Investigation of The Relationship Between Vaginal Dysbiosis with The Activation of Genital and Intestinal Inflammation during The Pregnancy in an in Vivo Mouse

by

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Graduate School of Health Sciences

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To my family...

ABSTRACT

Investigation of The Relationship Between Vaginal Dysbiosis with The Activation of Genital and Intestinal Inflammation During Pregnancy in an *in vivo* Mouse Model

Gülin Özcan Kuyucu

Doctor of Philosophy in Medical Microbiology

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The vaginal microbiota dysbiosis is represented by a low rate of *Lactobacillus* and several facultative pathogens such as *Gardnerella, Atopobium*, and *Prevotella*. A low abundance of *Lactobacillus crispatus* and a high abundance *Gardnerella vaginalis* and *Atopobium vaginae* species are significantly associated with preterm birth.

The host-microbiota has been shown to regulate maternal and fetal immune interaction and birth outcomes. Bacterial taxa associated with dysbiosis have been found to stimulate proinflammatory cytokines that induce preterm labor.

Progesterone treatment is one of the targeted therapies to prevent premature birth. The study aims to investigate the role of dysbiotic microbiota in the inflammatory process in a mouse model and also the effect of progesterone treatment on immune modulation in the genital and intestinal systems during pregnancy.

Our mouse model revealed that vaginal exposure to dysbiotic microbiota showed an increasing trend of proinflammatory cytokine levels in the uterus and pathological inflammation through macrophage accumulation, resulting in 28% preterm birth. After the progesterone treatment in the dysbiosis group, TNFa and IL-6 levels were decreased. Besides, the macrophage density in the uterus was reduced, and less cellular damage in the placenta was observed. Analyzing the vaginal microbiota before or during pregnancy may support the decision for initiation of progesterone therapy. Besides, our results may guide new strategies like personalized treatments with novel drugs for preventing preterm birth.

Keywords: Vaginal microbiota, dysbiosis, pregnancy, preterm, inflammation, progesterone

ÖZETÇE

Vajinal mikrobiyota disbiyozunun genital ve intestinal inflamasyonun aktivasyonu ve gebelik süreci ile ilişkisinin in vivo fare modelinde incelenmesi

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Tıbbi Mikrobiyoloji, Doktora

3 Ağustos, 2023

Vajinal mikrobiyota, farklı bakteri türlerini barındıran dinamik bir ortamdır. Genel olarak, üreme çağındaki sağlıklı kadınların vajinal mikrobiyotasında *L. iners, L. cripatus, L. gasseri ve L. jensenii* türleri baskındır. Disbiyosis, düşük *Lactobacillus* oranı ve *Gardnerella, Atopobium* ve *Prevotella* gibi çeşitli fakültatif patojenlerle temsil edilmektedir. Düşük *Lactobacillus crispatus* oranı ve artan *Gardnerella vaginalis* ve *Atopobium vaginae* kolonizasyonu erken doğum ile önemli ölçüde ilişkilidir.

Mikrobiyotanın maternal ve fetal bağışıklık etkileşimini ve doğum sonuçlarını etkilediği gösterilmiştir. Vajinal mikrobiyotadaki değişiklikler ile bağışıklık tepkisi arasında önemli bir ilişki vardır. Disbiyosis ile ilişkili bakteri türlerinin, erken doğumu tetikleyen proinflamatuar sitokinlerin ekspresyonu ile ilişkili olduğu bulunmuştur.

Progesteron tedavisi, erken doğumu önlemek için uygulanan tedavilerden biridir. Bu çalışma, gebe fare modelinde disbiyotik mikrobiyotanın enflamatuar süreçteki rolünü ve ayrıca progesteron tedavisinin genital ve bağırsak sistemindeki immün yanıt üzerindeki etkisini araştırmayı amaçlamaktadır.

Fare modelinde, disbiyotik mikrobiyota'nın uterusta proinflamatuar sitokin değerlerinde artış eğilimi ile makrofaj birikimi yoluyla patolojik inflamasyon gösterdiğini ve bunun da %28 erken doğumla sonuçlandığı gösterilmiştir. Disbiyoz grubunda progesteron tedavisi sonrası TNFa ve IL-6 seviyelerinde anlamlı bir düşüş gözlemlenmiştir. Ayrıca progesteron tedavisi sonucu uterusta makrofaj yoğunluğunun azaldığı ve plasentada daha az hücresel hasar olduğu tespit edilmiştir. Gebelik öncesi veya sırasında vajinal mikrobiyotanın incelenmesi, progesteron tedavisine başlama kararını destekleyebilir. Ayrıca, sonuçlarımız erken doğumu önlemek için yeni ilaçlarla kişiselleştirilmiş tedaviler gibi yeni stratejilere yol gösterebilir.

Anahtar Kelimeler: Vajinal mikrobiyota, disbiyozis, gebelik, erken doğum, inflamasyon, progesteron

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ABBREVIATIONS

PTB	Preterm Birth
CST	Community State Types
IL-1β	Interleukin-1β
IL-6	Interleukin -6
TNFα	Tumor necrosis factor-a
TLRs	Toll-like receptors
LPS	Lipopolysaccharide
PP’s	Peyer’s patches

Chapter 1

INTRODUCTION

1.1 Vaginal Microbiota

Microbiota plays an important role in maintaining the balance between health and disease. The vaginal microbiota is an important part of the human microbiome, which is a dynamic environment. Various factors such as gestational status, menstrual cycle, and sexual activity can create changes in microbial communities [1] [2]. Vaginal microbiota has a higher stability than gut microbiome. Microorganisms in the vaginal microbiota live in a relationship with the host and protect it from colonization of pathogenic bacteria that cause bacterial vaginosis, urinary tract infections, candida infections, and sexually transmitted diseases [3].

The normal vaginal microbiota is frequently dominated by lactobacilli [4]. Generally, *L. iners, L.cripatus, L.gasseri, and L.jensenii* dominate the vaginal microbiota of healthy women of reproductive age [5]. The microbial taxonomic community state types (CST) were divided into five groups. Four of these have Lactobacillus species predominantly; CST I (*Lactobacillus cripatus*), CST II (*Lactobacillus gasseri*), CST III (*Lactobacillus iners*), and CST V (*Lactobacillus jensenii*), CST IV has a lower proportion of Lactobacillus and represented by several facultative pathogen groups such as *Prevotella, Dialister, Atopobium, Gardnerella, Megasphaera, Peptoniphilus, Aerococcus* [6] (Figure 1.1).

Vaginal microbiota also varies between different races. The rate of lactobacilli in the vaginal microbiota of African women is lower than that of European women, and the species diversity in the vaginal microbiota has increased [7].

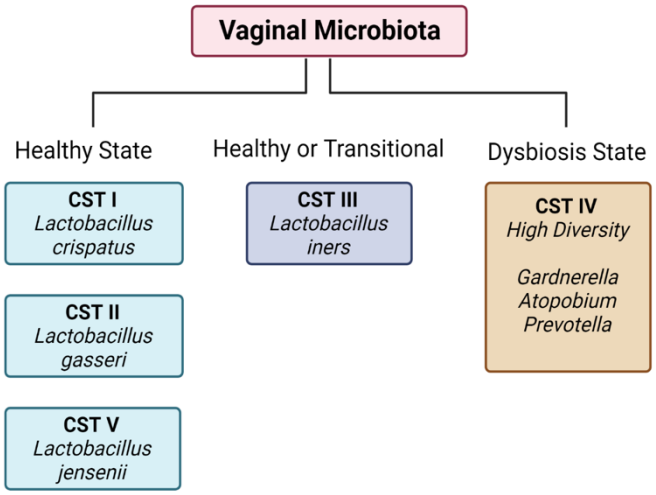

Figure 1.1: Vaginal microbiota classification with community state types (CST).

Lactobacilli has important protective features in the vaginal environment, like maintenance of vaginal homeostasis and regulation of host immunity [8]. *Lactobacilli* bind to the vaginal epithelial surface and prevent the colonization of other microorganisms [9]. They also convert glycogen products deposited in the human vagina to lactic acid. Acidic pH inhibits the growth of other bacteria [10]. Moreover, they produce bacteriocins, and especially *Lactobacillus crispatus* and *L. jensenii species* produce hydrogen peroxide that inhibits the colonization of pathogenic bacteria [11].

The eubiosis term reflects a healthy and balanced microbiota composition. Dysbiosis is characterized by an imbalance of microbial homeostasis, changes in their function and metabolic activities.

In this condition, the relative abundance of the most dominant species decreases, and anaerobic pathogens increase [12]. Microbial dysbiosis can cause health problems by increasing the susceptibility of the host to inflammatory and metabolic disorders [13].

The vaginal microbiota changes with age, and *Lactobacillus* species become dominant after puberty. Pregnancy, menstrual cycle, hormonal changes, and antibiotics affect the microbiota composition [14]. The decrease in the abundance of lactobacillus species leads to an increase in pH (> 4.5) in the vagina and the overgrowth of anaerobes such as *Gardneralla vaginalis, Atapobium vaginae, Mycoplasma hominis, Mobiluncus spp., and Prevotela spp*. [15].

The gut and genital systems microbiota are very complex systems, and communication between these two ecosystems affects the homeostasis of the host immune response. In mouse models, it has been suggested that dysbiotic vaginal microbiota may induce a similar concept in the intestine [16].

The mechanism of preterm birth has been linked with different mechanisms like intrauterine infection, uterine ischemia, short cervix, microorganisms and related products, and inflammation. Approximately 40% of PTB is caused by intrauterine infection [17]. Despite the obstetric follow-up, such as pregnancy history, biochemical

markers, and ultrasonography of the cervix to prevent preterm labor, the rate is still high. The vaginal dysbiosis was found to be associated with preterm delivery and newborn health [18]. Women with Lactobacillus-dominated vaginal flora in the first trimester of pregnancy have a lower risk of preterm than those with dysbiotic microbiota containing *Gardnerella vaginalis* and *Atopobium vaginae* [19]. Also, the species diversity of *Lactobacilli* is a critical factor in determining the pregnancy outcome. *L. iners* is the predominant species associated with abnormal flora and preterm labor in pregnant women. [20] [21]. In contrast, *L. crispatus*, is associated with a stable and healthy vaginal microbiota and has a lower risk of PTB [22] [23].

Another factor related to dysbiosis and preterm is the short cervix. The cervix has a protective role against bacteria both mechanically and chemically [24]. The bacterial infection that progresses from the vagina to the uterine cavity is thought to be one of the leading causes of spontaneous preterm birth. A short cervix is less than 25mm (2.5cm) long at around 20 weeks of pregnancy. Pregnant women with a short cervix (< 25 mm) in the 2nd trimester are at higher risk of preterm delivery [25]. It was shown that women with a short cervix and CST IV group microbiota have a high risk of preterm delivery [26].

1.2 The vaginal dysbiosis, inflammation, and preterm birth

It has been shown that the host microbiota and metabolites regulate inflammatory processes between mothers and newborns [27] [28]. Microorganisms and their products are recognized by Toll-like receptors (TLRs) expressed in the myometrium and placenta

during pregnancy [29]. Activation of TLRs induces the expression of proinflammatory cytokines IL-1b, IL-6, IL-8, and tumor necrosis factor-a (TNFα).

Lactic acid produced by *Lactobacillus* species has been shown to reduce the inflammatory response, including IL-6, IL-8, and tumor necrosis factor (TNFα) [9]. In vitro studies have also shown that *L. Crispatus* and *L. jensenii* stimulate the release of immune modulatory mediators from vaginal epithelial cells [30].

Bacterial vaginosis and dysbiosis-related bacteria *G. vaginalis* and *A. vaginae* induce proinflammatory responses, primarily interleukin-1b (IL-1b), IL-6, and TNF-α. IL-17 and IL-22 produced by TH17 cells have important roles in immunity against bacterial infection. In addition, pathological inflammation by microbial products can trigger tissue damage associated with preterm birth by neutrophil, macrophage, and lymphocyte infiltration [31]. Increased inflammatory molecules in uterine components such as IL-1b, TNFa, and IL-6 have been noted as markers of PTB [32].

In a pregnant murine model, pro-inflammatory cytokines in the uterus were increased, although there was no clear correlation between the murine vaginal microbiota and proinflammatory cytokine levels [33]. In a study, elevated IL-6 in the vaginal fluid was associated with spontaneous preterm labor [34]. Another study with a pregnant mouse model, cervicovaginal *G. vaginalis* colonization, reported an increased inflammation with overexpression of IL-6 in the cervix [35]. The interleukin (IL)-1β, IL-6, IL-8,

tumor necrosis factor (TNF)-α, and IL-17A were found to be higher in women with preterm [21].

Besides the activation in inflammatory reactions, a reduction of anti-inflammatory mechanisms with downregulation in IL-10 response was found to be associated with dysbiotic vaginal microbiota [36]. Vaginal dysbiosis rat model results indicated that vaginal microbiota transplantation and probiotic combination significantly reduce inflammation in the uterine wall and decrease pro-inflammatory cytokines IL-1β and TNFα in vaginal tissue [37].

1.3 Progesterone treatment

Progesterone is increasingly used for the elimination of the surgery risk in the treatment of cervical shortening. Another advantage of progesterone is the lack of adverse outcomes on the newborn (Iams 2014). It has a demonstrable effect on the prevention of premature birth when administered to women in the second trimester of pregnancy [38]. Progesterone reduces the risk of preterm birth by 40% in women with a short cervix. Progesterone maintains the pregnancy with the placenta and decreases cervical inflammation [39]. The mechanism of action of vaginal progesterone regarding the prevention of preterm labor is unknown. It has been suggested that it promotes anti-inflammatory responses in the uterus or reduces uterine contractility. In an in vitro study, progesterone prevented IL-6 secretion by peripheral blood mononuclear cells when stimulated with lipopolysaccharide or lipoteichoic acid [40]. In another study

CD4+ CD25+ T-regulatory cells were increased peripherally in the uterus of progesterone-treated mice [41].

In a bacterial endotoxin-induced mouse model, promegestone inhibited uterine contractions and prevent systemic preterm delivery by reducing systemic and placental inflammation [42].Currently, the vaginal microbiome changes during progesterone therapy is not clarified. In the literature, there is only one study that reported the effect of progesterone treatment on vaginal microbiota with no change in the microbiota composition [26].

The first aim of this thesis is to assess the vaginal microbiota compositions of pregnant women with normal and short cervical lengths and the effect of progesterone on the vaginal microbiota. The second aim of the study is to investigate the role of dysbiotic microbiota in the inflammatory process in a pregnant mouse model and also the effect of the progesterone treatment on pro-inflammatory cytokine levels in the uterus and intestine.

This thesis addresses the gap in the literature regarding the link between preterm birth induced by vaginal dysbiosis and the microbiome-related immune modulatory effect of progesterone treatment during pregnancy.

Chapter 2

MATERIALS AND METHODS

2.1 Study cohort and collection of samples

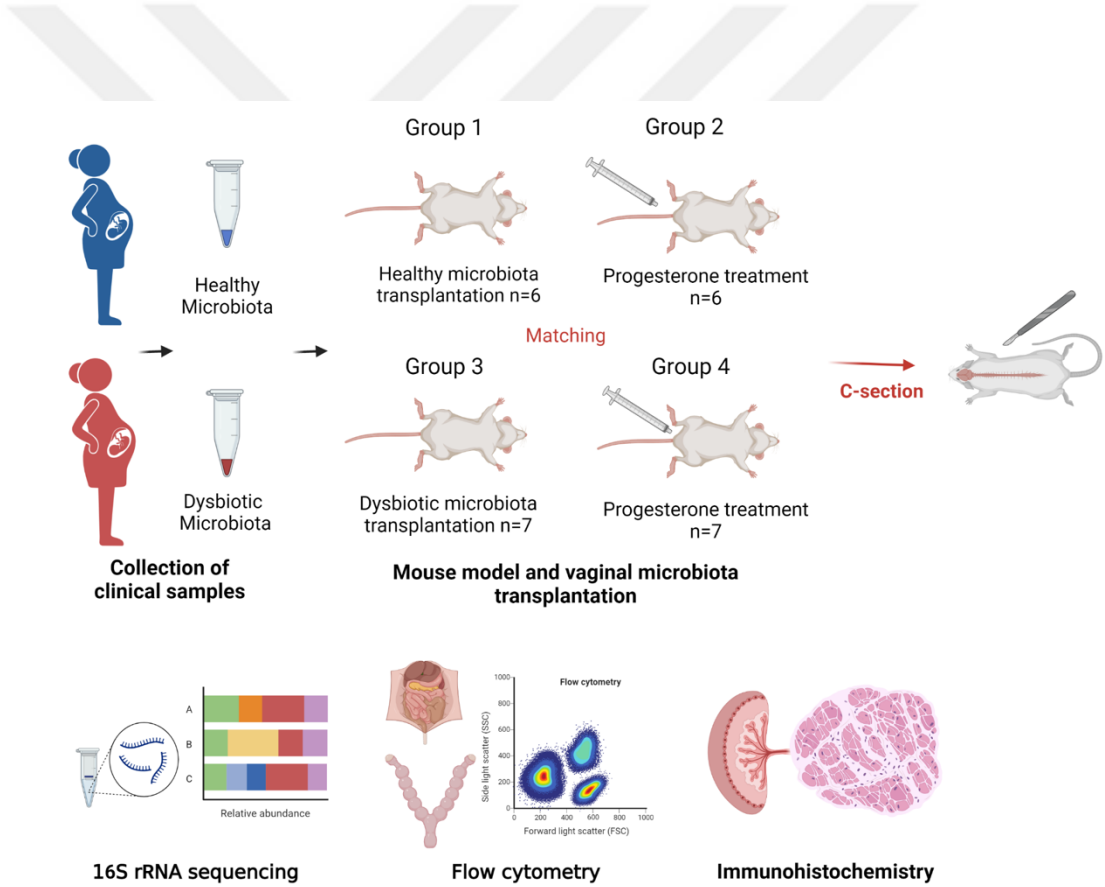

Figure 2.1: Summary of experimental design

Pregnant women followed up in Koc University Hospital Gynecology and Obstetrics Clinic were included in the study. The maternal demographic characteristics, including age, height, weight, obstetric history, and pregnancy outcome, were recorded. The pregnancy outcomes were obtained from hospital records. Gestational age was

determined based on the maternal last menstrual history and further confirmed through the measurement of fetal crown-rump length during a first-trimester scan. The vaginal samples were collected from the posterior fornix using REMEL BactiSwab at gestational ages 11-13, 20-24, and 28-34 weeks. Then, all samples were immediately frozen and stored at −80°C until extraction.

Additionally, the transvaginal measurement of cervical length was done at each visit. Women with singleton pregnancy and short cervical length (< 25mm) were included. Exclusion criteria were as follows: maternal age < 18 years, presence of major fetal defects or chromosomal abnormalities, multiple pregnancies, cervical cerclage in situ, uterine anomaly, recent used antibiotics or antifungals within 2 weeks before the sample collection, sexual intercourse within 72-hour before the sample collection and ongoing progesterone treatment.

Inclusion criteria in the study;

1. Those with one viable singleton pregnancy
2. Pregnancy follow-up at Koç University Hospital Gynecology and Obstetrics Clinic
3. Pregnant women who are 18 years or older
4. Pregnant women who gave consent to participate in the study

Exclusion criteria from the study;

1. Pregnant women under the age of 18
2. Those who did not agree to participate in the study
3. Multiple pregnancies
4. Finding a dead fetus

5. Finding fetal congenital anomaly or chromosomal abnormality

6. Uterine anomaly

7. Previous cervical surgery

8. Having cervical cerclage

9. To have used antibiotics or antifungals 2 weeks before sample collection

10. Having sexual intercourse 72 hours before sample collection

11. Presence of vaginal bleeding during sample collection

12. Pregnant women with fetal loss before 20 weeks of gestation will be excluded from the study.

2.2 DNA isolation from vaginal swabs and 16S rRNA sequencing

DNA isolation was performed using the Qiagen DNeasy PowerSoil Kit (Qiagen), as described by the manufacturers. DNA concentration was quantified by Qubit (Thermo Fisher Scientific). Library preparation was performed using QIAseq 16S/ITS Panel Kit (Qiagen) for sequencing the V1−V9 region of the 16S rRNA bacterial gene. V1 to V9 of the bacterial 16S rRNA genes will be amplified by PCR from microbial genomic DNA. All PCR reactions will be performed with Master Mix, a mix containing forward primer, reverse primer, and 2 ng/μL template DNA. QIAseq Index kit was used for library preparation. DNA fragments were selected with magnetic beads. PCR amplification was done to increase the amount of DNA and to create the library. Library quantification was done using the QIAseq Library Quant Assay (Qiagen) kit following the manufacturer's instructions with Applied Biosystems QuantStudio 7 Flex Real-Time PCR (Applied Biosystems Inc.). Sequencing was performed with the Illumina MiSeq platform using the MiSeq v3 Reagent Kit (Illumina).

2.3 Bioinformatics

FASTQ files were demultiplexed by the different regions using the module in the GeneGlobe Data Analysis Center (https://geneglobe. qiagen.com/tr/analyze). The resulting paired-end FASTQ files containing V1−V2 region sequences were used to profile the microbiota of the samples with Mothur (v.1.45.3). High-quality sequences were aligned with SILVA bacterial reference database (v.138.1).14 Chimeric sequences were removed using the VSEARCH program embedded in the Mothur. Then, the sequences were assigned with taxonomic annotation using the Wang approach implemented in the Mothur. Silva (v.138.1) was used as the reference database for the assignment. Finally, sequences with no more than 3% dissimilarity were clustered into one Operational Taxonomic Unit for the analysis of diversity and composition. The classification of all vaginal trimester samples was identified based on the Community State Types suggested by Ravel et al. (VaginaL community state type nearest centroId classifier).

2.4 Establishment of a mouse model and vaginal microbiota transplantation

2.4.1 Ethics statement

This study was approved by the Koç University Biomedical Research Ethics Committee (2022.HADYEK.039). Human samples and data were obtained by written informed consent in accordance with the ethics committee requirements (2019.093.IRB2.030).

2.4.2 Selection of vaginal microbiota samples

After analyzing the microbiota composition of vaginal swabs from pregnant women with normal and short cervical lengths. 8 pregnant women with a dysbiotic vaginal microbiota CST IV (low *Lactobacillus* ratio and high anaerobic pathogen *Gardnerella vaginalis* or *Atopobium vaginae ratio*) and 8 pregnant women with normal vaginal microbiota composition CST 1 (90% *Lactobacillus crispatus*) were selected for the animal model study. Vaginal swabs were stored at −80 °C until transplantation to mice.

2.4.3 Animals

C57BL/6j 10-week-old female mice were housed in Koç University experimental animal laboratory under a 12 h light/day photoperiod 50-80% humidity and 25 °C temperature conditions with ad libitum water and breeding diet. Timed pregnancies were established by introducing a breeder male to a cage housing two females within one hour before lights off. Males were removed, and copulation plugs were checked. Noon on the day the plug was observed was considered embryonic day 0.5 (E0.5).

2.4.4 Maternal antibiotic exposure

Antibiotic treatment started after the definition of pregnancy with vaginal plug. The existing microbiota was destroyed by oral administration with drinking water (ad libitum) mixture containing ampicillin, gentamycin, and metronidazole (1 g/L), 3% sucrose, and 1% glucose for 7 days [43].

2.4.5 Microbiota transfer

From the 11th day of pregnancy, vaginal microbiota was transplanted to pregnant mice vaginally for 5 days [37]. Vaginal swabs were solved in PBS and 20 µL of selected human vaginal samples for each mouse were stored at −80 °C. Inoculation was achieved by pipetting with 20 µL of fluid containing human vaginal microbiota at the vaginal opening of the mouse.

2.4.6 Progesterone treatment

One of the matched pregnant mice was treated with progesterone intraperitonally (1 mg) twice, and the other mouse group was followed without progesterone. Progesterone treatment was done after the transfer of the vaginal microbiota.

2.4.7 Vaginal microbiota sampling

To investigate the effect of progesterone on microbiota with 16S rRNA sequencing, vaginal samples were collected on the 18.5th day of pregnancy from mice.

2.4.8 Cesarean section

Mouse groups followed up for the time for delivery and if preterm delivery was not observed, the cesarean section was performed on the 18.5th day of pregnancy; placenta, cervix, uterus, and intestine tissues (peyer's patches) were collected.

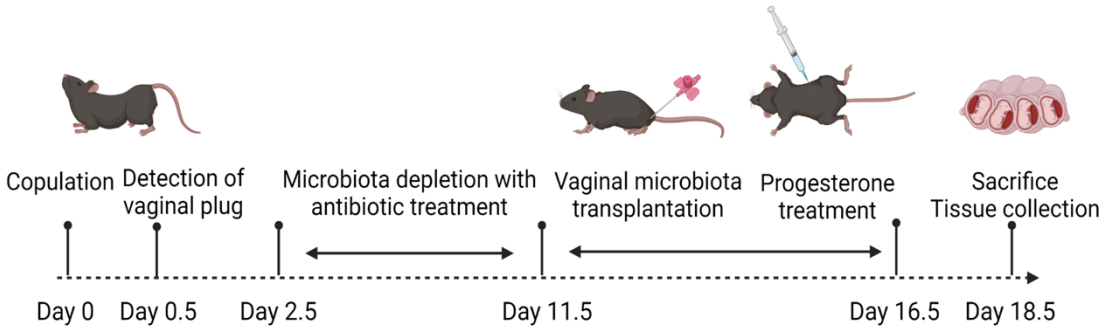

Figure 2.2: Experimental design scheme of pregnant mouse model

2.5 Monitoring of inflammatory processes

2.5.1 Flow cytometry

2.5.1.1 Isolation of lymphocytes from Peyer's patches and uterus

PPs located on the anti-mesenteric side of the intestine and uterus tissue were excised and placed in cold Hank's Balanced Salt Solution (HBSS). PP's transferred into the complete medium with 1 mg /ml collagenase IV (Sigma) to enzymatic dissociation and incubated for 30 min at 37 °C shaking at 100 rpm. For lymphocyte isolation from the uterus, uterus tissue was cut into small pieces with sterile scissors and put in gentleMACS™ cell dissociation tube containing 1mg/ml collagenase, A complete medium. Then, cells were incubated for 1 hour at 37 °C using gentleMACS™ Tissue Dissociator (Miltenyi Biotec) to generate a single-cell suspension. Then, cells were placed on a sterile 70 µm nylon mesh cell strainer. The cell suspension was centrifuged for 5 min at 500 x g. Supernatant resuspended in the complete medium.

2.5.1.2 Stimulation of lymphocytes

Cells were incubated with a cell activation cocktail with Brefeldin A (BioLegend) for 4 hours in the incubator at 37 °C to stimulate the cells.

2.5.1.3 Antibody labeling of lymphocytes

After the incubation, cells were stained with viability dye (1:500 in PBS) and incubated for 20 min. Then, 50 µL FACS buffer per well of master mix was prepared containing pre-titrated amounts of the antibodies (CD69, CD25, CD19, CD3, and CD4), plus 5 µl/mL of α-CD16/32-Ab (clone 2.4G2). The master mix was added to the cells, resuspended, and incubated for 20 minutes in the dark. Stained cells were fixed and permeabilized with BD Cytofix/Cytoperm Fixation/Permeabilization and BD Transcription Factor buffer set. Intracellular staining was performed with the RORgt and cytokines IL-6, IL-17, IL-22, TNFα, and IL-1B overnight at 4 °C. Cells were centrifugated for 5 min at 500 x g and washed with Facs buffer. Cells were resuspended in 700 ul Facs buffer (to eliminate other cells, the cell suspension was placed on a 40 um nylon mesh cell strainer). Samples were run using Attune Flow cytometer (Attune NxT Flow Cytometer). Analysis was performed with FlowCo Software.

2.5.2 Immunohistochemistry

Cervix, uterus, and placenta tissues from mice embedded in paraffin and 4-6 µm sections were taken. Tissue sections were deparaffinized and rehydrated using xylene and ethanol. Antigen retrieval was performed with citrate buffer (Ph 6.0) for ten minutes. Protein block was done to reduce non-specific binding. Sections were incubated with macrophage marker anti-Iba1 antibody (ab178846) diluted in PBS

1/4000 for 45 min at room temperature. Then, sections were incubated with a secondary antibody for 15 min using Mouse and Rabbit-specific detection kit (ab236466). Counterstains were done using Mayer hematoxylin. Histologic examination was performed.

2.5.3 Statistical analysis

The alpha diversity, beta diversity, and vaginal microbiota composition of pregnant women were analyzed. Alpha diversity indices were calculated by the summary. single command embedded in the Mothur. Beta-diversity was defined using the Bray−Curtis distance and generated using the dis. shared command in the Mothur. The evaluation of differences in the alpha diversity metrics and microbiota composition was performed by Wilcoxon signed-rank test using Python 3.7. The significance of group dissimilarity based on the Bray−Curtis distance matrix was evaluated by the analysis of molecular variance (AMOVA) test using Python. For comparison of flow cytometry results between control and dysbiosis groups were calculated with the Mann-Whitney test. To evaluate the effect of progesterone treatment Wilcoxon signed-rank test was used. Statistical significance was set as $p< 0.05$. Statistical data were visualized with GraphPad Prism 8.0.2

Chapter 3

RESULTS

3.1 Demographics of the study population

A total of 58 pregnant women who met the entry criteria were included in the study. The maternal and neonatal characteristics and pregnancy outcomes are presented in Table 1. The preterm birth rate was nearly 10 percent in the short cervix group. Some of the patients with preterm risk used progesterone treatment (n=18).

Table 3.1: The characteristics of study participants

Demographics	Control group (n=27)	Short cervix group (n=31)
Maternal age, mean ± SD	30,9 ± 3,8	32,4 ± 4,6
Body mass index, mean ± SD	22,5 ± 3,7	23,7 ± 3,9
Gravida, median (IQR)	2 (1 - 3)	2 (1 - 3)
Para, median (IQR)	0 (0 - 2)	1 (0 - 2)
Cervical length, mean ± SD	36,1 ± 5,2	21,4 ± 4,6
Birth week, median (IQR)	38,9 ± 0,78	36,9 ± 3,15
Birth weight, mean ± SD	3348,8 ± 411,5	3041.9 ± 700,0
Preterm rate (%)	0%	10%

3.2 Analyses and classification of vaginal microbiota

3.2.1 Vaginal microbiota composition and CST Classification

A total of 3319713 high-quality 16S rRNA gene sequences were identified. The mean number of sequences obtained per sample was 44262. In total, 4682 OTUs from 73 bacterial families were identified in the dataset.

The characteristics of bacterial communities during pregnancy were examined by assessing their alpha and beta diversity. The analysis of beta diversity using the Bray-Curtis distance metric did not demonstrate any significant differences throughout the trimesters (Figure 3.1A). The analysis revealed that the Shannon diversity index did not show significant differences among the trimesters ($p=0.17$), indicating a similar level of microbial diversity throughout pregnancy. Similar to the Shannon index, the Chao index value did not show significant changes among trimesters ($p=0.886$) (Figure 3.1B). The diversity of vaginal microbiota is sustained during all trimesters of pregnancy.

The most abundant taxa of the three trimesters are shown in Figure 3.2. The vaginal microbiota of the three trimesters was dominated by Firmicutes and Actinobacteria. Bacteriodata was abundant in the first trimester (Figure 3.2A).

At the genus level, *Lactobacillus* was consistently dominant throughout all trimesters of pregnancy. Other taxa, such as *Gardnerella* and *Prevotella,* were relatively rare in comparison (Figure 3.2B). There was no significant difference at the species level

throughout all trimesters. *Lactobacillus crispatus* (40.6%, 40.8%, and 44.4%, respectively) was dominant in all trimesters ($p=0.081$). It was followed by *Lactobacillus*

*iners (*28.5%, 31%, 25.04%, respectively) (p=0.311)*, Lactobacillus gasseri* (16.1%, 14.7%, 15.8%, respectively) (p=0.959), and low amounts of *Lactobacillus jensenii* (7.1%, 5.8%, 5.6%, respectively) (p=0.898). The remaining species had a lower abundance in the gestational ages, including *Lactobacillus vaginalis*, *Gardnerella vaginalis, Prevotella bivia, and Prevotella timonensis* with no differences throughout the trimesters (Figure 3.2C).

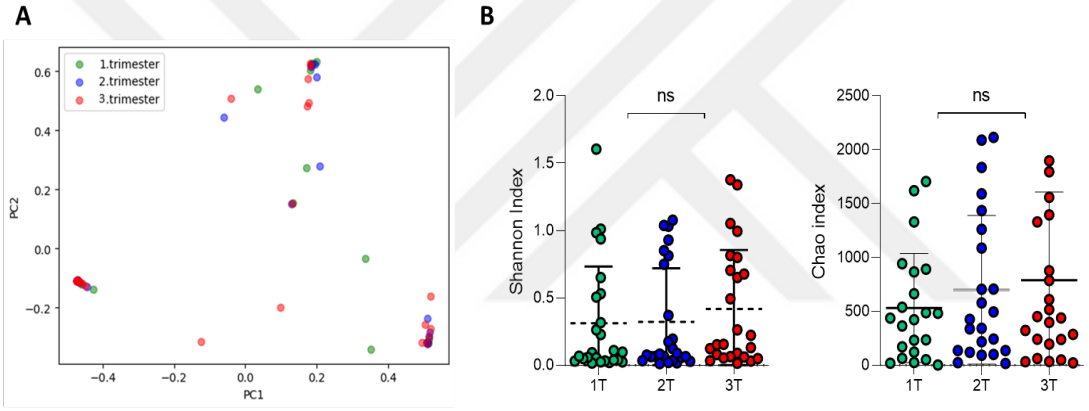

Figure 3.1: The comparison of beta diversity and alpha diversity of the vaginal microbiota among three trimesters (n=25). (A) Beta diversity was analyzed using Bray−Curtis distances. (B) Wilcoxon signed rank test is performed for comparison of alpha diversity metrics.

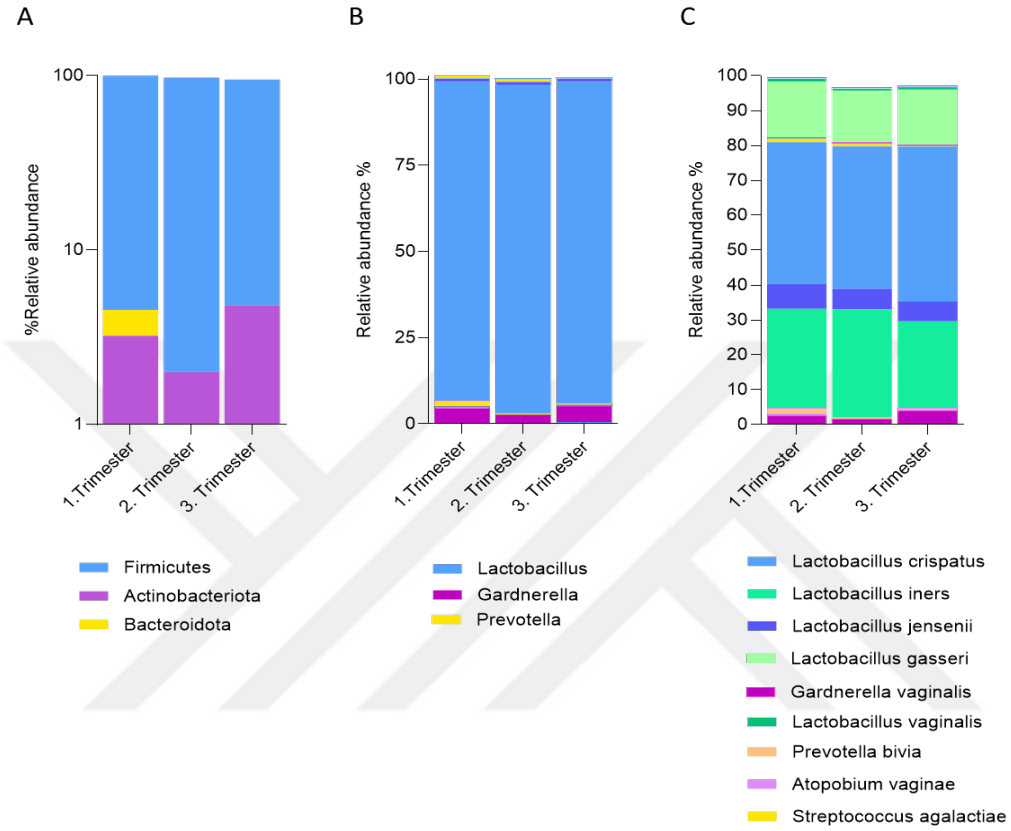

Figure 3.2: The vaginal microbiota composition of pregnant women during three trimesters (A) The most abundant bacteria at the phylum level are represented in three trimesters (B) Abundance at the genus level are represented in three trimesters (C) The relative abundance in species level in three trimesters.

The distribution of community-state types of trimesters is represented in Figure 3.3. In 40-48% of the pregnant women, *L. crispatus* dominated CST I-A and was identified in all trimesters. Also, 20-24% of the pregnant women harbored *L. gasseri* dominated CST-II, while 4-8% of them had *L. iners* dominated CST III-A or CST III-B, and 4-8% had *L. jensenii* dominated CST-V in all trimesters.

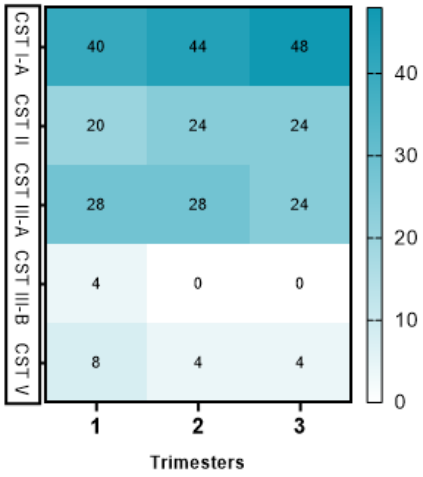

Figure 3.3: CST I, II, III-A -III-B and V during pregnancy were characterized by dominant Lactobacillus species typically represented by *L. crispatus, L. gasseri, L. iners, L. jensenii* respectively.

3.2.2 Differences in vaginal microbiota of women with normal and short cervical length

During the second trimester of pregnancy, a short cervix was associated with vaginal dysbiosis, indicated by an increased alpha diversity Shannon index and Chao index, significantly higher in women with short cervix than in the control group (p=0.018) (Figure 3.4). Beta diversity was similar between the two groups (p=0.657).

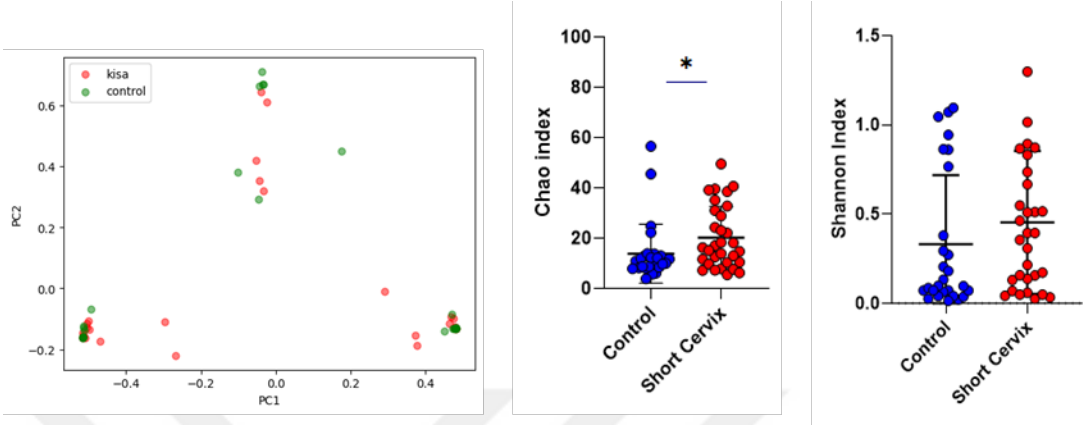

Figure 3.4: The comparison of alpha diversity of the vaginal microbiota between women with the normal cervix (n=27) and short cervix (n=31). Mann−Whitney U rank test is performed for comparison of alpha diversity metrics. Data are presented as mean the p-value less than 0.05 is accepted as significant (*p< 0.05).

There was a decrease in Firmicutes (90.68% vs. 97.6%; p=0.041) with an increase in Actiobacteriota (7.11% vs. 1.87%; p=0.497), Bacteroidota (1.73% vs. 0.4; p= 0.004) and Proteobacteria (0.2% vs. 0.01%; p=0.007) in women with short cervix than those women with a normal cervical length.

Lactobacillus (88.8% vs. 95.5%; p=0.089) abundance was decreased in the short cervix group however, *Gardnerella* (5.65% vs.1.5%; p=0.510), *Atopobium* (1.07% vs. 0.06 p=0.524), *Prevotella* (1.72% vs.0.4%; p=0.013), *Aerococcus* (0.34% vs.0.01%; p=0.812), and *Escherichia-Shigella* (0.267% vs.0.0009; p=0.022) ratios significantly increased.

In the species level, the relative abundance of *Lactobacillus gasseri* (4.9% vs. 13.8%; p=0.023) was lower, however *Lactobacillus iners* (%37.7 vs. 32.2%; p=0.553), *Gardnerella vaginalis* (5.6% vs. 1.5%; p=0.520), and *Atopobium vaginae* (1% vs.

0.06%; p=0.599), *Prevotella spp.* (1.39% vs.0.31%; p=0.254) were higher in women with short cervix compared to women with control (Figure 3.4). There was no significant difference in the abundance of *Lactobacilus crispatus* (40.2% vs. 41.2%; p=0.574).

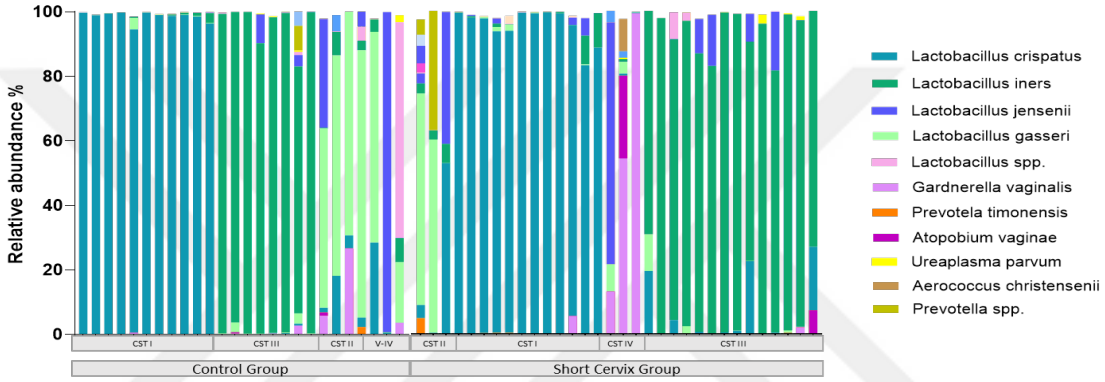

Figure 3.5: The second-trimester vaginal microbiome composition of the control group and (n =27) women with short cervical length (n =31).

3.2.3 Changes in Microbiota composition during pregnancy in women with short cervix

Since a short cervix develops in the second trimester of pregnancy, vaginal microbiota composition in the first trimester can contribute to cervical shortening. Therefore we compared the vaginal microbiota composition of women with short cervix during the first and second trimesters. The mean relative abundance of *L. crispatus* decreased from 55% to 36.1% after cervical shortening. In contrast, *L. iners, L. jensenii* and *A. vaginae* ratio increased from 20.1%, 5.91%, 2.09% to 30.9%, 16.5%, 3.30% in the second trimester, respectively (Figure 3.5).

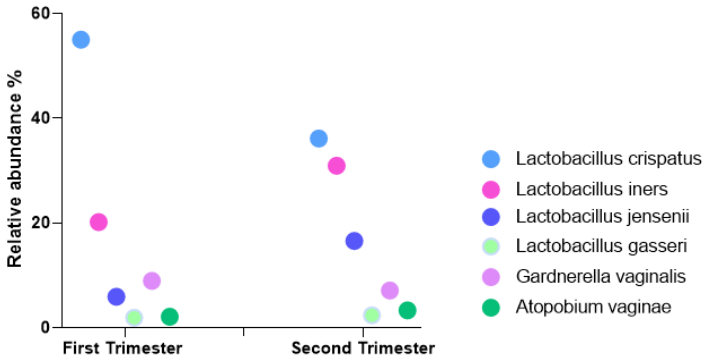

Figure 3.6: The vaginal microbiome composition of the women with short cervical length in the first and second trimesters.

3.2.4 Effect of progesterone treatment on vaginal microbiota in women with short cervix

Vaginal progesterone treatment had no significant effect on vaginal bacterial diversity of the short cervix group regarding species richness and alpha diversity indices Shanon index (p=0.283). Progesterone did not significantly change the mean relative abundance of bacterial taxa. Before treatment *L. crispatus* ratio was 44.4%, while after the treatment, it was 41.2% (p=0.141), and *L. iners* was 33.9% vs. 37.3% (p=0.167) (Figure 3.6).

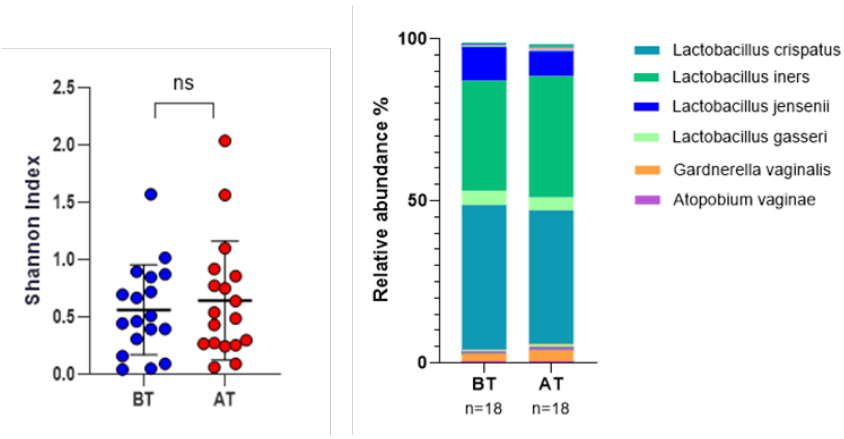

Figure 3.7: Vaginal microbiota comparison of women with short cervix before and after progesterone treatment (n=18). BT; Before treatment, AT; After treatment.

3.2.5 Effect of progesterone treatment on vaginal microbiota in women with normal cervix

Vaginal progesterone treatment also had no significant effect on women with normal cervix. However, in some patients, vaginal microbiota dynamics slightly changed after treatment. *Gardnerella vaginalis* and *Atopobium vaginae* abundance decreased after treatment, as shown in the Figure for Patient 4 and Patient 6 (Figure 3.7).

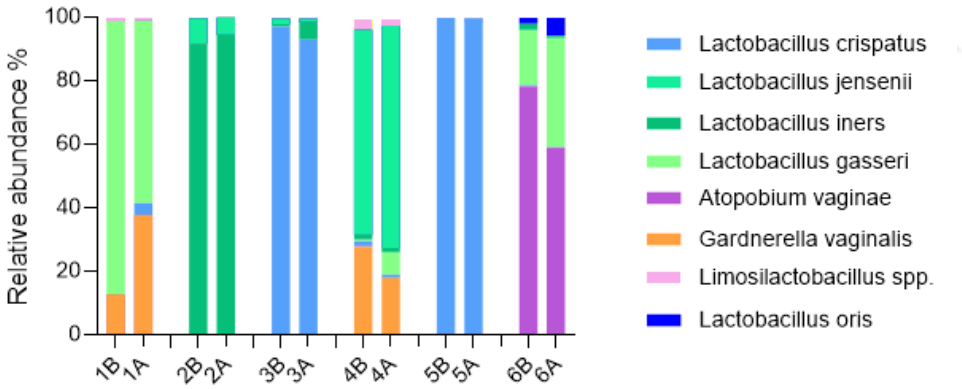

Figure 3.8: Vaginal microbiota comparison of women with normal cervix before and after progesterone treatment. 1; Patient number B; before treatment, A; After treatment

PART II

3.3 Establishment of a mouse model and vaginal microbiota transplantation

3.3.1 Classification and selection of human vaginal microbiota samples for transplantation

Community state types were established using VALENCIA (Vaginal community state type nearest centroId classifier) method for select samples and transplantation of human microbiota to mice. Six CST I *Lactobacillus crispatus-dominated* samples were selected for transplantation to the mice control group, and seven CST IV *Gardnerella vaginalis* or *Atopobium vaginae* dominated samples were selected for transplantation to the mice dysbiosis group (Figure 3.8).

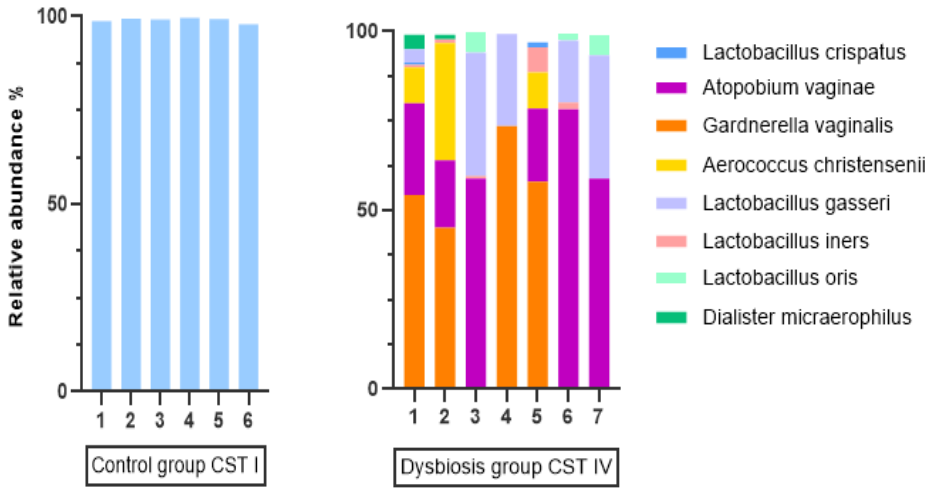

Figure 3.9: Compositions of vaginal microbiota transplanted into mice. Control group; *Lactobacillus crispatus* dominated CST I type microbiota, Dysbiosis group; *Gardnerella vaginalis* or *Atopobium vaginae* dominated CST IV type microbiota.

3.3.2 Animal experiments

Samples were collected by cesarean section at 18.5 days of pregnancy (Figure 3.9). However, in the dysbiotic group, among ten fetuses, one fetal intrauterine death and three abortus were seen in the two dysbiotic mice group, and two preterm birth (28.5%) at 17.5 days of pregnancy was seen in the dysbiotic group belonging to Atopobium vaginae dominated dysbiotic microbiota (Table 3.2). Pups were exterminated after exposure to cold.

Table 3.2: Mouse model pregnancy outcomes

Groups	Microbiota transplantation (n)	Term birth n / %	Preterm birth n / %	Intrauterine fetal death	Abortus
Dysbiosis	n= 7	5 / 71.43 %	2 / 28.57%	1*	3**
Dysbiosis with progesterone treatment	n=7	7/ 100%	0 / 0	0	1**
Control	n=6	6 / 100%	0 / 0	0	0
Control with progesterone treatment	n=4	4 / 100%	0 / 0	0	0

*among 10 fetuses one intrauterine fetal death was seen (multiple births)

**among 10 fetuses three abortus was seen (multiple births)

**among 10 fetuses one abortus was seen (multiple births)

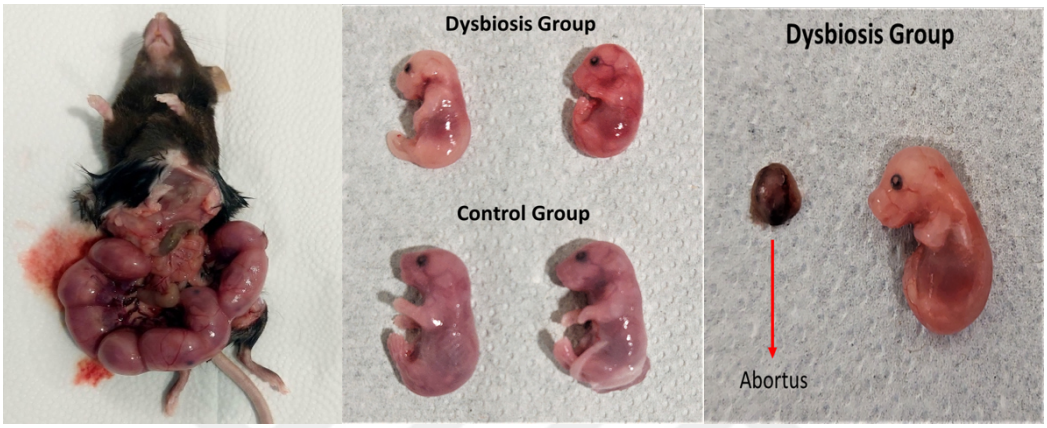

Figure 3.10: C-section and fetal anomalies in dysbiosis group.

3.3.2.1 Confirmation of vaginal microbiota transplantation to mice and investigation of the effect of progesterone treatment on dysbiotic microbiota composition

As a result of the 16S rRNA analysis of mouse vaginal samples to confirm the sustainability of vaginal microbiota transplantation at the time of delivery, we observed that the transferred microbiota were in the similar composition in all mouse groups. We also confirmed the successful depletion of the mice microbiota. We detected low rates (<%1) of mouse microbiota-related species such as *Rodentibacter pneumotropicus* (Figure 3.10).

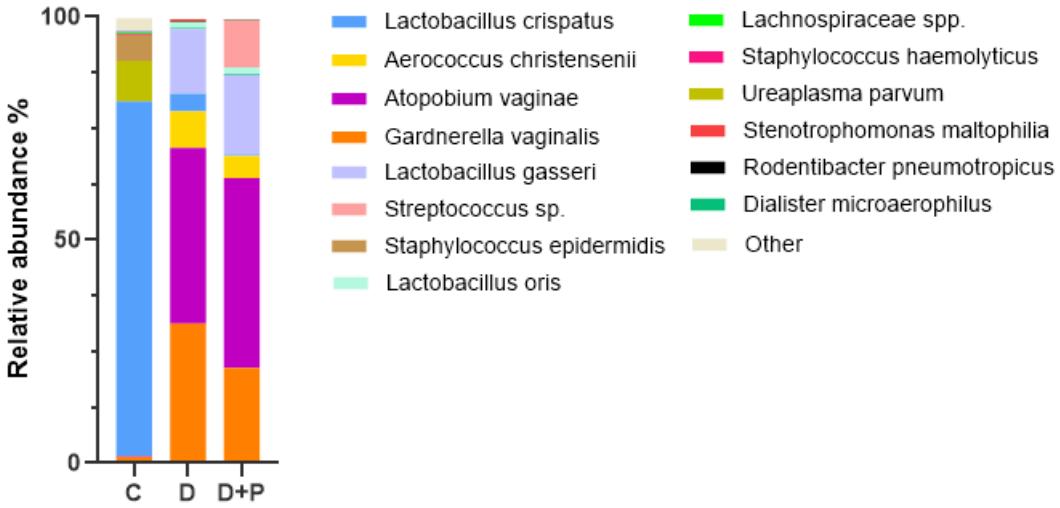

Figure 3.11: Relative abundance of the bacterial species detected in the mice vagina on 18.5 days of the pregnancy. C; Healthy vaginal microbiota (CST-I) transferred mice, D; Dysbiotic vaginal microbiota (CST-IV) transferred mice, D+P; Dysbiotic vaginal microbiota (CST-IV) transferred mice after progesterone treatment.

3.4 Monitoring of inflammatory processes

3.4.1 Flow cytometry

Lymphocytes were isolated from the uterus and peyer patches (Figure 3.6.). First, pro-inflammatory factors, including IL-6, IL-1β, and TNFα in the uterus, were examined to effect of vaginal dysbiosis (n=7) compared to healthy vaginal microbiota (n=6). There was no significant difference between the IL-6+ (25.460% vs. 29.279%) and IL-1β+ (26.5% vs. 20.5%) percentages of the two groups. TNFα+ cell ratio increased in the dysbiosis group from 39.910% to 48.546% (p=0.6296) compared to the control group.

CD3+CD4+ T cells (3.39% vs. 3.84) and CD25+CD69+ (18.561% vs. 16.835%) cells were similar between the two groups. Second, IL-6, IL-1β, and TNFα were examined

for the verify therapeutic effect of progesterone on vaginal dysbiosis. IL-6+ cells ratio decreased from 29.279% to 21.216% (p=0.0167), and TNFα decreased from 49.085% to 31.274% (p=0.0313) after the progesterone treatment in the dysbiosis group, respectively (Figure 3.11).

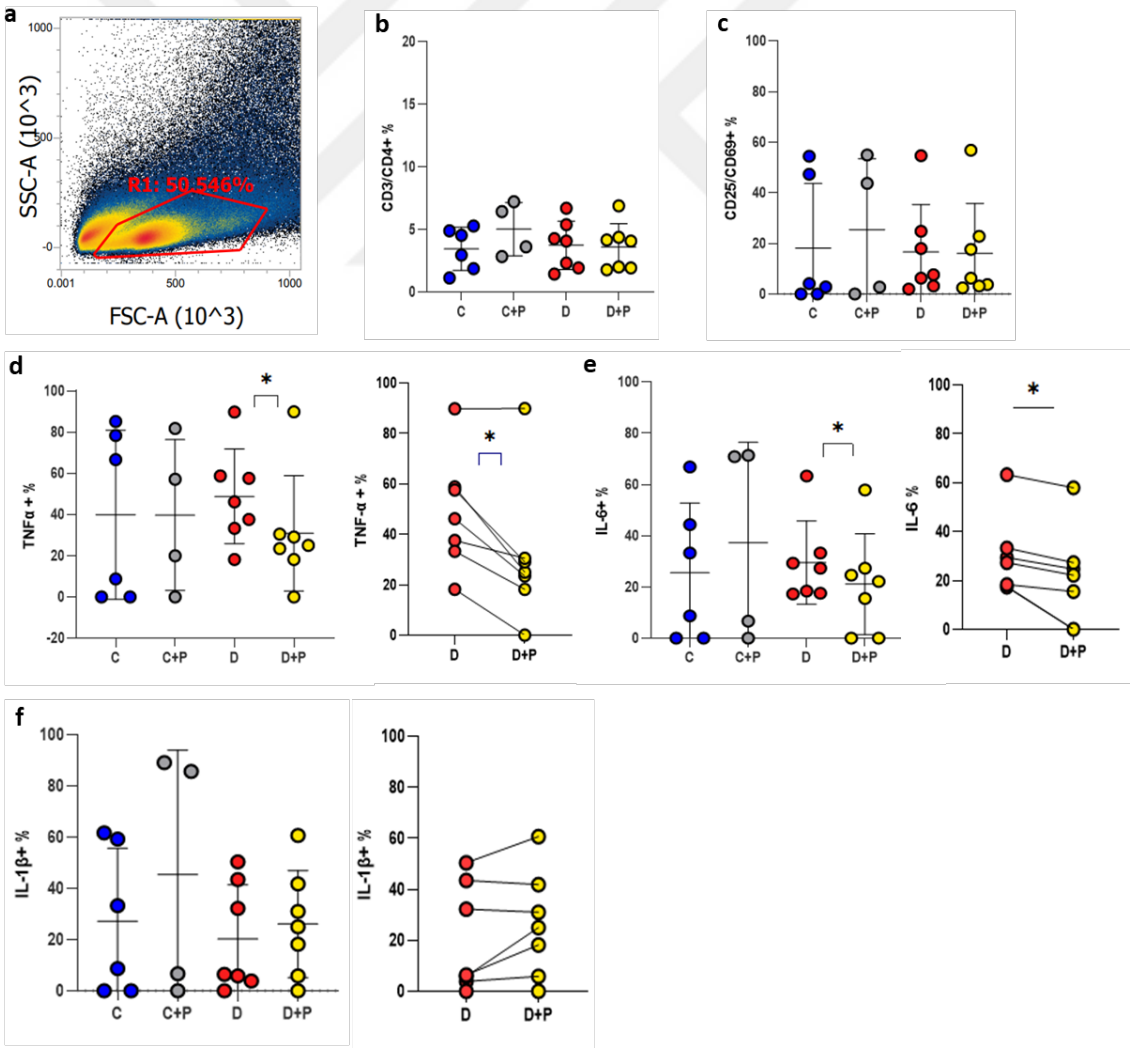

Figure 3.12: Lymphocyte cells isolated from the uterine tissue (a). Effect of human dysbiotic vaginal microbiota and progesterone treatment on the production of

CD3/CD4+ immune cells in uterine tissues (b) CD25/CD69+ cells, (c) tumor necrosis factor-alpha (TNFα) and (d) pro-inflammatory cytokines interleukin-6 (IL-6) (e), interleukin-1β (IL-1β), (f). C(Control Group); healthy vaginal microbiota transplanted

mice, D(Dysbiosis group); vaginal dysbiosis microbiota transplanted mice, (C+P, D+P) Progesterone treatment; mice receiving 1mg/ml progesterone.

In the intestine, there was no significant difference between the control group (n=5) and the dysbiosis group (n=5) in the production of interleukin IL-17 (16.456% vs. 18.565%), CD3+CD4+ (17.698% vs. 15.971) and CD25+CD69+ (24.143% vs. 18.331%) T cells. IL-22 (17.620% vs. 0.92%) was lower in the dysbiosis group than in the control group (Figure 3.8). After the progesterone treatment, CD3+CD4+ T cells decreased from 16.403% to 10.791% in the dysbiosis group (Figure 3.8). There was no significant change in the CD25+CD69+ T cell and IL-17+ and IL-22+ production after the progesterone treatment (Figure 3.12).

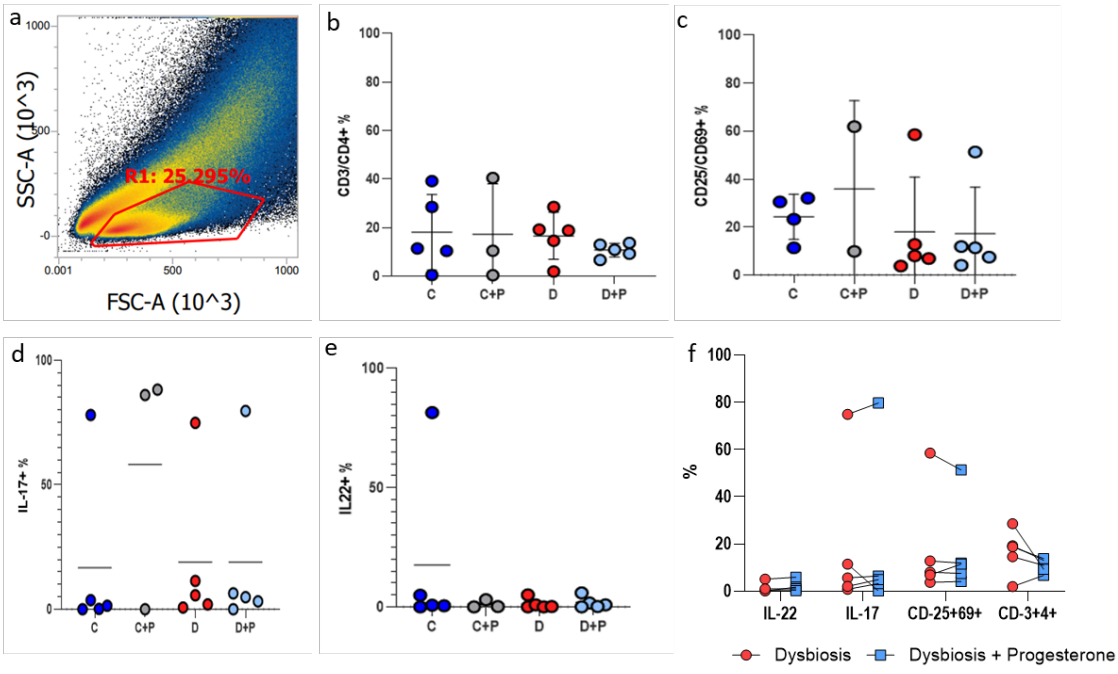

Figure 3.13: Lymphocyte cells isolated from the Peyer's patches (intestine) (a). Effect of human dysbiotic vaginal microbiota and progesterone treatment on the production of CD3/CD4+ immune cells in Peyer patch's (b) CD25/CD69+ cells (c) IL-17 (d) IL-22

(e) IL-22, IL-17, CD25+69+, and CD3+CD4+ levels before and after progesterone treatment (f). C (Control Group); healthy vaginal microbiota transplanted mice, D (Dysbiosis group); vaginal dysbiosis microbiota transplanted mice, (C+P, D+P) Progesterone treatment; mice receiving 1mg/ml progesterone.

3.4.2 Immunohistochemistry

Immunohistochemical evaluation demonstrated that significant difference between the control and dysbiosis group samples. While no cell damage was observed in the placenta tissue of the healthy vaginal microbiota transferred control group, cell damage was detected in the dysbiotic microbiota transferred group. In the dysbiosis group, it was observed that the damage decreased in progesterone-treated matched mouse tissues (Figure 3.13A).

According to the cellular damage scoring, the placental tissue of the dysbiosis group had a higher damage score (score 2; moderate/high) than the control group (42.9% vs.0%). In the dysbiosis group with progesterone treatment, the damage score was represented by scores 1 and 0 (occasional/mild and absent/rarely) (Figure 3.13B). The three dysbiotic groups of samples that had a damage score of 2 (moderate/high) before treatment decreased to score of 0 (absent/rarely) after progesterone treatment. The one dysbiotic group sample that had a damage score of 1 (occasional/mild) decreased to score of 0 (absent/rarely) also after progesterone treatment.

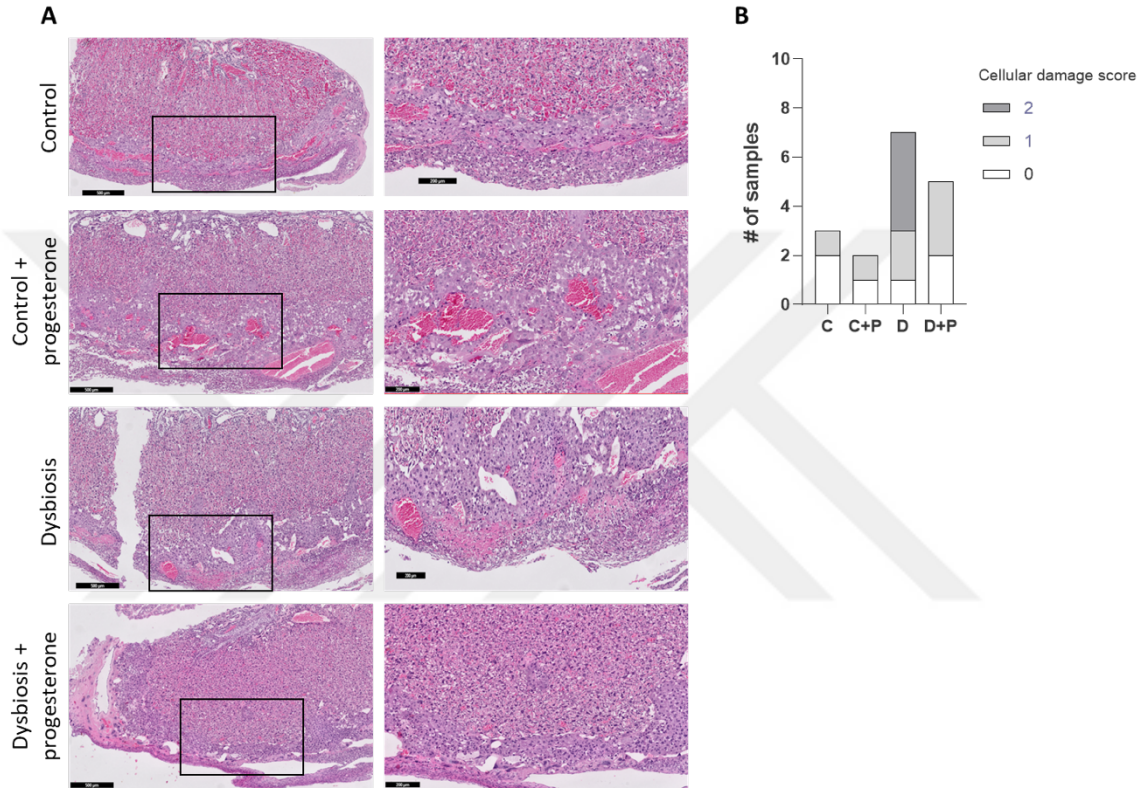

Figure 3.14: H&E-stained formalin-fixed mouse placental section (A) scoring of the placental cellular damage examination. The area in the boxed regions are shown at higher magnification. C; Control group n=3, C+P; Progesterone treated control group n=2, D; Dysbiosis group n=7, D+P; Progesterone treated dysbiosis group n=5 (B). Cellular damage score; 0: absent/barely, 1: occasional/mild, 2: moderate/high.

Iba1 macrophage marker in uterine tissue was evaluated in healthy control and dysbiotic microbiota transplanted groups. In the control group and progesterone-treated control group, no macrophage infiltration was observed. While macrophage density was high in the dysbiosis group, infiltration tended to decrease in some of the paired mouse groups treated with progesterone (Figure 3.14).

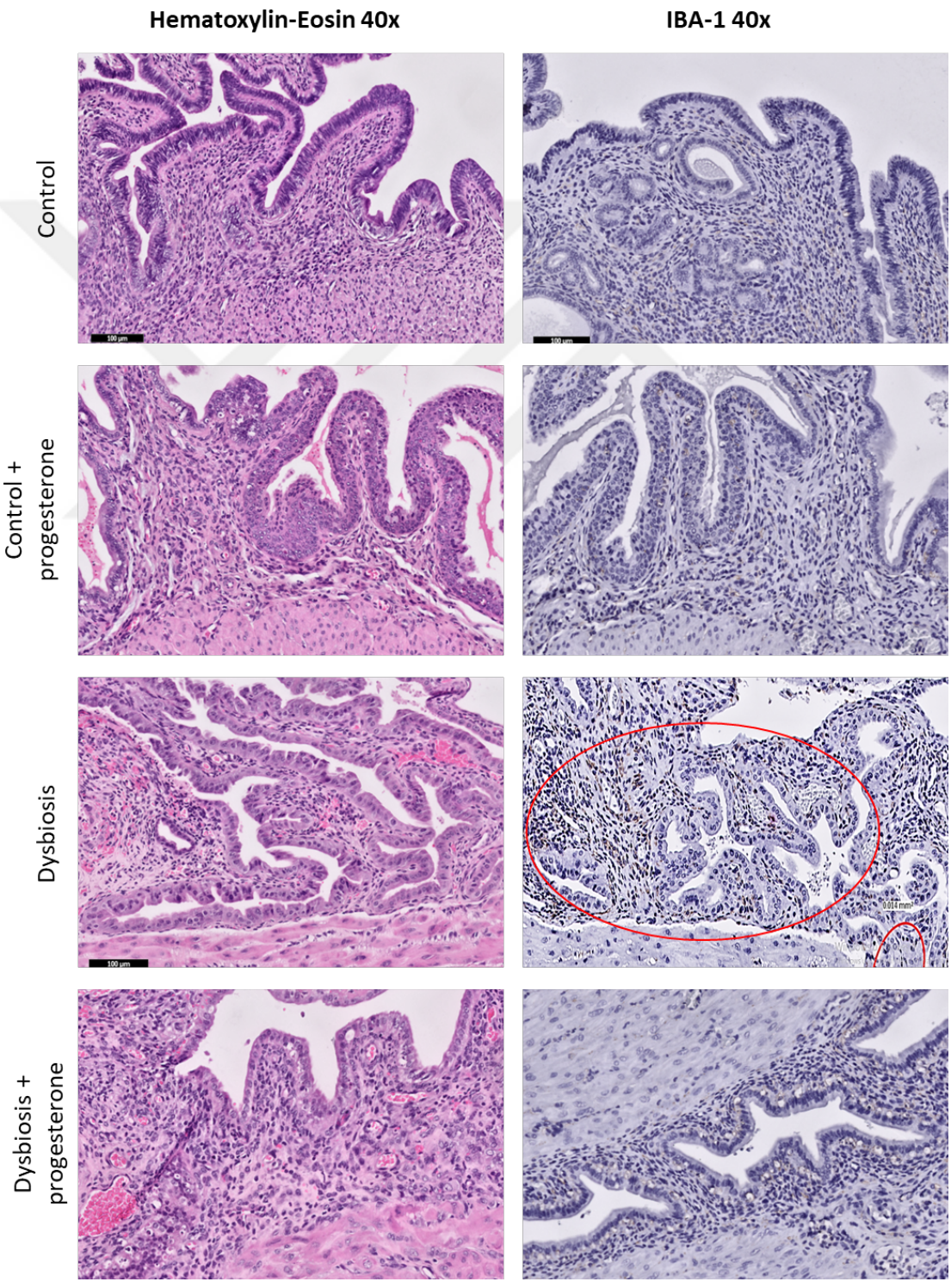
Hematoxylin-Eosin 40x
IBA-1 40x
Control
Control + progesterone
Dysbiosis
Dysbiosis + progesterone

Figure 3.15: Evaluation of macrophage infiltration with IBA-1 antibody in mouse uterus sections. Hematoxylin-eosin sections (left panel) anti-IBA-and 1 staining (right panel). Macrophage accumulation was marked in the red circle area.

Chapter 4

DISCUSSION

In this thesis, we showed an increased inflammation in the uterus of pregnant mice colonized with dysbiotic microbiota and a significant recovery of pathological findings after progesterone treatment. Premature birth is a major cause of perinatal mortality and morbidity worldwide, and an estimated 13.4 million babies were born prematurely in 2020 (World Health Organization. (2012). Born too soon: the global action report on preterm birth. World Health Organization). Preterm birth causes immediate health complications in infants and increases long-term health problems. It greatly affects developmental disabilities such as neurodevelopmental abnormalities, immature organ systems, behavioral disorders, and quality of life [44]. There are various reasons that lead to preterm birth, particularly intrauterine infections are considered one of the main causes of the reasons because they trigger inflammation in the uterus. When we compared the effect of a healthy vaginal microbiota CST I type (>90% *Lactobacillus crispatus*) and CST IV type dysbiotic microbiota (*Gardnerella vaginalis* and *Atopobium vaginae* dominant) on genital and intestinal immune response, our findings showed that the dysbiotic vaginal microbiota can stimulate preterm birth, abortus, developmental anomalies, and pathological deformations by increasing uterine inflammation. In addition, we have shown that progesterone reduces the risk of preterm

through suppressing inflammation in the mice uterus by decreasing TNFa and IL-6 responses, lowering cell damage in the placenta, and macrophage density in the uterine tissue.

In our study, the women with a short cervix group have shown vaginal microbiota dysbiosis with an increasing alpha diversity index compared to controls (p=0.018) and higher relative abundance of *G. vaginalis (*p=0.520*), A.vaginae* (p=0.599) *and L.iners (*p=0.553). There was a significant decrease in *L. gasseri* ratio (p=0.023). The preterm birth rate was 10% in the short cervix group. Vaginal dysbiosis is one of the important factor associated with maternal and neonatal diseases and birth outcomes [45]. Infection and increased inflammation caused by bacteria colonizing the lower genital tract responsible for preterm birth of nearly 40% [46]. *Gardenerella vaginalis,* one of the dominant bacteria in the CST IV group microbiota, has been shown to be associated with a short cervix [47]. CST IV-type dysbiotic microbiota showed a negative association with gestational delivery [2]. Furthermore, studies link vaginal dysbiosis, especially CST IV-type microbiota, and short cervix to preterm birth [25] [48]. Whereas the role of *L. iners* in the vaginal microbiota is not clear, it has been identified both in normal conditions and in the case of vaginal dysbiosis, and some studies have shown that *L. iners* is a risk factor for preterm delivery [26]. We also found that the relative abundance of *Lactobacillus iners* (%37.7 vs. 32.2%; p=0.553) was higher in women with short cervix compared to women with normal cervix.

Progesterone reduces myometrial contractility by inhibiting prostaglandin and cytokine production through anti-inflammatory action, thus preventing preterm labor [49].

In the clinical trials, prophylactic progesterone administered to women with a previous preterm birth has been shown to reduce the recurrence rate of preterm labor by fifty percent and reduce spontaneous preterm birth risk in women with cervical lengths (<25mm) by attenuating the rate of cervical shortening [50] [38]. In our short cervix cohort, the preterm rate was around 10%, while there was no preterm birth in the control group. Our mice model confirmed the clinical data. There was no preterm delivery in healthy vaginal microbiota-transferred mice; however, the preterm delivery rate was 28.57% in the dysbiotic microbiota-transferred mice group. Additionally, there was no preterm delivery in the progesterone-treated matched dysbiotic mice group

In the pregnant patients of our study, progesterone treatment did not change the microbial composition in control and short cervix groups. In the literature, there is only one study related to the effect of progesterone on vaginal microbiota. Similar to our study, they showed that progestin oral treatment does not significantly change vaginal microbiota [26]. Likewise, there was no difference in the microbiota composition after progesterone treatment in our mice model. These results suggested us that the preventative effect of progesterone on preterm is not related to the modification of bacterial taxa in vaginal microbiota.

Increasing levels of infection-mediated pro-inflammatory cytokines, including TNFα, IL-1B, IL-6, and IL-2, have been linked to chorioamnionitis and adverse pregnancy outcomes [52] [53]. Maternal-fetal interaction has mechanisms that lower the effects of

this pro-inflammatory response and maintain pregnancy [54]. Therefore, we investigated the expression of

TNFα, IL-1B, and IL-6 in the uterus for healthy (*L. crispatus* dominated) and dysbiotic microbiota-transferred pregnant mice. We found a significant correlation between inflammation in the uterus and dysbiotic microbiota through increased TNFα and IL-6 expression. In the immune histochemistry examination, macrophage density in the uterus and placental cellular damage was higher in the dysbiotic group compared to controls. The dysbiotic microbiota composition is important in the level of inflammatory response. *Gardnerella vaginalis* riched microbiota (CST IV) showed higher proinflammatory cytokine levels compared to *Lactobacillus crispatus* dominated microbiota (CST I) in the uterine tissue [55] and PTB [31]. In contrast, the *G. vaginalis* and *P. bivia* microbiota caused no histologic inflammation in vaginal tissue [56].

We observed premature birth and abortion only in the two *Atopobium vaginae*-dominated microbiota-transferred mice. A human vaginal epithelial cell model showed that *A. vaginae* increase pro-inflammatory cytokines, chemokines, and antimicrobial peptides [57]. Our results indicated the importance of Atopobium vaginae in dysbiotic microbiota in triggering inflammation. In accordance with the literature, we suggest that the pathogenesis of vaginal dysbiosis should be more intensively studied with different types of dysbiotic vaginal microbiota composition [45].

Progesterone has been shown to significantly reduce proinflammatory cytokines, including IL-6, TNF-α, IL-8, and macrophage activation triggered by lipopolysaccharides [58] [59].

Also, one study showed that in the systemic endotoxin-induced mice model, promegestone prevented preterm birth by decreasing systemic inflammation [42]. However, in a clinical trial, 17α-hydroxyprogesterone caproate treatment was shown to reduce IL1B but not IL-6, IL-8, and TNFα. Therefore, the anti-inflammatory mechanism of progesterone is still not understood. We showed that the IL-6+ cells ratio (from 29.279% to 21.216% (p=0.0167)) and TNFα (from 49.085% to 31.274% (p=0.0313)) decreased after the progesterone treatment in the dysbiotic vaginal microbiota-induced group. Also, macrophage density and cellular damage were lower after progesterone treatment. Previous studies with E.coli and LPS induction have shown that progesterone reduces inflammation in the genital tract. Our study is the first study with disbiotic microbiota. According to our data, progesterone reduces macrophage accumulation while proinflammatory cytokines decrease. These results are important to determine the mechanism of action of progesterone in the treatment of dysbiosis to develop strategies for preventing adverse pregnancy outcomes.

Gut and vaginal microbiota can interact with other systems, influence health conditions, and their interconnection can stimulate local and systemic immune responses [60]. *G. vaginalis* can stimulate NF-κB and TNF-α expression in the vagina and gut simultaneously [61]. Therefore, we also aimed to see the impact of different vaginal microbiota compositions on the gut immune response. We couldn't detect any changes and differences in the intestinal immune system between healthy and dysbiotic vaginal

microbiota groups. This may be related to short-term (five-day) vaginal microbiota transfer in our cohort that could cause inadequate bacterial colonization in the gut.

Studies investigating the relationship between *G. vaginalis* infection-associated BV and PTB show controversial results since this bacterium can be found in also healthy women [51]. This suggests that *G. vaginalis* alone is not the only cause in triggering preterm labor; therefore, transfer of pure G.vaginalis culture in mouse models for assessment of preterm delivery may not be appropriate. In our mouse model, we transferred a complete healthy or dysbiotic vaginal microbiota (rich for *Gardnerella vaginalis, Atopobium vaginae,* and *Aerococcus christensenii*) obtained from different pregnant patients. The sustained microbiota colonization at the time of delivery indicates that our experimental design is an appropriate mouse model for vaginal microbiota studies, which is one of the strongest part of our study.

The limitation of the study we didn’t analyze mycobiota in our study.

Chapter 5

CONCLUSION

In our study, we first showed that a short cervix is related to vaginal microbiota dysbiosis and increases preterm birth risk. Our mouse model revealed that vaginal exposure to healthy (CST I)) and dysbiotic (CST IV) type microbiota had different effects during pregnancy. In the dysbiotic microbiota transplanted mice group, uterine proinflammatory cytokine levels were in the increasing trend, accompanied by pathological inflammation through macrophage accumulation, resulting in 28% preterm birth. After the progesterone treatment, there was a significant decrease in the TNFa and IL-6 levels with low macrophage density in the uterus. Also, cellular damage scores in the placenta samples were lower than in the non-treated group. These immunological and clinical effects of dysbiotic microbiota during pregnancy enabled us to assess the mechanisms in the pathogenesis of preterm birth. Analyzing the vaginal microbiota before or during pregnancy may support the decision for initiation of progesterone therapy. Besides, our results may guide new strategies like personalized treatments with novel drugs for preventing preterm birth.

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