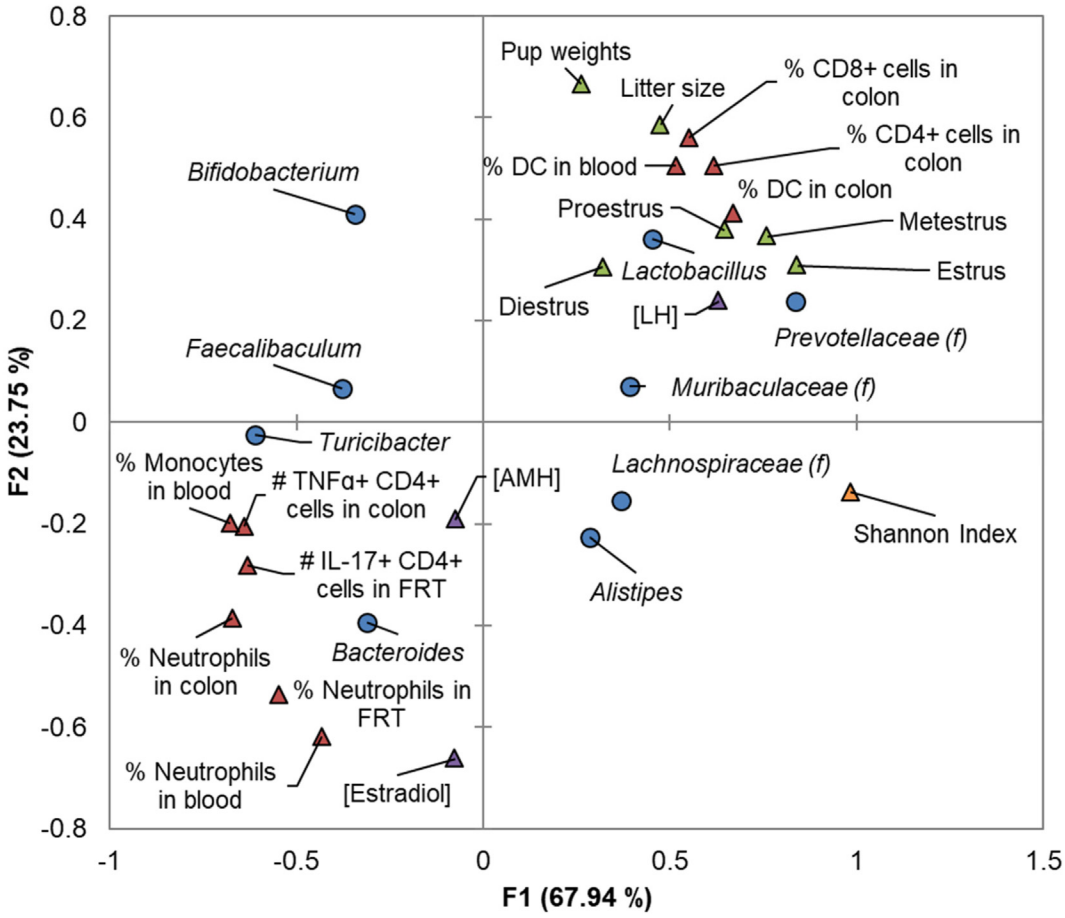

Figure 6. Relationship between fecal microbiota, DSS-induced colitis severity, and reproductive cycling, and reproductive hormones at parturition. CCA of predominant bacterial genera and fertility variables, reproductive hormones, and immune cells in fecal samples of SPF and DSS-treated mice. Correlation of various reproductive (green triangles), hormone (purple triangles), immune (red triangles), and microbiological variables (orange triangles) with bacterial taxa (blue circles) present in fecal samples of SPF and DSS-treated mice. Axes represent a combined 91.69% of the data.

established a mouse model of IBD to characterize the association between chronic gut inflammation and reproductive outcomes in females. The creation of a mouse model offered unique opportunities to dissect the dynamics and associations among changes in (1) the reproductive cycle; (2) circulating reproductive hormones; (3) the innate and adaptive immune system in the peripheral blood, colon and FRT; (4) reproductive outcomes; and (5) the gut microbiota.

Our DSS model successfully induced chronic gut inflammation in the form of significant weight loss, altered stool consistency, rectal bleeding and reduced colon lengths, weights, and ratios of lengths to weights.[11] Our model also successfully mirrored the proinflammatory cytokine state described in women with IBD during preconception.[9] Additionally, the model demonstrated decreased microbial diversity, which is supported by existing IBD literature.[27,28] Moreover, in our model of gut inflammation, pup weights and litter size were smaller compared to controls. Similarly, women who have active IBD during pregnancy have babies who are small for gestational age.[29]

Patients with IBD exhibit intestinal dysbiosis, characterized by greater temporal fluctuation and decreased alpha diversity compared to healthy controls.[30–34] Additionally, prior research suggests a bidirectional relationship between the gut microbiome and estrogen.[35] Under normal conditions, various enzymatic reactions occur in the intestinal lumen, notably the deconjugation of estrogen from glucuronic acid via β-glucuronidase, producing a deconjugated, active form of circulating estrogen.[36,37] β-glucuronidase-producing bacteria include *Lactobacillus* and *Alistipes*.[38] However, gut dysbiosis may disrupt the activity of estrogen-metabolizing bacterial enzymes, leading to dysregulated estrogen signaling and potentially driving estrogen-mediated conditions, such as altered reproductive cycling and infertility.[36,39] Our findings extend this concept by demonstrating that DSS induces microbial dysbiosis, including increases in *Turicibacter* and *Bacteroides*, and decreases in *Lactobacillus* and *Alistipes*. Moreover, DSS-associated genera were positively correlated with serum estradiol levels in DSS mice, while *Lactobacillus* was negatively correlated, suggesting that gut microbial dysbiosis associated with intestinal inflammation may alter estrogen-mediated signaling, contributing to changes in reproductive outcomes.

Circulating estrogen, along with other reproductive hormones, plays a key role in regulating the reproductive cycle. We found that mice treated with DSS spent fewer days in the estrus phase of their reproductive cycle, which is considered the time of greatest fertility. Indeed, women with IBD have been reported to have altered menstrual cycle changes, although data are very limited.[6,40]

Conclusion

Currently, there are no prospective data regarding fertility in women with IBD, but retrospective data suggest that active IBD is associated with infertility.[2,41,42] The cause of this remains a wide knowledge gap that precludes adequate counseling and fertility care for women with IBD. Besides altered reproductive cycling, our study also supports decreased ovarian reserve as a potential mechanism through which chronic gut inflammation may decrease fertility. We found that mice treated with DSS had significantly reduced levels of AMH, suggesting decreased ovarian reserve. Similarly, lower levels of AMH in women with Crohn's disease have been reported.[7,8] It is important to note that age is known to be one of the primary determinants of fertility.[43] Our study design controlled for this factor by ensuring that all mice were the same age at the time of mating.

Reproductive outcomes observed in this study are likely multifactorial and not driven solely by dysbiosis. Our immunologic analyses suggest that proinflammatory changes in chronic gut inflammation expand beyond the peripheral blood and colon, and involve the FRT. Specifically, increased neutrophils and IL-17+ CD4 cells in the FRT strongly correlated with reproductive cycling, litter size, pup weights and specific gut microbiota. The inflammatory state of the FRT has not been characterized in women, but may be a potential mechanism for decreased fertilization and implantation in women with IBD undergoing assisted reproductive technology described in literature.[44]

The main limitation of this study is that the associations made between the specific gut microbial taxa, the proinflammatory changes in the colon, FRT, and circulation with changes in reproductive hormones, cycling and outcomes are largely descriptive. Future studies are required to test the causal links among them. The absence of histological data limits our ability to directly assess the severity of colitis induced in this study. Additionally, food consumption data were not collected, which could help differentiate the effects of colonic inflammation from those of generalized illness on pregnancy outcomes. Finally, microbial analysis of fecal samples from DSS-treated mice at midpregnancy and postpregnancy timepoints was restricted by small sample sizes resulting from DSS-induced morbidity and mortality. Further studies should incorporate these methodologies and refine the experimental model of DSS-induced colitis to better investigate the relationship between IBD and pregnancy.

Although the use of DSS in mice as a model for IBD in humans is well established, it has never been used as a model for reproductive outcomes. We established a mouse model of chronic gut inflammation to help understand the basic mechanisms of pathogenesis of reproductive changes in IBD. Specific gut microbiota and proinflammatory changes in the FRT and circulation are associated with changes in reproductive hormones, cycling and outcomes. We hope that the insights generated by our findings may be applied to further mechanistic investigations to elucidate the pathogenesis of altered reproductive outcomes in the setting of chronic gut inflammation in human patients.

Supplementary Material

Material associated with this article can be found, in the online version, at https://doi.org/10.1016/j.gastha.2025.100670.

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Eugenia Shmidt: Study conceptualization. Vaiva Vezys: Study conceptualization. Alexander Khoruts: Study conceptualization. Christopher Staley: Study conceptualization. Clare F. Quarnstrom: Performed the mouse experiments, collected samples, and performed analyses of DSS severity, immunological, reproductive cycling, fertility outcomes, and reproductive hormone data. Maria Martell: Microbiome and correlative analyses, writing – original draft.

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Experimental procedures were approved by the University of Minnesota Institutional Animal Care and Use Committee and performed following the Office of Laboratory Animal Welfare guidelines.

Data Transparency Statement:
Raw data are deposited in the Sequence Read Archive (URL:https://www.ncbi.nlm.nih.gov/sra) under BioProject accession SRP337962. Additional raw data can be made available upon request.

Reporting Guidelines:
ARRIVE.