acta microbiologica hellenica

MDPI

Systematic Review

Endometrial Microbiome and Its Correlation to Female Infertility: A Systematic Review and Meta-Analysis

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Abstract: The endometrial cavity was considered sterile until the second half of the 20th century. Through modern technological advances and the sequencing of the bacterial 16S rRNA gene, it was proven that the area possesses its own unique microbiome, which can be categorised into two types, *Lactobacillus*-dominant (LD, with a *Lactobacillus* spp. abundance percentage greater than 90%) and non-*Lactobacillus*-dominant (non-LD, with a *Lactobacillus* spp. abundance percentage smaller than 90%), with other species like *Bifidobacterium*, *Gardnerella*, *Prevotella*, and *Streptococcus* also being prominent. The aim of this study was to investigate the possible correlation of the endometrial microbiome to female infertility, through the identification and appraisal of studies published in the databases PubMed, Web of Science, and Scopus. Moreover, 12 studies met the research criteria, including the analysis of endometrial fluid or tissue samples from infertile women through PCR, culturomics-based, or NGS methods. According to most of these studies, a eubiotic LD-type microbiome seems to be best for maximising endometrial receptivity and pregnancy chances, whereas a dysbiotic non-LD-type microbiome, with increased α-diversity and a higher number of pathogens, has a harmful effect. There were few studies that presented contradictory results without, however, a satisfactory explanation. Thus, more time and a greater number of studies are required to clarify contradictions and achieve more certain results.

Keywords: microbiome; endometrium; infertility; systematic review; *Lactobacillus*

Citation: Foteinidou, P.; Exindari, M.; Chatzidimitriou, D.; Gioula, G. Endometrial Microbiome and Its Correlation to Female Infertility: A Systematic Review and Meta-Analysis. *Acta Microbiol. Hell.* **2024**, *69*, 14–28. https://doi.org/10.3390/amh69010004

Academic Editor: Thomas Walsh

Received: 30 January 2024
Revised: 17 February 2024
Accepted: 20 February 2024
Published: 26 February 2024

1. Introduction

1.1. The Microbiome

Humans have co-evolved with trillions of microorganisms that colonise the human body and create a complex, adaptive and dynamic ecosystem, which is fully attuned to the constantly changing physiology of the host [1]. The human microbiome is defined as the trillions of commensal microbial cells hosted by every human—primarily bacteria, but also archaea, viruses, bacteriophages and fungi—and their genomes [2,3]. There has been some confusion in the definition of the human microbiome, due to the very subtle difference in the terms "microbiota" (the microbial taxa associated with humans) and "microbiome" (the catalogue of these microbes and their genes), though in most cases, the two terms are used interchangeably [2]. A microbiome can be found in every niche of the human body that has been examined [3], even in organs that were traditionally considered sterile, such as the lungs [4] and the stomach [5], and the largest microbiome in terms of the number of microorganisms it contains is found in the intestinal tract and, more specifically, mainly in the large intestine [2]. In contrast to the host's genome, which remains relatively stable, the microbiome and its genome are, as previously mentioned, dynamic, and undergo changes during a human's development due to environmental factors, such as their diet, use of antibiotics, way of birth, past infections, etc., but also as a response to disease [6]. The recent advances in DNA sampling and sequencing techniques have given answers to a number of questions regarding the composition of the microbiome of every niche of the human body,

the formation of microbial communities of various degrees of complexity, their correlation to the microbiomes of other species [7], and, most importantly, their correlation to different diseases [6]. Dysbiosis in a microbial community can be described as a disturbance in the balance of the community's ecology which causes or intensifies a health problem. Thus, dysbiosis in the human microbiome has been associated with a plethora of diseases and conditions, such as irritable bowel syndrome, diabetes, allergies, asthma, and even cancer [1]. More specifically, in the case of reproduction and fertility-related conditions, like endometriosis, studies have shown that the gastrointestinal, neuroendocrine and immune system interact and play an important part. The gut microbiome has been shown to influence the progression of endometriosis, with the endometriotic microbiome consisting of the genera *Prevotella*, *Bautia*, and *Bifidobacterium* [8]. A dysbiotic gut microbiome has also been shown to affect the pathological process of polycystic ovary syndrome (PCOS), with lower α- and β-diversities and an increased abundance of *Bacteroides*, *Parabacteroides*, and *Clostridium* [9]. The characterisation of the human microbiome and the factors that affect the composition and evolution of the microorganisms that it is composed of was the main goal of the Human Microbiome Project (HMP) as a logical extension of the Human Genome Project (HGP) [10]. The HMP, which started in 2007 and was set up by the National Health Institutes of the U.S.A., has produced a total of 2.3 Tb of metagenomic data about the 16S ribosomal RNA (16S rRNA). The use of the 16S rRNA gene in the identification and taxonomy of bacteria began in the 1980s by C. Woese, as it was proven that it makes for an excellent molecular chronograph. It is defined by a great degree of conservation, which stems from the importance of the gene as a crucial element of cell function, and very few other genes are as conserved as this [11]. It is clear that big projects like the HMP and its European equivalent, MetaHIT, already offer a deeper understanding of the biology and the clinical importance of the human microbiome and its genome [12].

1.2. The Endometrial Microbiome

The data that originally described the healthy microbiome of the female reproductive system were derived from studies performed exclusively on the vagina and how its microbiome changes throughout the reproductive years of a woman's life and during the menstrual cycle [13]. As a result, the endometrial cavity was considered to be a sterile field until the second half of the 20th century [14], and the prevailing opinion at the time was that it was protected from chemical and mechanical trauma and the invasion of microorganisms by the cervical mucus plug [15]. However, recent studies on the area, and on the upper reproductive tract in general, prove that the endometrium possesses its own unique microbiome, which has greater species diversity than that of the lower reproductive tract [16]. The results of studies on women of reproductive age who underwent hysterectomy led to the categorisation of the endometrial microbiome into two main types, according to their microbial composition: *Lactobacillus*-dominant (LD, with a *Lactobacillus* spp. abundance percentage greater than 90%) and non-*Lactobacillus*-dominant (non-LD, with a *Lactobacillus* spp. abundance percentage smaller than 90%). Other species prominent in the samples include *Bifidobacterium*, *Gardnerella*, *Prevotella*, and *Streptococcus* [14]. Further analysis and organisation of the aforementioned species in communities showed a negative correlation of *Lactobacillus* with *Gardnerella*, *Bifidobacterium*, and *Atopobium* and a positive correlation with the commensal genera *Clostridium* and *Streptomyces* [14]. The endometrial microbiome, as shown by the analyses of samples from women before the start of in vitro fertilisation (IVF), plays an important role in the reproductive process, as women with an LD-type microbiome had higher chances of a successful implantation, full-term pregnancy and live birth, while women with an abundance of other genera and a decreased presence of *Lactobacillus* in their microbiome were significantly less likely to achieve pregnancy and more likely to suffer a miscarriage [17]. Moreover, it has been proven that the presence of a non-LD-type microbiome is also associated with pathological conditions and diseases of the endometrium, such as endometriosis [18] and chronic endometritis [19]. Nonetheless,

an agreement on the microbial composition of the healthy microbiome, or the existence of a core microbiome, has not yet been reached [18].

1.3. Analysis of the Endometrial Microbiome

All data published until now concerning the analysis of the endometrial microbiome, and mainly the bacteria found in it, have been extracted using one of two method types: culture-based or sequencing-based methods. Culture-based methods, which are the ones traditionally used, include cultivation of the samples in appropriate conditions and identification of the bacteria found, either by the substances they produce or via characterisation of conserved genes in their 16S rRNA. However, analyses of the vaginal microbiome have shown that several microorganisms are impossible to cultivate, and unsuccessful cultivation, in many cases, fails to fully reveal the diversity of the microorganisms present in the sample [20]. For these reasons, methods based on sequencing the 16S rRNA gene are increasingly being used lately. This gene's sequence is approximately 1550 base pairs (bp) long, consisting of conserved and variable regions, and the many intraspecific polymorphisms present are sufficient for identifying and classifying strains all the way to the sub-species level [11]. The most common sequencing methods are next-generation sequencing (NGS) or high-throughput sequencing methods, which allow the sequencing of large DNA or RNA molecules, up to 30,000–50,000 bp long, in real time. NGS methods are based on using an engineered DNA polymerase attached to the DNA to be sequenced at the bottom of a well, which incorporates nucleotides, labelled with different phosphor-linked fluorophores for differential detection, to the growing chain. When a nucleotide is incorporated into the growing chain, light is emitted, which enables the identification of the nucleotide due to its colour, and then the fluorophore is released, allowing for the incorporation of the next nucleotide. The whole process occurs simultaneously in up to one million wells on a single microchip, producing sequences of a total length of 10,000–15,000 bp (equivalent to a data volume of up to 7.6 Gb) [21]. In the case of the 16S rRNA gene in microbiome analyses, universal primers are used, which are complementary to the conserved region at the beginning of the gene and also to either the region at approximately 540 bp or the end of the gene, whereas the variable regions in between [11], mainly the V4 hypervariable region [22,23], are used for bacterial differentiation. In recent years, there has also been an increase in the use of fourth-generation sequencing methods, which include the identification of the individual nucleotides of a single-strand DNA molecule as it passes through a small-diameter nanopore, according to the different electrical signal each one produces. This way, the production of even larger data volumes is possible, utilising less time and space [21,24]. However, in the case of the endometrium, which is characterised by a microbiome of relatively low biomass and a high risk of DNA from contamination being present, an appropriate protocol, which combines both high reliability and low cost, has not yet been designed [24].

2. Methods and Materials

2.1. Information Sources and Search Strategy

This systematic review was conducted according to the PRISMA guidelines (preferred reporting items for systematic reviews and meta-analyses. The literature search about the endometrial microbiome and infertility was performed using the databases PubMed, Scopus, and Web of Science, with the last search date being October 2023. The search strategy was accordingly modified for each database, taking into consideration alternate spellings, synonyms for the keywords used, and changes in terminology over the years, and MeSH (medical subject headings) terms were also used where possible, in order to increase the specificity of the search. Furthermore, the references of the relevant studies were hand-searched to ensure a more thorough coverage of the topic.

The search strategy formed for the PubMed database was as follows: ("Microbiota" [MeSH terms] OR microbiot* [All fields] OR microbiom* [All fields]) AND ("Endometrium" [MeSH terms] OR endometrial [All fields] OR endometrium [All fields]) AND ("Infertility"

[MeSH terms] OR infertil* [All fields] OR fertil* [All fields] OR steril* [All fields]). The number of retrieved results was 177.

For the Scopus database, the search strategy was formed as follows: (endometrium OR endometrial) (Title, Abstract, Keywords) AND (microbiot* OR microbiom*) (Title, Abstract, Keywords) AND (infertil* OR fertil* OR steril*) (Title, Abstract, Keywords). The number of retrieved results was 261.

Finally, for the Web of Science database, the search strategy was formed as follows: (endometrium OR endometrial) (Topic) AND (microbiot* OR microbiom*) (Topic) AND (infertil* OR fertil* OR steril*) (Topic). The number of retrieved results was 230.

2.2. Results Screening and Eligibility Criteria

First, the retrieved results were imported into the reference management software EndNote Online Classic (Clarivate Analytics (former Thomson Reuters), Philadelphia, U.S.A.), where duplicate results were identified and deleted. Then, the relevant results were screened by their titles and abstracts, and the full texts of all eligible studies were retrieved, as well as those of articles where eligibility could not be decided based solely on their title and abstract. The final stage of the results management was picking the studies that met all the eligibility criteria after studying their full texts when necessary. The eligibility criteria set for this review were as follows:

- Only articles of studies;
- Publication date: 2020 or later;
- Language: English;
- Subjects: humans;
- Analysis of the endometrial microbiome either exclusively or in combination with the microbiome of other parts of the reproductive tract;
- Correlation to infertility and/or the outcome of IVF treatment.

Exclusionary criteria, corresponding to the aforementioned eligibility criteria, were:

- Reviews, systematic reviews, books or chapters of books and other types of text;
- A publication date of 2019 or earlier;
- Language of the article other than English;
- Animal species as subjects of the studies;
- Analysis and focus on the microbiome of other organs and systems of the human body besides that of the female reproductive tract;
- Correlation of the microbiome to pathological conditions or diseases of the female reproductive system (e.g., endometriosis, endometritis, etc.).

2.3. Data Extraction

The data extraction from each study was conducted using the Microsoft Excel program. The relevant data extracted were:

- Author(s);
- Publication date;
- Country where the study was conducted;
- Aim of the study;
- Basic demographic data;
- Sample types;
- Analysis method;
- Basic data and results from the analyses;
- Correlation to infertility and/or IVF treatment outcome.

3. Results

3.1. Search Results

The search strategy described previously retrieved a total of 668 results. After combining the results from all databases and deleting duplicates, there were 338 unique results

left, which were then screened for their relativity to the search criteria. Then, the irrelevant results were also removed, and the full texts of the 258 remaining articles were retrieved, which were then further assessed for eligibility according to the criteria described in Section 2.2. Finally, 12 studies' articles were chosen to be included in this review, which contained all the necessary data about the endometrial microbiome analysis on infertile women and its potential correlation to pregnancy achievement or the outcome of IVF treatment, and all of the relevant data were extracted for further studying. The full selection and retrieval process is shown in Figure 1.

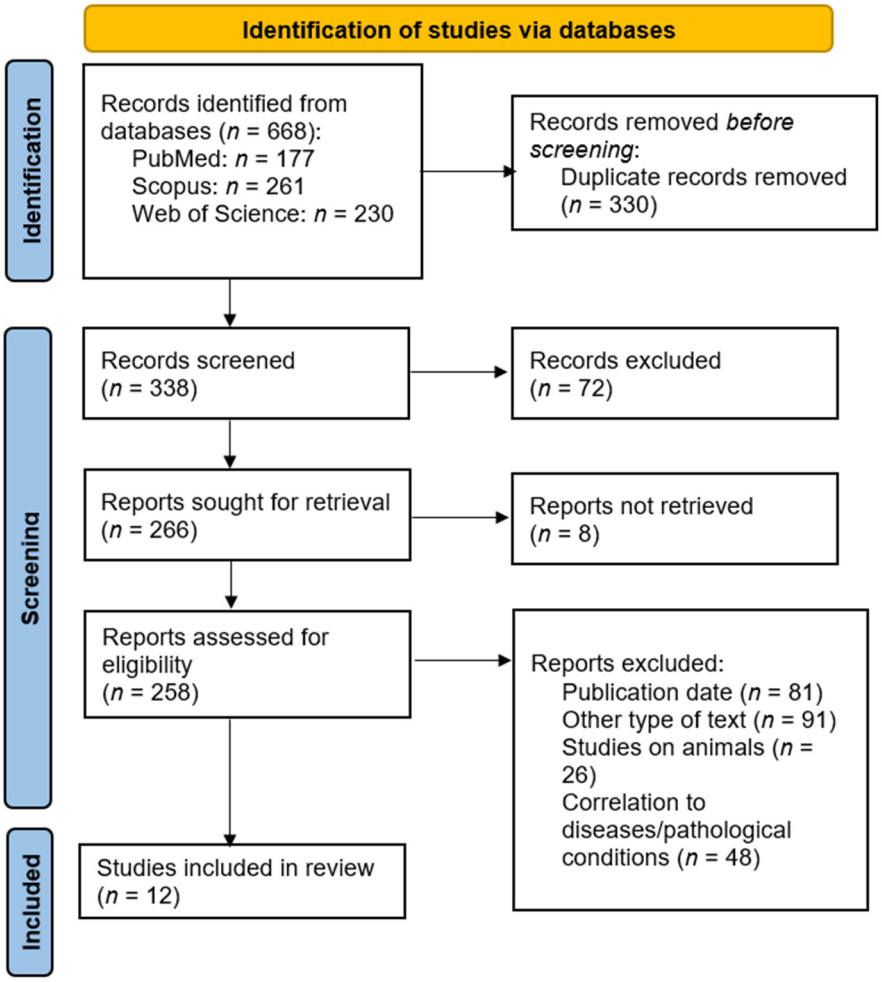

Figure 1. PRISMA flow diagram.

3.2. Publication Characteristics

From 2020 until October 2023, 12 studies have been published about endometrial microbiome analysis on infertile women and its correlation to pregnancy achievement and/or the outcome of IVF treatment and were therefore included in this review. Five of them (41.67%) were published in 2023, four (33.33%) in 2022, two (16.67%) in 2021, and only one (98.33%) in 2020. With regard to the country where each study was conducted, three studies each (25%) were conducted in Japan and Italy, two (16.67%) in Spain, and one each (8.33%) in Turkey, Russia, China and the U.S.A. The main goal of these studies was the analysis and study of the endometrial microbiome, either exclusively or along with the vaginal and/or cervical microbiome, in women with infertility, and mainly in cases with repeated implantation failure (RIF). In some studies, a comparison to the microbiome of healthy (fertile) women was performed, with the ultimate goal always being the investigation of its microbial composition and its potential correlation to pregnancy achievement and the

outcome of IVF treatment or embryo implantation in cases where these were performed. The basic publication characteristics of each study are shown in Table 1.

Table 1. Basic publication characteristics of the studies included.

Author(s)	Year	Country	Aim	Reference Number
Shunsaku Fujii, Takaaki Oguchi	2023	Japan	Evaluation of the correlation of age and microbiome to endometrial receptivity.	[25]
Takuhiko Ichiyama et al.	2021	Japan	Identification of specific microbial communities in the vaginal and endometrial microbiomes as potential biomarkers for implantation failure.	[23]
Ozlem Sezer et al.	2022	Turkey	Correlation of the disruption of the vaginal and endometrial microbiome to unexplained infertility.	[26]
Francisca Maria Lozano et al.	2023	Spain	Comparison of the endometrial microbiome between patients with and without RIF before undergoing IVF.	[27]
Mark Jain et al.	2023	Russia	Comparison of the qualitative and quantitative species abundance of bacteria, viruses and fungi in vaginal, cervical and endometrial fluid samples of infertile women.	[28]
Maho Miyagi et al.	2022	Japan	Investigation of the effect of the balance between *Lactobacillus* and other pathogens in the vaginal and endometrial microbiome on IVF outcomes of infertile patients.	[29]
Marco Reschini et al.	2022	Italy	Comparison of the vaginal and endometrial microbiomes of women undergoing IVF, and correlation to the possibility of a successful pregnancy.	[30]
Immaculada Moreno et al.	2022	U.S.A.	Investigation of the possible effect of the composition of the endometrial microbiome on the reproductive result.	[17]
Federica Cariati et al.	2023	Italy	Use of culturomics-based methods for endometrial microbiome analysis, and correlation to pregnancy rates.	[31]
Maria del Carmen Diaz-Martinez et al.	2021	Spain	Description and comparison of the vaginal and endometrial microbiomes between women with and without a successful pregnancy after IVF, as well as between women with and without RIF.	[32]
Lucia Riganelli et al.	2020	Italy	Investigation of structural differences between the vaginal and endometrial microbiome to define potential biomarkers related to implantation failure.	[33]
Yixuan Zou et al.	2023	China	Recording of the endometrial microbiome profiles of women with RIF, and investigation into the use of antibiotics on these patients.	[34]

3.3. Population Characteristics

The studies in this review included a total of 1229 infertile patients, and three of them also included a total of 65 control patients (not facing fertility problems). All studies excluded patients with secondary infertility, which could be attributed to pathological causes such as endometriosis, polyps and other masses and lesions in the endometrial

cavity, underlying infections, etc. The age range of the patients was between 18 and 50 years, and no studies presented any further demographic data. In every study, the patients were characterised either as infertile, meaning unable to achieve clinical pregnancy after 12 consecutive months of natural efforts, or as infertile with RIF, meaning infertile with a history of at least three failed IVF attempts and good-quality embryo transfers. Moreover, the studies can also be split into those where IVF or embryo transfer was performed as part of them and those that only included microbiome analysis. In total, five studies were conducted on infertile patients with a simultaneous IVF attempt, including 549 patients and 26 controls, while four studies were conducted on patients with RIF with a simultaneous IVF attempt, including 387 patients and 18 controls. One study was conducted with a simultaneous IVF attempt on patients with and without RIF. With regard to the studies not including an IVF attempt, one of them was conducted on a total of 100 infertile patients and one on a total of 145 patients with RIF. The basic population characteristics of each study are shown in Table 2.

Table 2. Basic population data of the studies.

Reference Number	Patients Total	Controls	Age (Years)	Patients' Characterisation	IVF Attempt
[25]	185	No	25–47	With RIF	Yes
[23]	145	21	N/A	With RIF	No
[26]	26	26	20–45	Infertile	Yes
[27]	27	18	<45	With RIF	Yes
[28]	100	No	N/A	Infertile	No
[29]	35	No	N/A	Infertile	Yes
[30]	53	No	N/A	Infertile	Yes
[17]	342	No	<50	Infertile	Yes
[31]	93	No	29–47	Infertile	Yes
[32]	48	No	18–50	With and without RIF	Yes
[33]	34	No	22–43	With RIF	Yes
[34]	141	No	<40	With RIF	Yes

3.4. Sample Types and Analysis Methods

The main type of sample used for microbiome analysis was endometrial fluid, which was used in eight studies (66.67%). In three studies (25%), an endometrial tissue sample was taken after a biopsy, and in one study (8.33%), samples from both endometrial fluid and tissue were used. There were some studies that also used vaginal and/or cervical samples for analysis and comparison, but for the purposes of this review, only the endometrial samples were taken into consideration. A graph with the percentage distribution of the sample types used in the included studies is shown in Figure 2.

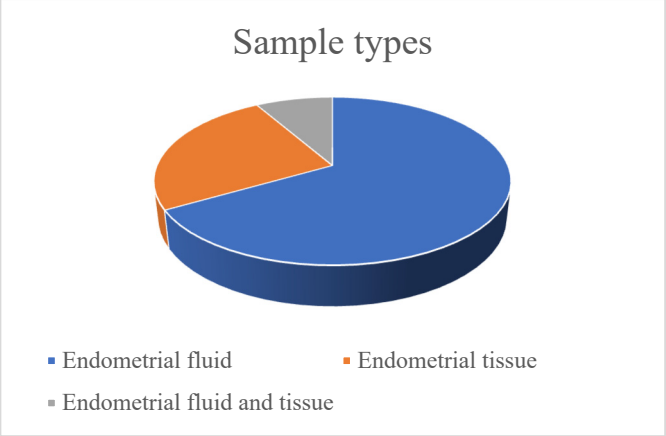

Figure 2. Sample types used in the studies.

Regarding the methods used for DNA analysis after its extraction, the majority of the studies, and more specifically nine of them (75%), used NGS methods for sequencing the 16S rRNA gene. Real-time PCR, wide-spectrum PCR, and MALDI-TOF (matrix-assisted laser desorption/ionisation—time of flight) mass spectrometry after culture were used in one study each. A graph with the percentage distribution of the DNA analysis methods used in the included studies is shown in Figure 3.

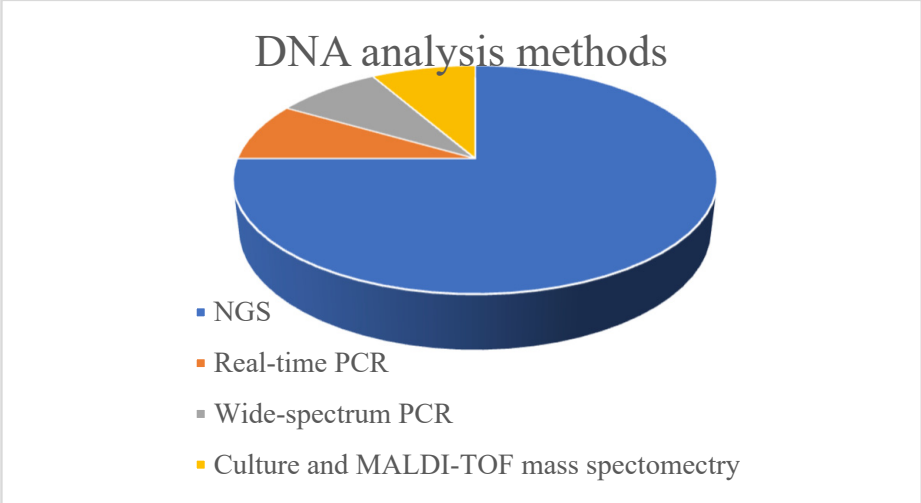

Figure 3. DNA analysis methods used in the studies.

3.5. Data and Results from the DNA Analysis

According to the data from the analysis of the microbial DNA extracted from the samples, a non-LD-type microbiome was found in most women with infertility or RIF, with low percentages of *Lactobacillus* and a higher abundance of pathogenic bacterial genera, primarily *Prevotella*, *Gardnerella*, *Atopobium*, *Pseudomonas*, *Streptococcus* and *Staphylococcus*. In general, the main trend in patients with infertility problems seems to be the presence of a dysbiotic microbiome, with looser and disorganised connections among microbial species in its communities, while it also seems that the β-diversity, meaning the composition of the microbial communities of the endometrium, plays a more important role in endometrial receptivity and pregnancy rates rather than the α-diversity, meaning the number of species in the communities, as the α-diversity did not significantly differ between infertile patients and the controls. Regarding the outcome of IVF attempts, in the studies where they were performed, in most cases, there were successful pregnancies in women with an LD-type microbiome, with a higher *Lactobacillus* abundance and low percentages of pathogens. However, this is not absolute because, in some cases, it was proven that the abundance of *Lactobacillus* was not as important for pregnancy achievement as it did not differ greatly between patients who got pregnant and those who did not, but rather, the presence of pathogens was of greater importance, which seems to decrease the chances of a successful IVF attempt and pregnancy. Moreover, it is worth noting that in one study, the high abundance of species other than *Lactobacillus* seemed beneficial, as there were higher pregnancy rates in women with this microbiome type, while another study presented a higher number of pregnancies in women with a complete absence of *Lactobacillus* and a higher IVF failure rate in women with an LD-type microbiome. The data from the DNA analysis and the basic results of each study are shown in Table 3.

Table 3. Analysis data and studies' results.

Reference Number	Analysis Data	Results
[25]	Patients' categorisation into five distinct microbial profiles: 40 patients with profile 1 (normal, with *Lactobacillus* percentage >90% and absence of pathogens), 8 patients with profile 2 (abnormal, with *Lactobacillus* percentage <90% and absence of pathogens), 32 patients with profile 3 (abnormal, with *Lactobacillus* percentage <90% and presence of pathogens), 49 patients with profile 4 (mildly dysbiotic), and 56 patients with profile 5 (very low biomass).	The patients with profile 1 had a more receptive endometrium, whereas those with profile 5 had a less receptive endometrium. In general, a non-receptive endometrium was correlated to low *Lactobacillus* levels and the presence of a dysbiotic microbiome.
[23]	A total of 131 microbial species were detected in the endometrial microbiome. In the RIF group, 14 genera (*Atopobium*, *Gardnerella*, etc.) were in higher abundance than in the control group, whereas the *Lactobacillus* abundance did not significantly differ between the two.	The endometrial α-diversity was higher than the vaginal one, and dysbiosis in the vagina was always noticed along with dysbiosis in the microbiome (the 14 genera indicative of dysbiosis are the same in both the vagina and the endometrium, so they are likely transferred upwards), while an abundance of *Lactobacillus* does not necessarily correlate to an unsuccessful implantation and pregnancy.
[26]	Patients with unexplained infertility had more dysbiotic microbiomes, with lower *Lactobacillus* and higher pathogen percentages than the control group.	The percentages of a disorganised microbiome in the reproductive tract differed significantly between fertile and infertile women, with the key factor being the reduction in *Lactobacillus* abundance.
[27]	Control group: higher abundance of *Lactobacillus* (specifically *L. iners*) and lower abundance of *Prevotella*. RIF group: high abundance of *Lactobacillus*, but also high percentages of *Prevotella*, *Gardnerella* and *Ralstonia*. In general, there were no differences in the α-diversity but a great difference in β-diversity between the two groups.	Negative correlation of *Lactobacillus* to pathogens, and an abundance of species related to implantation failure in the RIF group.
[28]	The total bacterial loads were lower in the endometrial samples (6.3×10^3 genome copies), with 16% of the samples not containing any bacterial DNA. The endometrial samples had a very low α-diversity compared to the vaginal and cervical ones, and the abundance of *Gardnerella vaginalis*, *Prevotella bivia*, and *Porphyromonas* spp. influenced the abundance of *Lactobacillus*.	The endometrium of infertile patients has a distinct microbial profile compared to the vagina and the cervix, and the immunological and biochemical interactions among members of the microbial communities possibly play an important role in regulating its receptivity.
[29]	Categorisation of patients into four microbial profiles: 22 patients with profile 1 (high *Lactobacillus*, low pathogens), 3 patients with profile 2 (high *Lactobacillus* and high pathogens), 2 patients with profile 3 (low *Lactobacillus* and low pathogens) and 6 patients with profile 4 (low *Lactobacillus* and high pathogens). Furthermore, 77.3% of patients with profile 1 had a successful pregnancy, while 83.3% of patients without a successful pregnancy had profile 4 microbiome.	Higher chances of pregnancy in high *Lactobacillus* and low pathogen levels, and lower chances in high pathogen and low *Lactobacillus* levels.
[30]	In total, 8% of patients were found with an LD-type microbiome, with high percentages of *Pelomonas*, *Probionabacterium*, *Pseudomonas*, *Streptococcus* and *Escherichia* as well. 30% of patients achieved pregnancy, with 53% of them having an LD-type microbiome.	Relatively low frequency of LD patients, with a higher species diversity rather than *Lactobacillus* abundance which is beneficial for pregnancy achievement.
[17]	The dominant genus in both sample types was *Lactobacillus*, with other genera such as *Atopobium*, *Bifidobacterium*, and *Gardnerella*, etc., also being quite common. Differences were noted in the bacterial networks between the two sample types: fluid samples had two connected communities, while tissue samples had four, the fluid samples networks were more tightly connected than the tissue sample ones, and finally, *Lactobacillus* was both positively and negatively correlated to neighbouring genera in fluid samples but only negatively correlated in tissue samples.	Patients with live births had denser and more tightly connected microbial communities, and some connections were only present in these cases, which shows their importance in the IVF outcome. On the other hand, these connections were absent in patients with failed attempts, who had looser and more disorganised microbial networks.

Table 3. *Cont.*

Reference Number	Analysis Data	Results
[31]	In total, 74% of patients had a positive culture of at least one bacterial species, whereas 26% presented no growth. The most common phyla were *Firmicutes* (87.76% of patients), *Proteobacteria* (27.94% of patients), *Actinobacteria* (10.29% of patients) and *Ascomycota* (8.82% of patients). The most dominant genus was *Lactobacillus* (37% of pregnant patients versus 5% of non-pregnant ones), while the phylum *Actinobacteria* was only present in non-pregnant patients. There was also a correlation of the families *Staphylococcaceae* and *Enterobacteriaceae* to failed IVF attempts.	An LD-type microbiome correlated to higher pregnancy rates, whereas pathogens, and specifically the genus *Staphylococcus*, were more common in patients with failed IVF attempts.
[32]	There were no differences in α- and β-diversities between pregnant and non-pregnant women, and pregnant women had a higher abundance of *Lactobacillus, Gardnerella, Burklhodena,* and *Anaerobacillus*, while non-pregnant ones had a higher abundance of *Streptococcus, Ralstonia, Prevotella,* and *Delfia*. Regarding the history of RIF, there was a higher α-diversity in women without RIF, with differences in β-diversity also being present. The dominant genus in women with RIF was *Prevotella* and the species *L. iners* and *L. jensenii,* while in women without RIF, the dominant genus was *Ralstonia* and the species *L. helveticus* and *Sneathia amnii*.	In total, 21 women achieved pregnancy, of which 38.9% had a history of RIF compared to 70% of those who did not achieve pregnancy. There was a higher diversity in the endometrial microbiome compared to the vaginal one, and a non-LD-type microbiome correlated to lower rates of successful implantation, pregnancy and live births.
[33]	The endometrial microbiome presents ahigh species heterogeneity, specifically with the presence of species, like *Kocuna dechagenensis*, not found before in the reproductive tract. The microbiome of pregnant women presented a total absence of *Lactobacillus* and a high abundance of Lachnospiraceae and Enterobacteriaceae, while that of non-pregnant women presented *Lactobacillus* dominance and a higher α-diversity.	The presence of *Lactobacillus* in the endometrium is possibly caused by upwards migration from the vagina, and it creates an unfavourable environment for embryo implantation and a successful IVF attempt.
[34]	In total, 20 patients were found with an LD-type microbiome and 121 with a non-LD-type one. 11.3% of patients did not have any pathogenic bacteria in their microbiome, and 88.7% of them did. The most common species were *Streptococcus* (72.3% of patients), *Staphylococcus* (51.8% of patients) and *Neisseria* (47.5% of patients)	In total, 69 patients achieved pregnancy, of which 63 had pathogens in their microbiome. A non-LD-type microbiome and pathogens were noted in most patients with RIF. Apart from the presence of pathogens and the decrease in *Lactobacillus*, the co-occurrence of most of these pathogens seems to play an important role in the disturbance of the microbial ecosystem of the endometrium and the decrease in successful implantation and pregnancy chances.

4. Discussion

4.1. Population Characteristics and Demographics

With regards to the age of the patients recruited for the studies, it is only mentioned in half of the studies. The patients cover almost the entire range of the reproductive ages (18–50 years old), with the majority of them, however, being 25 to 40 years old. As the studies investigate the correlation of the microbiome of the endometrium and, in some cases, of other organs of the reproductive system to female infertility, we can safely deduce that the rest of the patients whose age is not included in the studies, are also of reproductive age. Other demographic data are also omitted, apart from the countries where the studies were conducted, which also constitute the patients' countries of origin. Regarding these, half of the studies were conducted in Asian countries and the rest in European countries (and more specifically, in countries of the European south), apart from one which was conducted in the U.S.A. It is obvious that the number of countries is quite limited, which could potentially lead to misleading results. For this reason, it is suggested that subsequent studies on the subject include patients from a larger number of countries, especially African, American and Oceanian countries, which have not so far been represented. This way, the results can be more representative, and there can be further investigation on whether

the country of origin and the lifestyle and standard of living there plays a role in the composition of the microbiome and infertility. Finally, the population sample sizes were quite small, with the average being 103 patients per study and the median being 73 patients per study, which is mainly due to the complexity of the process and the time required to choose and recruit the appropriate patients and perform all the analyses. Nonetheless, studies with a significantly larger sample size, to the extent that this is feasible, would lead to safer and more complete results and would help us decipher if any unusual or unexpected results are really statistically important or just the result of chance.

4.2. Sample Types

The main sample type used in the studies was fluid from the endometrial cavity, which, in most cases, was taken using a double-lumen catheter [23,26,28,30–32]. With this device, the internal catheter easily passes through the external one without making contact with the vaginal or cervical epithelium, and the fluid is drawn through the syringe on the other end [30]. In only two cases, the sample was taken using a cell-collecting brush, specifically a Tao Brush (Cook Medical, Madrid, Spain) in one [27] and a Yuino Brush (Asuka Pharmaceuticals, Tokyo, Japan) in the other [29]. In the studies which used tissue from a biopsy as the preferred sample type, this was taken using a Pipelle-type device (Laboratoire CDD, Paris, France). This device is a flexible polypropylene tube with an external diameter of 3.1 mm and an internal diameter of 2.6 mm. By removing an internal piston, negative pressure is created, and the tissue is aspirated in the cannula [35]. All the aforementioned modern techniques are significantly less invasive than traditional ones, causing minimal pain and discomfort to the patients and eliminating the need for anaesthesia. Moreover, through the simultaneous ultrasound guidance performed by experienced technicians, the sample is taken quickly from the exact anatomical spot [30].

Any unusual or unexpected results did not occur only in studies using one or the other sample type, and thus it cannot be safely said that either sample type is more or less reliable than the other. The most important point during the sample collection process is avoiding contamination from the lower reproductive tract organs. For this reason, the vagina and cervix should be washed out with plenty of saline solution prior to the sample collection and then carefully dried using a sterile gauze or cotton pieces [29,34]. Also, extra precaution should be taken during the insertion and removal of the catheter or brush into and out of the endometrial cavity to avoid contact with the vaginal walls [36]. Lastly, proper storage of the samples until further analysis is also crucial, and it should be carried out using sterile containers and appropriate buffer solutions. The samples should then be stored either in the refrigerator (4 °C) or the freezer (−80 °C), according to how much time there will be between the collection and analysis stages.

4.3. Sample Analysis Methods

The three types of methods used in the studies are, as previously mentioned, culturomics-based methods, which include the mass culture of microorganisms and identification using MALDI-TOF mass spectrometry, PCR-based methods and NGS methods. First, MALDI-TOF mass spectrometry allows for the identification of microorganisms by species-specific peptide and protein mass profiles. This method can identify microorganisms up to the species level, with a high level of accuracy and reliability even in samples with a very low microorganism biomass. However, it is a method with a high workload as it requires cultivation and incubation of the microorganisms from the samples, which subsequently increases the total time and cost of the process. Furthermore, the methods that make use of the various different forms of PCR achieve detection, identification and quantification of the microbial species in the samples, in a quick, highly accurate and effective way. Their main disadvantage, however, is the fact that they require knowledge of the sequences of interest of the microorganisms under investigation in order for the appropriate primer sequences to be designed. Lastly, NGS methods, along with the evolution of bioinformatics, allow for DNA

sequencing straight from the sample, even if only a small amount is available, produce a high data volume in real time and only require a little amount of space.

From these, it becomes clear that modern molecular methods are the most advantageous, defined by their high degree of sensitivity, specificity and reliability. However, the endometrial cavity is a niche of the human body whose microbiome is only recently starting to get more thoroughly explored. Thus, a universal protocol for sample analysis has not yet been created, and therefore it is up to each researcher to choose the desired methods, according to the available funds and materials and the specific needs and expected results of each study.

4.4. Analysis Results and Correlation of the Microbiome to Infertility

In accordance with most of the earlier studies conducted on the topic, the majority of the studies included in this review present a eubiotic endometrial microbiome, with a percentage of species of the genus *Lactobacillus* greater than 90% and very few or no other species of pathogenic bacteria present, as the most beneficial for increasing the chances of a successful IVF attempt and pregnancy. The genus *Lactobacillus* was positively correlated to the commensal bacteria and negatively correlated to pathogenic ones, and the microbial networks of patients who achieved a successful pregnancy were found to be denser and with more and denser microbial relations compared to those of patients with RIF. According to two of the studies, the mechanisms through which the eubiotic microbiome of the vagina and the endometrium alike affects endometrial receptivity and chances of a successful embryo implantation are most possibly immunological, as the immune tolerance of some cells participating in the immune response (e.g., T-regulatory lymph cells) potentially affects implantation. When bacteria invade the endometrium and stimulate the pattern recognition receptors (PRRs) of the epithelial cells, these cells secrete cytokines that affect the local lymph cell population. Bacteria of the genus *Lactobacillus* prevent pathogens from entering by acting on the PRRs of the mucosal cells to regulate the immune response necessary for embryo implantation [17,29]. Furthermore, bacteria of the genus *Lactobacillus* secrete lactic acid, thus creating an acidic environment that inhibits pathogen growth [31].

On the other hand, a dysbiotic microbiome, defined by a decrease in the percentage of *Lactobacillus* and other commensal bacteria and an increase in pathogen levels, has negative effects in IVF outcome. Almost all studies presented similar results regarding the composition of the dysbiotic microbiome, with the most common genera appearing as biomarkers of RIF being *Gardnerella*, *Prevotella*, *Megasphaera*, *Atopobium*, *Streptococcus* and *Staphylococcus*, with most of these coexisting in the microbiome. As the different bacteria interact and the dense community formed by the *Lactobacillus* species and other commensal bacteria is necessary for the stability of the local "ecosystem" in the endometrium, the presence of pathogens, which also interact with each other, plays an important role in the disorganisation of this "ecosystem" in patients with RIF. However, it is not clear whether the presence of a non-LD-type microbiome facilitates the entrance of pathogens in the endometrium or if the entrance of pathogens is what causes the decrease in the *Lactobacillus* percentage [34]. In general, it seems that the increase in α- diversity in a dysbiotic, non-LD-type microbiome has harmful effects on endometrial receptivity and the chances of a successful pregnancy, as the lack of a dominant species potentially facilitates colonisation by many bacterial species, especially pathogens, and creates an adverse environment related to infertility [32].

Nevertheless, there were studies that presented contradictory results. One of these showed that the microbiome of patients with lower levels of *Lactobacillus* was not as highly correlated to endometrial receptivity compared to patients with a complete lack of microbiomes, which may indicate that it is the quantity rather than the percentage ratio of *Lactobacillus* that affects endometrial receptivity [25]. Another study also agrees with these results, in which the microbiome of all patients with a successful pregnancy was found completely lacking the *Lactobacillus* species, with high levels of *Lactobacillus* found in non-pregnant women [33]. In a third study, the *Lactobacillus* abundance did not significantly

differ between control patients and ones with RIF, with the successful pregnancy rates also being similar between patients with a *Lactobacillus* percentage greater and smaller than 90% [23]. Finally, one study showed a higher α- diversity in the microbiome of pregnant patients than non-pregnant ones, which led the researchers to assume that more diverse microbiomes are more beneficial for endometrial receptivity than a non-LD-type one [30]. Therefore, it seems that in some cases, the absence of *Lactobacillus* and the presence of more diverse microbiomes does not necessarily lead to dysbiosis, while higher *Lactobacillus* levels could even prove to be detrimental to pregnancy chances. However, these studies are only a small percentage of the total, and as none of them provide a satisfactory explanation, a larger number of studies are required in order to prove if these results are indeed statistically important or just the result of chance.

5. Conclusions

This review investigated the composition of the endometrial microbiome and its potential correlation to female infertility and the outcome of IVF treatment based on the most recent studies on the topic. The strength of this study is that it examines a very interesting topic that only recently started getting more attention from researchers, and since female infertility is a problem many people are struggling with, we feel that this study would be valuable to researchers and practitioners working in the field of human reproduction. According to the majority of the studies included in this systematic review, as well as most of the earlier ones, a eubiotic LD-type microbiome seems to be best for maximising endometrial receptivity and the chances of a successful pregnancy, whereas a dysbiotic non-LD-type microbiome, with increased α- diversity and a higher number of pathogens present, has a harmful effect. On the other hand, there were few studies that presented contradictory results, without, however, a satisfactory explanation. Thus, also taking into consideration the fact that studies on the endometrial microbiome are still in the early stages, there is only a small number of them, from few countries and with small population sizes, and it is clear that more time and a larger number of studies are needed in order to decipher contradictions and produce more certain results.

Author Contributions: Conceptualization, P.F. and G.G.; methodology, G.G.; software, P.F.; validation, P.F., M.E., D.C. and G.G.; formal analysis, P.F.; investigation, P.F. and G.G.; resources, P.F. and G.G.; data curation, P.F.; writing- original draft preparation, P.F.; writing-review and editing, P.F., M.E., D.C. and G.G.; visualisation, P.F., M.E., D.C. and G.G.; supervision, M.E., D.C. and G.G.; project administration, M.E., D.C. and G.G. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

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

References

1. Lloyd-Price, J.; Abu-Ali, G.; Huttenhower, C. The healthy human microbiome. *Genome Med.* **2016**, *8*, 51. [CrossRef]
2. Ursell, L.K.; Metcalf, J.L.; Parfrey, L.W.; Knight, R. Defining the human microbiome. *Nutr. Rev.* **2012**, *70*, S38–S44. [CrossRef] [PubMed]
3. Manos, J. The human microbiome in disease and pathology. *APMIS* **2022**, *130*, 690–705. [CrossRef] [PubMed]
4. Dickson, R.P.; Erb-Downward, J.R.; Martinez, F.J.; Huffnagle, G.B. The Microbiome and the Respiratory Tract. *Annu. Rev. Physiol.* **2016**, *78*, 481–504. [CrossRef] [PubMed]
5. Bessède, E.; Mégraud, F. Microbiota and gastric cancer. *Semin. Cancer Biol.* **2022**, *86*, 11–17. [CrossRef] [PubMed]
6. Amon, P.; Sanderson, I. What is the microbiome? *Arch. Dis. Child. Educ. Pract. Ed.* **2017**, *102*, 258–261. [CrossRef]
7. Davenport, E.R.; Sanders, J.G.; Song, S.J.; Amato, K.R.; Clark, A.G.; Knight, R. The human microbiome in evolution. *BMC Biol.* **2017**, *15*, 127. [CrossRef]
8. Iavarone, I.; Greco, P.F.; La Verde, M.; Morlando, M.; Torella, M.; de Franciscis, P.; Ronsini, C. Correlations between Gut Microbial Composition, Pathophysiological and Surgical Aspects in Endometriosis: A Review of the Literature. *Medicina* **2023**, *59*, 347. [CrossRef]

9. Zhang, M.; Hu, R.; Huang, Y.; Zhou, F.; Li, F.; Liu, Z.; Geng, Y.; Dong, H.; Ma, W.; Song, K.; et al. Present and Future: Crosstalks Between Polycystic Ovary Syndrome and Gut Metabolites Relating to Gut Microbiota. *Front. Endocrinol.* **2022**, *13*, 933110. [CrossRef]
10. Turnbaugh, P.J.; Ley, R.E.; Hamady, M.; Fraser-Liggett, C.M.; Knight, R.; Gordon, J.I. The Human Microbiome Project. *Nature* **2007**, *449*, 804–810. [CrossRef]
11. Clarridge, J.E. Impact of 16S rRNA gene sequence analysis for identification of bacteria on clinical microbiology and infectious diseases. *Clin. Microbiol. Rev.* **2004**, *17*, 840–862. [CrossRef] [PubMed]
12. Cho, I.; Blaser, M.J. The human microbiome: At the interface of health and disease. *Nat. Rev. Genet.* **2012**, *13*, 260–270. [CrossRef] [PubMed]
13. Franasiak, J.M.; Scott, R.T. Endometrial microbiome. *Curr. Opin. Obstet. Gynecol.* **2017**, *29*, 146–152. [CrossRef] [PubMed]
14. Toson, B.; Simon, C.; Moreno, I. The endometrial microbiome and its impact on human conception. *Int. J. Mol. Sci.* **2022**, *23*, 485. [CrossRef]
15. Peric, A.; Weiss, J.; Vulliemoz, N.; Baud, D.; Stojanov, M. Bacterial colonization of the female upper genital tract. *Int. J. Mol. Sci.* **2019**, *20*, 3405. [CrossRef] [PubMed]
16. Canha-Gouveia, A.; Pérez-Prieto, I.; Rodríguez, C.M.; Escamez, T.; Leonés-Baños, I.; Salas-Espejo, E.; Prieto-Sánchez, M.T.; Sánchez-Ferrer, M.L.; Coy, P.; Altmäe, S. The female upper reproductive tract harbors endogenous microbial profiles. *Front. Endocrinol.* **2023**, *14*, 1096050. [CrossRef]
17. Moreno, I.; Garcia-Grau, I.; Perez-Villaroya, D.; Gonzalez-Monfort, M.; Bahçeci, M.; Barrionuevo, M.J.; Taguchi, S.; Puente, E.; Dimattina, M.; Lim, M.W.; et al. Endometrial microbiota composition is associated with reproductive outcome in infertile patients. *Microbiome* **2022**, *10*, 1. [CrossRef]
18. Medina-Bastidas, D.; Camacho-Arroyo, I.; García-Gómez, E. Current findings in endometrial microbiome: Impact on uterine diseases. *Reproduction* **2022**, *163*, R81–R96. [CrossRef]
19. Punzón-Jiménez, P.; Labarta, E. The impact of the female genital tract microbiome in women health and reproduction: A review. *J. Assist. Reprod. Genet.* **2021**, *38*, 2519–2541. [CrossRef] [PubMed]
20. Franasiak, J.M.; Werner, M.D.; Juneau, C.R.; Tao, X.; Landis, J.; Zhan, Y.; Treff, N.R.; Scott, R.T. Endometrial microbiome at the time of embryo transfer: Next-generation sequencing of the 16S ribosomal subunit. *J. Assist. Reprod. Genet.* **2016**, *33*, 129–136. [CrossRef] [PubMed]
21. Slatko, B.E.; Gardner, A.F.; Ausubel, F.M. Overview of Next-Generation Sequencing Technologies. *Curr. Protoc. Mol. Biol.* **2018**, *122*, e59. [CrossRef]
22. Kitaya, K.; Nagai, Y.; Arai, W.; Sakuraba, Y.; Ishikawa, T. Characterization of microbiota in endometrial fluid and vaginal secretions in infertile women with repeated implantation failure. *Mediat. Inflamm.* **2019**, *2019*, 4893437. [CrossRef]
23. Ichiyama, T.; Kuroda, K.; Nagai, Y.; Urushiyama, D.; Ohno, M.; Yamaguchi, T.; Nagayoshi, M.; Sakuraba, Y.; Yamasaki, F.; Hata, K.; et al. Analysis of vaginal and endometrial microbiota communities in infertile women with a history of repeated implantation failure. *Reprod. Med. Biol.* **2021**, *20*, 334–344. [CrossRef]
24. Oberle, A.; Urban, L.; Falch-Leis, S.; Ennemoser, C.; Nagai, Y.; Ashikawa, K.; Ulm, P.A.; Hengstschläger, M.; Feichtinger, M. 16S rRNA long-read nanopore sequencing is feasible and reliable for endometrial microbiome analysis. *Reprod. Biomed. Online* **2021**, *42*, 1097–1107. [CrossRef]
25. Fujii, S.; Oguchi, T. Age-and endometrial microbiota-related delay in development of endometrial receptivity. *Reprod. Med. Biol.* **2023**, *22*, e12523. [CrossRef]
26. Sezer, O.; Soyer Çalışkan, C.; Celik, S.; Kilic, S.S.; Kuruoglu, T.; Unluguzel Ustun, G.; Yurtcu, N. Assessment of vaginal and endometrial microbiota by real-time PCR in women with unexplained infertility. *J. Obstet. Gynaecol. Res.* **2022**, *48*, 129–139. [CrossRef] [PubMed]
27. Lozano, F.M.; Lledó, B.; Morales, R.; Cascales, A.; Hortal, M.; Bernabeu, A.; Bernabeu, R. Characterization of the Endometrial Microbiome in Patients with Recurrent Implantation Failure. *Microorganisms* **2023**, *11*, 741. [CrossRef] [PubMed]
28. Jain, M.; Mladova, E.; Dobychina, A.; Kirillova, K.; Shichanina, A.; Anokhin, D.; Scherbakova, L.; Samokhodskaya, L.; Panina, O. Comparison of microbial profiles and viral status along the vagina-cervix-endometrium continuum of infertile patients. *Syst. Biol. Reprod. Med.* **2023**, *69*, 310–319. [CrossRef] [PubMed]
29. Miyagi, M.; Mekaru, K.; Tanaka, S.E.; Arai, W.; Ashikawa, K.; Sakuraba, Y.; Nakamura, R.; Oishi, S.; Akamine, K.; Aoki, Y. Endometrial and vaginal microbiomes influence assisted reproductive technology outcomes. *J. Bras. Reprod. Assist.* **2023**, *27*, 267–281. [CrossRef] [PubMed]
30. Reschini, M.; Benaglia, L.; Ceriotti, F.; Borroni, R.; Ferrari, S.; Castiglioni, M.; Guarneri, D.; Porcaro, L.; Vigano', P.; Somigliana, E.; et al. Endometrial microbiome: Sampling, assessment, and possible impact on embryo implantation. *Sci. Rep.* **2022**, *12*, 8467. [CrossRef] [PubMed]
31. Cariati, F.; Carotenuto, C.; Bagnulo, F.; Pacella, D.; Marrone, V.; Paolillo, R.; Catania, M.R.; Di Girolamo, R.; Conforti, A.; Strina, I.; et al. Endometrial microbiota profile in in-vitro fertilization (IVF) patients by culturomics-based analysis. *Front. Endocrinol.* **2023**, *14*, 1204729. [CrossRef] [PubMed]
32. Diaz-martínez, M.D.C.; Bernabeu, A.; Lledó, B.; Carratalá-munuera, C.; Quesada, J.A.; Lozano, F.M.; Ruiz, V.; Morales, R.; Llácer, J.; Ten, J.; et al. Impact of the vaginal and endometrial microbiome pattern on assisted reproduction outcomes. *J. Clin. Med.* **2021**, *10*, 4063. [CrossRef] [PubMed]

33. Riganelli, L.; Iebba, V.; Piccioni, M.; Illuminati, I.; Bonfiglio, G.; Neroni, B.; Calvo, L.; Gagliardi, A.; Levrero, M.; Merlino, L.; et al. Structural Variations of Vaginal and Endometrial Microbiota: Hints on Female Infertility. *Front. Cell. Infect. Microbiol.* **2020**, *10*, 350. [CrossRef]
34. Zou, Y.; Liu, X.; Chen, P.; Wang, Y.; Li, W.; Huang, R. The endometrial microbiota profile influenced pregnancy outcomes in patients with repeated implantation failure: A retrospective study. *J. Reprod. Immunol.* **2023**, *155*, 103782. [CrossRef] [PubMed]
35. Terzic, M.M.; Aimagambetova, G.; Terzic, S.; Norton, M.; Bapayeva, G.; Garzon, S. Current role of Pipelle endometrial sampling in early diagnosis of endometrial cancer. *Transl. Cancer Res.* **2020**, *9*, 7716–7724. [CrossRef]
36. Vitale, S.G.; Ferrari, F.; Ciebiera, M.; Zgliczyńska, M.; Rapisarda, A.M.C.; Vecchio, G.M.; Pino, A.; Angelico, G.; Knafel, A.; Riemma, G.; et al. The role of genital tract microbiome in fertility: A systematic review. *Int. J. Mol. Sci.* **2022**, *23*, 180. [CrossRef]