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Association Between Endometrial Microbiome Composition and Local Immune Cell Distribution During the Window of Implantation

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Abstract

The aim of this study was to investigate the association between the endometrial microbiome and local immune cell composition during the implantation window. We conducted a single-center prospective observational study including endometrial samples from 58 women without endometrial pathology. All samples were obtained during the mid-secretory phase (day 5 post-ovulation). Endometrial stromal $CD3^+$ T cells, $CD4^+$ T helpers, $CD56^+$ NK cells and $CD68^+$ macrophages were identified by immunohistochemistry and quantified as a percentage of stromal cells using HALO image analysis software. Microbiome composition was assessed by 16S rRNA gene sequencing (V4–V5 region). *Lactobacillus* dominance (LD) was defined as the genus with the highest centered log-ratio value within each sample. Endometrial immune cell composition showed a median 2.28% $CD3^+$ T cells of stromal cells, 1.75% $CD56^+$ NK cells, 1.44% $CD68^+$ macrophages and 0.29% $CD4^+$ T helpers. *Lactobacillus*-dominated microbiota was identified in 51.7% (30/58) of samples. Correlation analysis showed that *Lactobacillus*-related taxa were negatively associated with NK/T ratios and positively with T helper-related ratios. LD samples exhibited reduced NK cell abundance (1.17% vs. 2.12%, $p = 0.006$, q = 0.023) and a lower NK/T ratio (0.52 vs. 1.05, $p = 0.004$, q = 0.023) compared to non-LD samples. This study provides evidence for a link between microbial composition and local immune regulation in the endometrium.

Keywords: endometrial microbiome; immune cells; NK cells; implantation window; *Lactobacillus*

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1. Introduction

The endometrium is a highly dynamic tissue, where successful implantation depends on a finely tuned balance between immunological, microbial, and hormonal factors [1–5].

Long considered sterile, the endometrial mucosa has been proven to present a distinct endometrial microbiome using next-generation sequencing (NGS) of the 16S rRNA gene [6]. This microbial community is often dominated by *Lactobacillus* species and is associated with successful embryo implantation in IVF cycles [7].

Many studies are reporting the influence of the endometrial microbiome composition on reproductive outcomes, including embryo implantation success [1,6–9]. In particular, a high relative abundance of intrauterine *Lactobacillus* has been proposed as a marker of endometrial health and has been associated with favorable IVF outcomes, whereas deviations from this profile may reflect dysbiosis and reduced receptivity [5,7,10]. Nevertheless, the precise ways in which the microbiome influences the local immune microenvironment to regulate endometrial receptivity are still not fully elucidated [1,6,11].

The mucosal microbiota is known to shape host immune responses through complex interactions involving both innate and adaptive immunity. This communication enables microorganisms to modulate local immune environments, influencing immune tolerance and activation. Such interactions may be particularly important in the context of implantation, where finely tuned immune regulation is essential for successful embryo acceptance and early pregnancy establishment [2,12]. In turn, the immune environment maintains the local homeostasis, with the balance between different immune cell populations being critical for endometrial function and receptivity. Our recent study pointed to the endometrial immune cell ratios (Th/T, NK/T, MΦ/T and Th/MΦ) during the implantation window as a determinant of the endometrial receptivity [12]. According to this study higher values for the ratios Th/T, NK/T, MΦ/T and Th/MΦ during the mid-secretory phase of the menstrual cycle were highly associated with successful embryo implantation. Many other authors have also proven that the quantities and ratios of stromal immune cells affect the tissue behavior [13,14]. In recent years a particular focus has been on endometrial NK cells, with studies reporting both increased and decreased NK cell counts in association with reproductive failure, including recurrent miscarriage and recurrent implantation failure [15–18]. Increased NK cells have been linked to acute or chronic inflammatory conditions, endometritis or viral infections and are often accompanied by a shift toward a more cytotoxic phenotype driven by cytokine signaling and interactions with other immune cells [19–22]. This pro-inflammatory and cytotoxic environment is considered incompatible with the immune tolerance required for successful embryo implantation [20–22].

Although both the endometrial microbiome and the local immune environment have been associated with reproductive outcomes, the relationship between these two components within the endometrial microenvironment remains poorly defined. While alterations in immune cell composition have been linked to endometrial receptivity, the factors driving these changes are not fully understood. The endometrial microbiome may contribute to the regulation of local immune responses, but data directly examining this association is limited.

While reproductive outcomes are clinically relevant, they are influenced by multiple systemic, embryonic, and clinical factors that may confound the interpretation of localized endometrial processes. Therefore, focusing on the association between the microbiome and immune environment represents a necessary step toward understanding the underlying biological mechanisms independent of downstream clinical endpoints.

Therefore, the aim of this study was to investigate the association between the endometrial microbiome and the local immune environment during the mid-secretory phase of the menstrual cycle, focusing on immune cell abundance and functionally relevant ratios. Understanding the interaction between these two crucial components may provide insight into the biological mechanisms underlying endometrial receptivity.

2. Materials and Methods

2.1. Patients

This single-center observational study includes endometrial samples from 58 women below 45 years old, collected between March 2022 and March 2024. All participants were undergoing unstimulated assisted reproduction therapy (ART) cycles with non-endometrial infertility cause.

Exclusion criteria were presence of endometrial pathology, such as endometriosis, endometrial polyps, adenomyosis, endometrial hyperplasia and carcinoma or presenting a bacterial infection. All participants signed written informed consent. This study was approved by the Institutional Review Board (No: 56/29.12.2022).

2.2. Sample Collection

All endometrial biopsies were obtained during the mid-secretory phase of the menstrual cycle, on day 5 after LH peak, measured by urinary test. The endometrial samples were taken using a Pipelle endometrial suction (CCD 1103000, Laboratoire CCD, Paris, France) under ultrasonographic guidance. The fresh tissue was separated into two equal parts. The first was placed in 4% formalin and paraffin embedded using the Leica TP1020 Benchtop Tissue Processor (Leica Biosystems, Nussloch, Germany) for immunohistochemical staining. The second part of the tissue was gently washed with sterile PBS (VWR, VWRRMS01OQ, Radnor, PA, USA) and instantly frozen at −80 °C for 16S rRNA amplicon sequencing.

2.3. Immunohistochemistry

The endometrial samples were paraffin-embedded and cut into 4 µm thick sections that were mounted on slides by 1 h incubation at 60 °C. After deparaffinization in xylene and rehydration in graded ethanol, endometrial sections were subjected to antigen retrieval in epitope retrieval buffer pH 9.0 (RE7119, Leica, Wetzlar, Germany) for 30 min in 60 °C water bath. Staining procedure was performed using Novolink polymer detection system (RE7280-K, Leica Biosystems) according to manufacturer's instructions in BOND-III Automated IHC (Leica Biosystems). Immune cells: $CD3^+$ T-cells, $CD4^+$ T-helpers, $CD68^+$ macrophages and $CD56^+$ NK cells were identified using antibodies against CD3 (BRB063, Zytomed Systems, Berlin, Germany), CD4 (IS649, Dako, Santa Clara, CA, USA), CD68 (IS613, Dako) and CD56 (A00121-0007, ScyTek, Logan, UT, USA), respectively.

2.4. Immune Cell Quantification

For each specimen, at least five high-resolution images were captured at 400× magnification using an Olympus EP50 digital camera (IDS Imaging Development Systems GmbH, Obersulm, Germany). Immune cell presence was assessed within the same five fields of view across all tissue samples, selected on consistent topological features.

Quantitative analysis was performed using HALO Image Analysis Software v3.4.2986.185 (Indica Labs, Albuquerque, NM, USA) with Cytonuclear Module v2.0.9. Cells were counted as individual objects based on predefined optical density thresholds and nuclear size parameters (6.82–20 µm). Positive staining was defined using optimized optical density thresholds (set manually in the range between 0.064 and 2.5) and color deconvolution parameters. Luminal and glandular epithelium were excluded from analysis. Quantification included the total number of stromal cells and the number of positively stained cells. Results are presented as percentage of positively stained stromal cells.

2.5. Metagenome Sequencing

The total DNA extraction and NGS was done as previously described by Parvanov et al., 2023 [8]. Briefly the DNA was extracted from endometrial samples using the Quick-DNA Fecal/Soil Microbe Microprep Kit (D6010, Zymo Research, Irvine, CA, USA) according to the manufacturer's instructions. DNA concentration and purity were assessed using a NanoDrop™ 2000c spectrophotometer (Thermo Scientific, Waltham, MA, USA).

The V4–V5 hypervariable regions of the 16S rRNA gene were amplified by PCR using barcoded universal primers 515F (5′-GTGCCAGCMGCCGCGGTAA-3′) and 907R (5′-CCGTCAATTCCTTTGAGTTT-3′) [23,24]. Amplicons were verified by electrophoresis on a 2% agarose gel, purified, and pooled in equimolar concentrations.

Sequencing libraries were prepared using standard Illumina protocols, including end-repair, A-tailing, and adapter ligation. Library quality and fragment size distribution were evaluated using a Qubit fluorometer (Thermo Fisher Scientific, Waltham, MA, USA), real-time PCR Instrument, QuantStudio 5 Real-Time PCR System (Thermo Fisher Scientific, Waltham, MA, USA) and Bioanalyzer, Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA).

Sequencing was performed by Novogene Co., Ltd. (Cambridge, UK) on the Illumina HiSeq platform (MiSeq System, Illumina, San Diego, CA, USA) using a 2 × 250 bp paired-end configuration, targeting approximately 30,000 reads per sample.

To monitor potential contamination, negative controls (including blank extraction controls and PCR controls) were processed alongside all samples throughout DNA extraction, amplification, and sequencing procedures. Sequences detected in negative controls were evaluated and considered during downstream analysis to minimize the impact of potential contaminant taxa, which is particularly critical for low-biomass samples such as endometrial tissue.

2.6. Bioinformatics Analysis

Raw sequencing data were processed using the QIIME pipeline (version 1.7.0). Paired-end reads were merged, quality-filtered, and chimeric sequences were removed to obtain high-quality clean reads.

Sequences were clustered into operational taxonomic units (OTUs) at a 97% similarity threshold using UPARSE (v7.0.1090) [25]. Representative sequences from each OTU were selected and used for taxonomic assignments.

Taxonomic classification was performed using QIIME (version 1.7.0) with the Mothur method against the SILVA SSU rRNA database (release 138) [26], applying a confidence threshold of 0.8–1.0 [27]. Taxonomic annotation was obtained across multiple levels, including phylum, class, order, family, genus, and species.

To account for differences in sequencing depth, OTU tables were normalized to the sample with the lowest sequencing depth. The normalized dataset was used for downstream diversity analyses.

Alpha diversity metrics, including the Shannon diversity index, observed features, and Pielou's evenness, were calculated to assess within-sample microbial diversity. Beta diversity was evaluated using unweighted UniFrac distances to assess differences in microbial community composition between samples.

2.7. Statistical Analysis

Data were analyzed using Python (v. 3.11.9) and SPSS (v. 23.0, IBM Corp., Armonk, NY, USA).

Taxonomic abundance tables were imported into Python (v. 3.11.9) for downstream analysis. To reduce sparsity, low-prevalence taxa were filtered by excluding genera present in fewer than 10% of samples. Given the compositional nature of microbiome data, abundance values were transformed using the centered log-ratio (CLR) transformation. A pseudocount of 1×10^{-6} was added to zero values prior to transformation. CLR transformation was performed on a per-sample basis using the geometric mean of all taxa within each sample.

Samples were classified as *Lactobacillus*-dominated (LD) or non-*Lactobacillus*-dominated (non-LD) depending on whether the genus *Lactobacillus* exhibited the highest centered log-ratio (CLR) value within a given sample. This approach was selected to account for the compositional nature of microbiome data and to avoid reliance on arbitrary relative abundance thresholds, which vary substantially across previous studies.

Alpha diversity indices (Shannon index, observed features, and Pielou's evenness) were calculated using QIIME (version 1.7.0). Differences in alpha diversity between LD and non-LD groups were assessed using the Mann–Whitney U test.

Beta diversity was calculated using Bray–Curtis dissimilarity in QIIME (version 1.7.0) and visualized using principal coordinates analysis (PCoA). Group differences were evaluated using permutational multivariate analysis of variance (PERMANOVA).

The normality of continuous variables was assessed using the Kolmogorov–Smirnov test. Comparisons between groups were performed using the Mann–Whitney U test. Correlations between microbial taxa and immune cell populations were assessed using Spearman's rank correlation coefficient. Multiple comparisons were corrected using the Benjamini–Hochberg false discovery rate (FDR) method. Adjusted p-values (q-values) < 0.05 were considered statistically significant. Data is presented as mean $\pm$ standard error (SEM) or median with interquartile range (IQR), as indicated.

3. Results

3.1. Baseline Characteristics of the Studied Patients

The mean age of participants was 36 $\pm$ 4 years. No significant differences were observed between LD (35 $\pm$ 5 years) and non-LD (36 $\pm$ 8) groups in age $p > 0.05$.

3.2. Endometrial Immune Characteristics

All investigated immune cell populations were detected within the endometrial stroma. Positive staining in the luminal or glandular epithelium was rare and excluded from further analysis.

Quantitative analysis demonstrated that $CD3^+$ T cells comprised 2.28% [2.34] of the stromal compartment, 0.29% [0.62] was $CD4^+$ T helper cells, 1.75% [1.85] $CD56^+$ NK cells and 1.44% [1.58] $CD68^+$ macrophages (Figure 1A–D), while immune cell ratios were 0.75 [1.00] NK/T, 0.16 [0.36] Th/T, 0.80 [0.90] MΦ/T and 0.23 [0.51] Th/MΦ (Figure 1E–H).

3.3. Endometrial Metagenomic Profile

Alpha diversity analysis indicated low microbial diversity across all samples (median Shannon index: 0.99 [IQR 1.13]), despite moderate richness (observed features: 27.5 [36.75]). Pielou's evenness was low (0.18 [0.25]), indicating a highly uneven community structure.

A subset of samples (51.72% (30/58)) was characterized by *Lactobacillus*-dominated microbiota, whereas others exhibited diverse microbial profiles dominated by: Acinetobacter 1.72% (1/58); Cupriavidus 5.17% (3/58); unclassified Muribaculaceae 3.44% (2/58); and others 37.93% (22/58). Samples were classified into two groups: *Lactobacillus*-dominated (LD) and non-*Lactobacillus*-dominated (non-LD) microbiota (Figure 2).

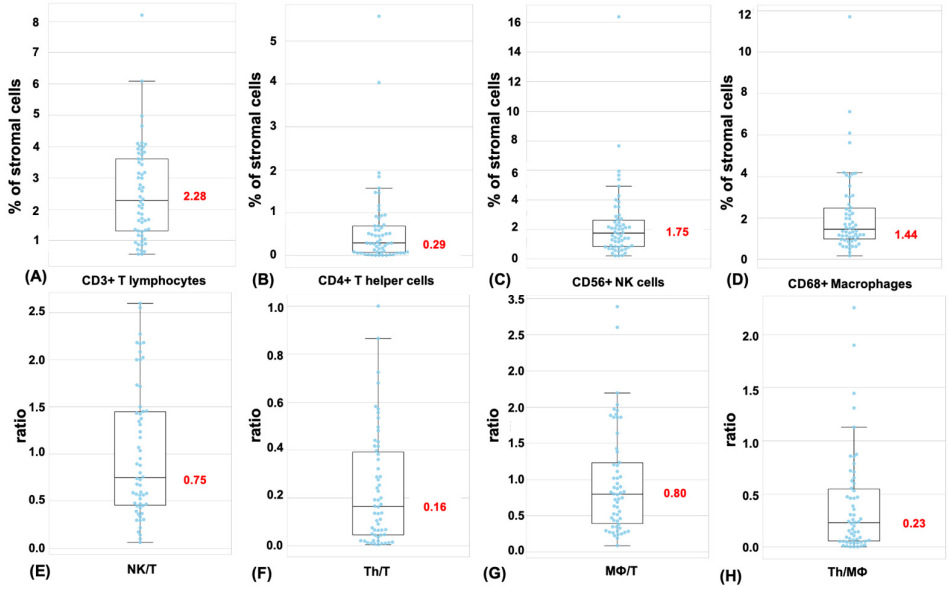


Figure 1. Dot plots and boxplots with median of (**A**) $CD3^+$ T cells; (**B**) $CD4^+$ T helper cells; (**C**) $CD56^+$ NK cells and (**D**) $CD68^+$ macrophages as percentage of the endometrial stroma; and the immune cell ratios (**E**) NK/T; (**F**) Th/T; (**G**) MΦ/T; (**H**) Th/MΦ.

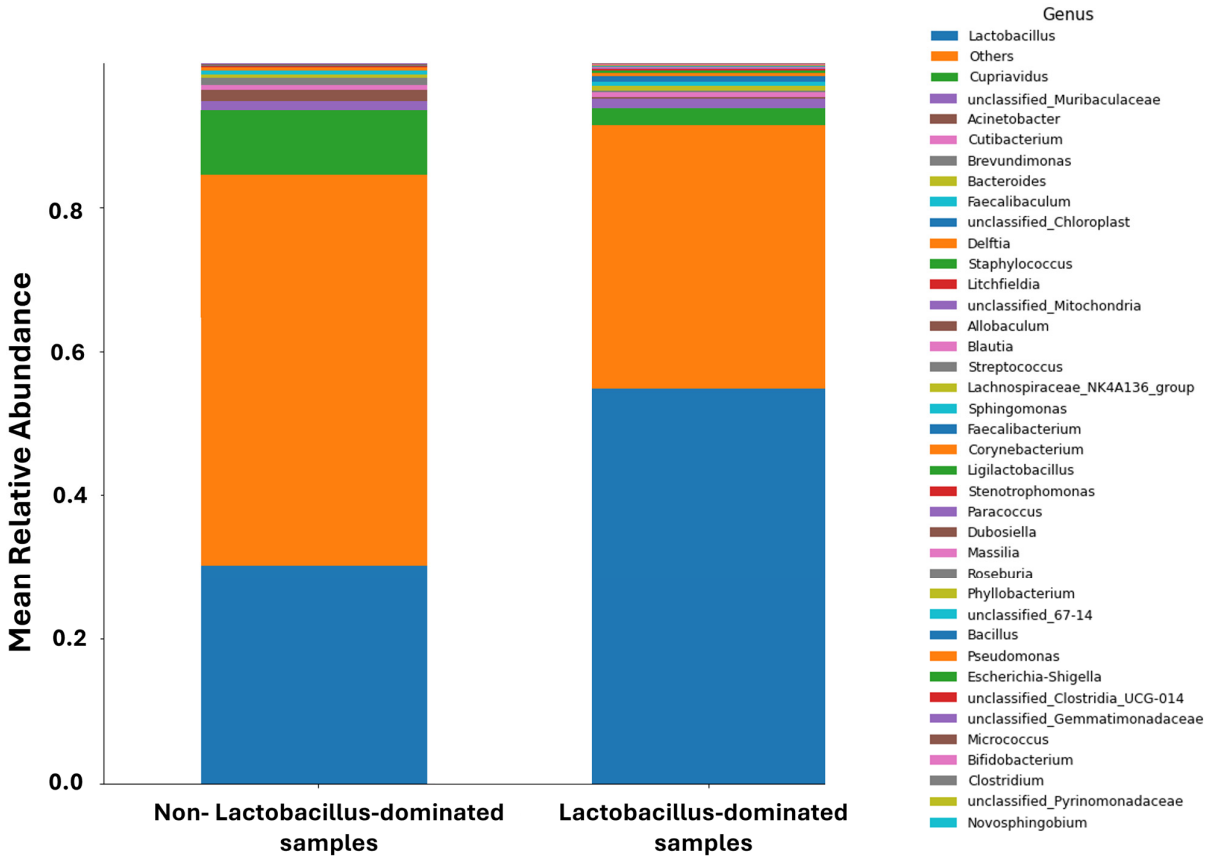


Figure 2. Stacked bar plot of the mean relative abundance of bacterial genera in samples grouped by *Lactobacillus* dominance.

When microbial diversity was compared between *Lactobacillus*-dominated and non-*Lactobacillus*-dominated samples, higher diversity was observed in the non-*Lactobacillus*-dominated group. Specifically, Shannon diversity was 1.20 [0.90] vs. 0.56 [0.94] ($p = 0.076$), observed features were 19.50 [8.50] vs. 57.50 [56.25] ($p < 0.001$) and Pielou's evenness was 0.28 [0.21] vs. 0.10 [0.16] ($p = 0.004$) (Figure 3A–C).

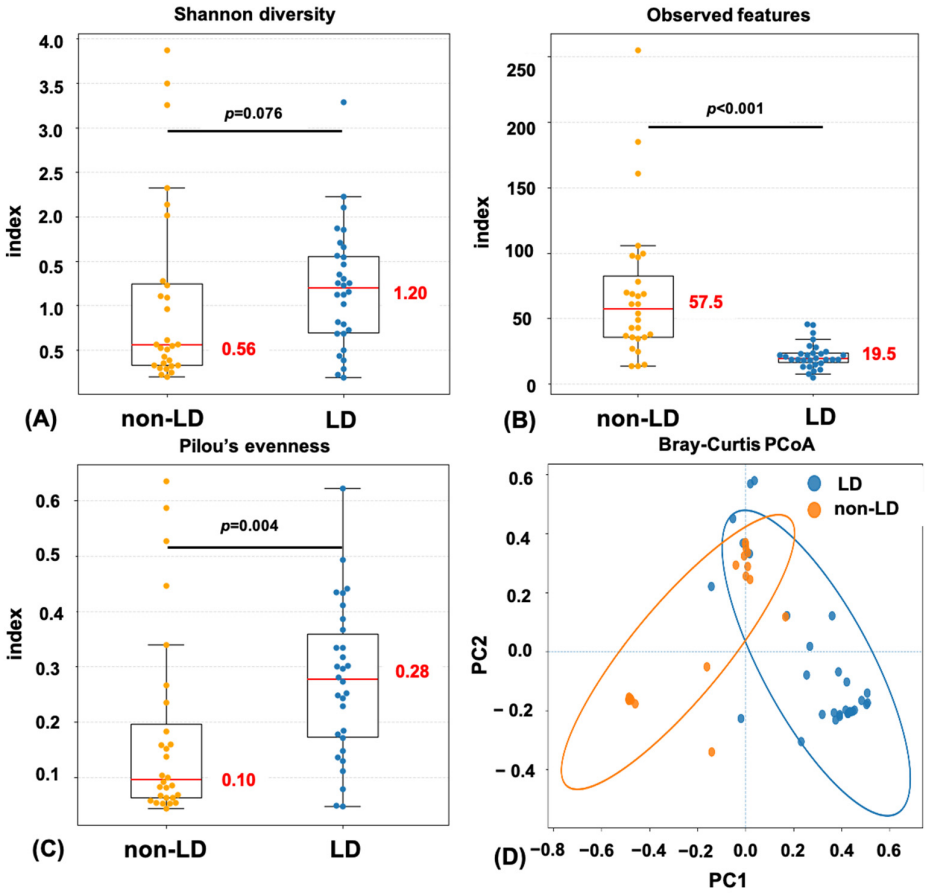


Figure 3. (**A**) Shannon diversity; (**B**) Observed features; (**C**) Pielou's evenness with median between non-*Lactobacillus* dominated (non-LD) and *Lactobacillus*-dominated (LD) samples and (**D**) Bray–Curtis PCoA plot of LD and non-LD samples.

Beta diversity based on Bray–Curtis dissimilarity differed significantly between *Lactobacillus*-dominated and non-*Lactobacillus*-dominated samples (PERMANOVA, pseudo-F = 21.609, $p = 0.0010$) (Figure 3D).

3.4. Association Between Microbiome and Immune Composition

Multiple significant correlations between microbial taxa and immune parameters were identified after FDR correction (Figure 4, Table A1).

FDR-significant correlations were consolidated across taxonomic levels, revealing consistent negative associations between *Lactobacillus*-related taxa and the NK/T cell ratio, and positive associations between microbial families: Actinomycetaceae, Neisseriaceae, Prevotellaceae and Rikenellaceae and $CD4^+$ T cell-associated immune ratios.

3.5. Lactobacillus *Dominance and Immune Composition*

When the immune cell parameters were compared between samples according to *Lactobacillus* dominance, LD samples demonstrated significantly lower NK cell abundance compared to non-LD samples (1.17% [1.44] vs. 2.12% [2.55], $p = 0.006$, q = 0.023). Similarly, the NK/T ratio was significantly reduced in LD samples (0.52 [0.91] vs. 1.05 [1.10], $p = 0.004$, q = 0.023) (Figure 5A,B, Table A2). All other immune cell parameters did not differ significantly between LD and non-LD (Table A2).

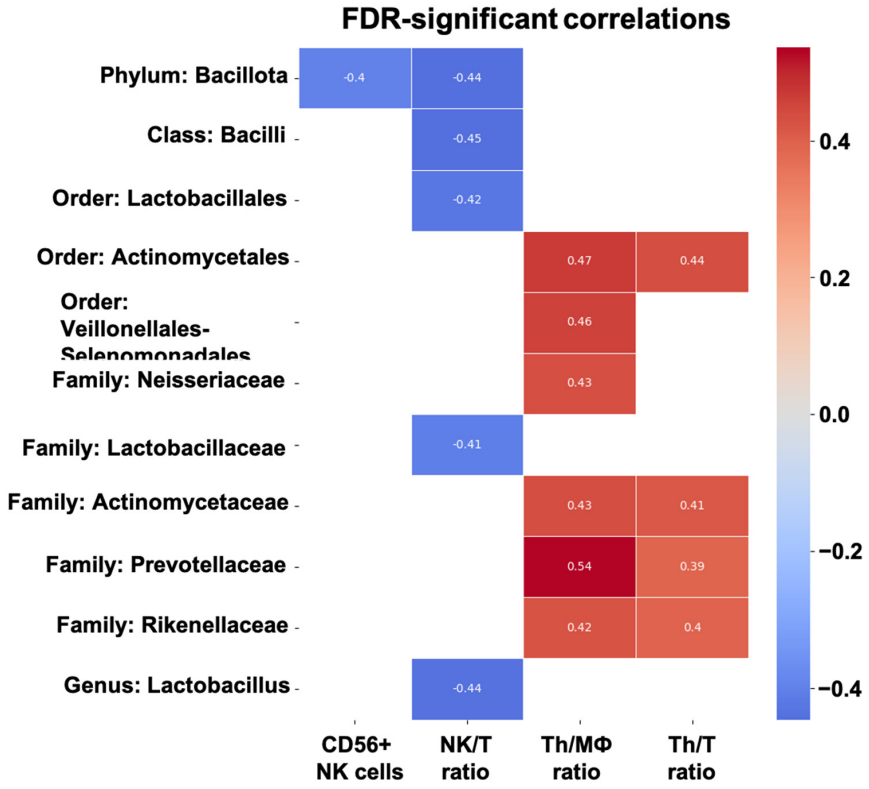


Figure 4. Correlation heatmap of the significant correlations after FDR correction between microbial taxa and immune cells and immune cell ratios.

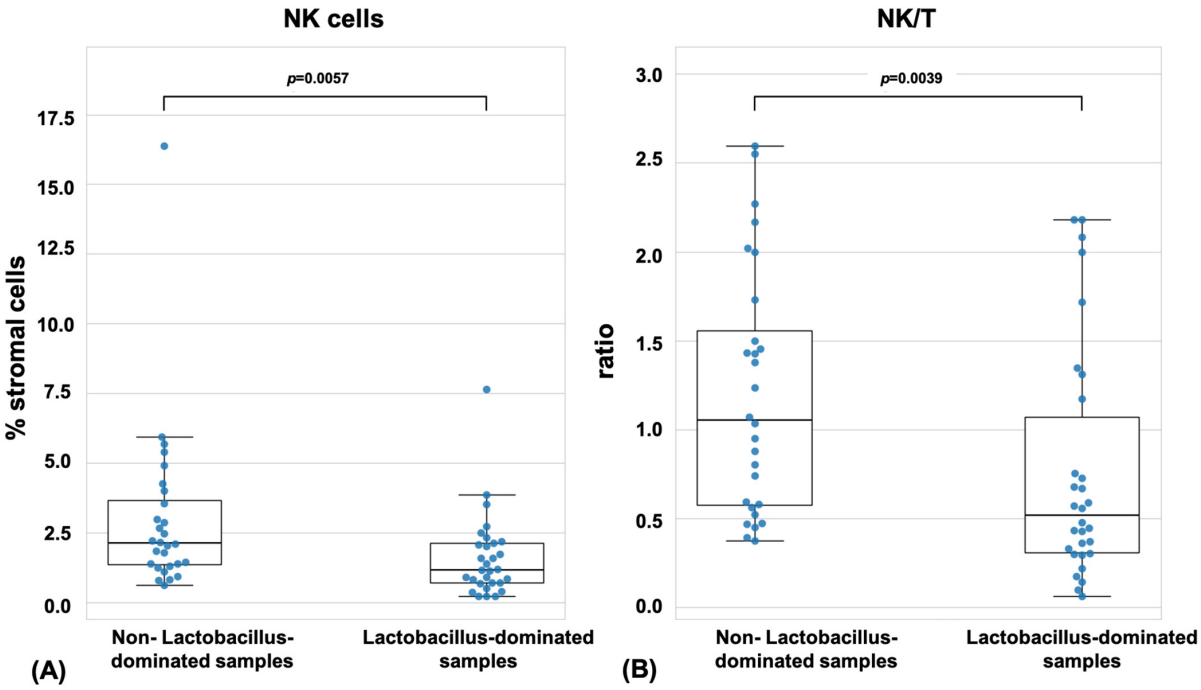


Figure 5. Dot plots and boxplots of (**A**) NK cell abundance and (**B**) NK/T ratio according to *Lactobacillus* dominance.

A summary of the principal findings of the study is presented in Table A3.

4. Discussion

While microbiome–immune interactions are well recognized across mucosal systems, data directly examining the relationship between the endometrial microbiome and local

immune cell composition during the implantation window remain limited. Specifically, *Lactobacillus*-related taxa were associated with lower NK/T ratios, whereas other microbial families such as Actinomycetaceae, Neisseriaceae, Prevotellaceae and Rikenellaceae were associated with higher $CD4^+$ T cell-related ratios. The endometrial microbiome exhibited low alpha and beta diversity, consistent with previous reports [28]. However, given the observational design of the study, direct causal relationships between microbiome composition and immune cell distribution cannot be established, and reverse interactions between the local immune environment and microbial composition are equally plausible.

In our study cohort approximately half of the samples exhibited the expected *Lactobacillus*-dominated profile. Prior studies have associated *Lactobacillus* dominance with favorable IVF outcomes [28–31], a pattern reported not only in the endometrium but also in the vaginal microbiome [28,32]. The non-*Lactobacillus*-dominated group comprised samples with diverse dominant taxa, including Acinetobacter, Cupriavidus, unclassified Muribaculaceae, and other low-abundance genera, reflecting inter-individual variability in microbial composition. In line with this, the non-*Lactobacillus*-dominated group demonstrated significantly higher diversity compared to *Lactobacillus*-dominated samples. In contrast to the gut microbiome, where increased diversity is generally considered beneficial, higher diversity in the endometrial environment may be consistent with instability and potential dysbiosis [33,34]. This loss of stability has been linked to reduced reproductive success, suggesting that, in the endometrium, microbial stability rather than diversity may be more critical for optimal function. Accordingly, non-*Lactobacillus*-dominated profiles may reflect a loss of microbial balance and a potentially altered endometrial immune–microbial environment.

This link was further supported by the correlation between the microbial taxa and immune parameters. We found a consistent negative association between *Lactobacillus*-related taxa and endometrial NK/T ratios, while other microbial families, such as Actinomycetaceae, Neisseriaceae, Prevotellaceae and Rikenellaceae, showed positive associations with Th-related ratios. Members of the families Actinomycetaceae and Neisseriaceae, which include potentially pathogenic species, and Prevotellaceae and Rikenellaceae, commonly associated with the gut microbiome, may be associated with the activation of the local adaptive immune response [33,35]. These microorganisms could activate pattern recognition receptors, such as Toll-like receptors, thereby promoting downstream signaling pathways that influence T helper cell distribution [36].

When comparing the endometrial immune cell distribution between LD and non-LD, significantly lower levels of NK cell abundance and NK/T ratios were observed in the *Lactobacillus*-dominated group, consistent with an immune profile previously associated with increased endometrial tolerance. Increased NK cell abundance during the mid-secretory phase has been associated with impaired endometrial remodeling, defective vascular transformation and repeated pregnancy loss [15,16]. In this context, the reduced NK cell abundance and lower NK/T ratios observed in *Lactobacillus*-dominated samples are consistent with immune profiles previously associated with favorable implantation outcomes.

Although direct evidence linking the endometrial microbiome to local immune cell organization remains limited, the present findings support a potential relationship between *Lactobacillus*-dominated microbial profiles and endometrial immune composition. Such effects may be mediated through cytokine signaling, pattern recognition or microbial metabolites that influence immune activity. Supporting this, previous studies suggest that *Lactobacillus* species can modulate uterine immune tolerance through cytokine regulation, including increased IL-10 and altered IFN-γ and TNF-α signaling, as well as through TLR/NF-κB and MAPK pathways that reduce inflammatory gene expression [37–39]. In murine models, *Lactobacillus gasseri* has also been shown to influence NK cell activity through IL-2

and IFN-γ signaling [40]. These mechanisms may potentially be associated with the lower NK cell abundance and NK/T ratios observed in *Lactobacillus*-dominated samples.

In contrast, non-*Lactobacillus*-dominated microbiota may be associated with an inflammatory profile, as these taxa can stimulate pattern recognition receptors and promote cytokine responses that influence local immune cell activity and NK cell expansion [33]. In line with this, conditions such as chronic endometritis and endometriosis are characterized by increased uterine NK cell infiltration and are associated with impaired reproductive outcomes [20,41]. Taken together, these observations suggest that non-*Lactobacillus*-dominated profiles, combined with higher NK cell abundance, may be associated with a less receptive endometrial environment. The relationship between these associations and reproductive outcomes remains to be elucidated in future studies.

Given that reproductive outcomes are influenced by multiple systemic and clinical factors, focusing on localized biological associations in this study allowed for a more controlled interpretation of the underlying biological interactions.

Despite these findings, several limitations should be considered when interpreting the results. The relatively small sample size may affect the generalizability of the findings. In addition, the absence of a fertile control group limits the ability to distinguish ART-associated findings from physiological microbiome–immune interactions in fertile women. In addition, microbiome profiling was based on 16S rRNA gene sequencing, which provides limited taxonomic resolution and does not allow for functional characterization of microbial communities. Furthermore, the immune analysis was restricted to phenotypic quantification of cell populations, without assessment of functional activity or cytokine profiles, which could provide further insight into the underlying mechanisms of immune regulation within the endometrium. Moreover, CD4 immunostaining identifies total helper T cell populations but does not distinguish functional subsets such as Th1, Th2, or regulatory T cells. Future studies integrating larger cohorts, higher-resolution microbiome analysis approaches, and functional immune assays, together with reproductive clinical outcomes will be essential to better define microbiome–immune interactions in the endometrium and clarify their potential value for diagnostic stratification. These findings support the hypothesis that the endometrial microbiome may act as an upstream regulator of immune cell composition, potentially influencing implantation-related immune balance.

5. Conclusions

This study demonstrates a significant association between endometrial microbiome composition and local immune cell distribution during the mid-secretory phase of the menstrual cycle. *Lactobacillus*-dominated microbiota was associated with lower NK cell abundance and reduced NK/T ratios, consistent with immune profiles previously associated with tolerogenic endometrial environments. Overall, these findings support the existence of an association between endometrial microbiome composition and local immune cell organization during the implantation window. These observations contribute to the growing understanding of microbiome–immune interactions in endometrial biology and provide a foundation for future studies investigating their potential relevance in reproductive medicine and endometrial diagnostics.

Author Contributions: Conceptualization, R.G. and M.R.; methodology, T.T., M.H., M.P. and M.S.; software, R.G., D.P. and S.K.; validation, T.T., S.C., D.M. and G.S.; formal analysis, R.G. and R.S.; investigation, T.T., M.R., M.P., M.S., S.C., J.S. and S.K.; resources, T.T., M.H., S.C., D.M. and G.S.; data curation, R.G. and D.P. writing—original draft preparation, R.G.; writing—review and editing, D.P., M.R. and M.H.; visualization, R.G., J.S., M.H. and M.R.; supervision, D.P. and G.S.; project administration, S.H., D.P. and B.R.; funding acquisition, S.H. and G.S. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: The study was conducted in accordance with the Declaration of Helsinki and approved by the Institutional Review Board of Nadezhda Women's Health Hospital (56/29.12.2022).

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: The original data presented in the study are openly available in zenodo.org at DOI: https://doi.org/10.5281/zenodo.19885173.

Conflicts of Interest: The authors declare no conflicts of interest.

Abbreviations

ART	Assisted Reproduction Treatment
bp	Base pairs
CD	Cluster of differentiation
CLR	Centered log-ratio
DNA	Deoxyribonucleic acid
FDR	False discovery rate
IFN-γ	Interferon γ
IL	Interleukin
IQR	Interquartile range
IVF	In vitro fertilization
LD	*Lactobacillus*-dominated
MAPK	Mitogen-activated protein kinase
MΦ	Macrophages
NF-κB	nuclear factor kappa B
NGS	Next-generation sequencing
NK	Natural killer
non-LD	Non-*Lactobacillus*-dominated
OTU	Operational taxonomic units
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
rRNA	Ribosomal ribonucleic acid
Th	T helper
TLR	Toll-like receptor
TNF-α	Tumor necrosis factor α

Appendix A

Table A1. Significant correlations between microbial taxa and immune cell ratios.

Taxa		Immune Cell Parameter	Spearman's ρ	*p*-Value	q-Value
Phylum	Bacillota	NK/T	−0.443	<0.001	0.006
	Bacillota	$CD56^+$ NK cells	−0.399	0.002	0.027
Class	Bacilli	NK/T	−0.446	<0.001	0.009
Order	Actinomycetales	Th/MΦ	0.469	<0.001	0.007
	Veillonellales-Selenomonadales	Th/MΦ	0.457	<0.001	0.009
	Actinomycetales	Th/T	0.438	0.001	0.014
	Lactobacillales	NK/T	−0.423	0.001	0.017

Table A1. *Cont.*

Taxa		Immune Cell Parameter	Spearman's ρ	*p*-Value	q-Value
Family	Prevotellaceae	Th/MΦ	0.536	<0.001	0.001
	Actinomycetaceae	Th/MΦ	0.435	0.001	0.024
	Neisseriaceae	Th/MΦ	0.429	0.001	0.024
	Rikenellaceae	Th/MΦ	0.422	0.001	0.024
	Actinomycetaceae	Th/T	0.413	0.001	0.028
	Lactobacillaceae	NK/T	−0.407	0.001	0.030
	Rikenellaceae	Th/T	0.396	0.002	0.039
	Prevotellaceae	Th/T	0.391	0.002	0.041
Genus	*Lactobacillus*	NK/T	−0.442	0.001	0.018

Table A2. Differences in immune cell parameters between *Lactobacillus*-dominated (LD) and non-*Lactobacillus*-dominated (non-LD) groups.

Immune Cell Parameter	*Lactobacillus*-Dominated (LD) Group	Non-*Lactobacillus*-Dominated (Non-LD) Group	*p*-Value	q-Value
$CD3^+$ T lymphocytes, %	2.52 [2.37]	2.46 [2.53]	0.870	0.944
$CD4^+$ T helper cells, %	0.30 [0.74]	0.29 [0.67]	0.613	0.817
$CD56^+$ NK cells, %	1.17 [1.44]	2.12 [2.55]	0.006	0.023 *
$CD68^+$ macrophages, %	1.34 [1.28]	1.96 [2.47]	0.300	0.736
NK/T, ratio	0.52 [0.91]	1.05 [1.10]	0.004	0.023 *
Th/T, ratio	0.15 [0.32]	0.15 [0.33]	0.460	0.736
MΦ/T, ratio	0.73 [0.73]	0.78 [0.97]	0.441	0.736
Th/MΦ, ratio	0.23 [0.60]	0.16 [0.38]	0.944	0.944

Data is presented as median [IQR]. * Significant value.

Table A3. Summary of the principal findings of the study.

Parameter	LD Group	Non-LD Group	*p*-Value	q-Value
$CD56^+$ NK cells (%)	1.17 [1.44]	2.12 [2.55]	0.006	0.023
NK/T ratio	0.52 [0.91]	1.05 [1.10]	0.004	0.023
Shannon diversity	0.56 [0.94]	1.20 [0.90]	0.076	—
Observed features	19.50 [8.50]	57.50 [56.25]	<0.001	—
Pielou's evenness	0.10 [0.16]	0.28 [0.21]	0.004	—
Beta diversity	Significant Bray–Curtis separation between LD and non-LD groups		PERMANOVA $p = 0.001$	
Significant correlations	*Lactobacillus*-related taxa negatively associated with NK/T ratio		q < 0.05	
Significant correlations	Actinomycetaceae, Neisseriaceae, Prevotellaceae and Rikenellaceae positively associated with CD4-related ratios		q < 0.05	

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