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Analysis of the endometrial microbiome and its impact on human reproduction

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Congresses

Congress 1

Title of the work: *In vitro* model of bacterial colonization for the study of the effect of the endometrial microbiota on the reproductive function

Authors: Moreno, I., Garcia-Grau, I., Silva, A., Vilella, F., Valbuena, D., Ramon, D., & Simon, C.

Name of the conference: 64th Annual Scientific Meeting of the Society for Reproductive Investigation (SRI)

Date of event: March 15-18, 2017

Place: Orlando, Florida (USA)

Type of presentation: Oral

Congress 2

Title of the work: Sensibility and specificity of the molecular vs classical diagnosis of chronic endometritis

Authors: Garcia-Grau, I., Cicinelli, E., Gonzalez, M., Vilella, F., Valbuena, D., Simon, C., & Moreno, I.

Name of the conference: 33rd Annual Meeting of European Society for Human Reproduction and Embryology (ESHRE)

Date of event: July 2-5, 2017

Place: Geneva (Switzerland)

Type of presentation: Poster

Congress 3

Title of the work: Endometrial microbiome sequencing, a new tool for the diagnosis of chronic endometritis

Authors: Garcia-Grau, I., Cicinelli, E., Gonzalez-Monfort, M., Valbuena, D., Moreno, I*., & Simón, C*.

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Name of the conference: 4th Biomarker Meeting in Personalized Reproductive Medicine

Date of event: April 12-13, 2018

Place: Valencia (Spain)

Type of presentation: Poster

Congress 4

Title of the work: Diagnóstico molecular de la endometritis crónica en pacientes infértiles

Authors: Garcia-Grau, I., Cicinelli, E., Gonzalez-Monfort, M., Bau, D., Vilella, F., Valbuena, D., Moreno, I*., & Simón, C*.

Name of the conference: 32° Congreso Nacional de la Sociedad Española de Fertilidad (SEF)

Date of event: May 16-18, 2018

Place: Madrid (Spain)

Type of presentation: Oral

Congress 5

Title of the work: Selection and validation of *Lactobacillus rhamnosus* CECT 8800 as an optimal probiotic strain for endometrial health

Authors: Chenoll, E*., Moreno, I*., Sánchez, M., Garcia-Grau, I., Silva, A., González-Monfort, M., Genovés, S., Vilella, F., Simón, C., Seco-Durbán, C., & Ramón, D.

Name of the conference: X Workshop of Sociedad Española de Probióticos y Prebióticos (SEPyP)

Date of event: February 6-8, 2019

Place: Gran Canaria (Spain)

Type of presentation: Poster

* These authors contributed equally to this article

Congress 6

Title of the work: Endometrial microbiome: a new frontier in reproductive medicine

Authors: Garcia-Grau, I.

Name of the conference: Société de médecine de la reproduction (SMR)

Date of event: June 5-7, 2019

Place: Aix-en-Provence (France)

Type of presentation: Lecture

Congress 7

Title of the work: The endometrial microbiome of clinical miscarriage, ectopic pregnancy and during early pregnancy in a successful live-birth

Authors: Garcia-Grau, I., Moreno, I., Perez-Villaroya, D., Bau, D., Gonzalez-Monfort, M., Vilella, F., & Simon, C.

Name of the conference: 75th American Society of Reproductive Medicine Scientific Congress (ASRM)

Date of event: October 12-16, 2019

Place: Philadelphia, Pennsylvania (USA)

Type of presentation: Poster

Congress 8

Title of the work: Diferencias en el microbioma endometrial de un aborto clínico, embarazo ectópico y embarazo a término con recién nacido vivo

Authors: Garcia-Grau, I., Moreno, I., Perez-Villaroya, D., Bau, D., Gonzalez-Monfort, M., Vilella, F., & Simon, C.

Name of the conference: X Congreso Asociación para el Estudio de la Biología de la Reproducción (ASEBIR)

Date of event: October 23-25, 2019

Place: Cáceres (Spain)

Type of presentation: Poster

* These authors contributed equally to this article

Congress 9

Title of the work: Upper genital tract microbiome in reproduction, does it matter?

Authors: García-Grau, I.

Name of the conference: 13 Congress of the European Society of Gynecology (ESG)

Date of event: October 13-19, 2019

Place: Vienna (Austria)

Type of presentation: Lecture

Congress 10

Title of the work: Composition of the endometrial microbiome is associated to reproductive outcomes in IVF patients

Authors: Moreno, I., Garcia-Grau, I., Bau, D., Perez-Vilarrolla, D., Gonzalez-Monfort, M., Valbuena, D., Vilella, F., & Simon, C.

Name of the conference: 36th Annual Meeting of of European Society for Human Reproduction and Embryology (ESHRE)

Date of event: July 5-8, 2020

Place: Virtual

Type of presentation: Oral

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RESUMEN

Introducción

El ser humano es un organismo holobionte que a lo largo de la evolución ha vivido en relación simbiótica con los microorganismos que le habitan (Theis et al., 2016). La mayoría de las comunidades microbianas coexisten en relaciones sinérgicas con los humanos y ejercen funciones esenciales en su organismo: influyen en la función metabólica, entrenan al sistema inmunitario y modulan las interacciones farmacológicas (Grice & Segre, 2012). Sin embargo, estas interacciones pueden convertirse en una relación parasitaria, predisponiendo al huésped a diferentes condiciones patológicas (Knight et al., 2017).

Los microorganismos que habitan el cuerpo humano se han identificado tradicionalmente por microscopía y métodos dependientes de cultivo. No obstante, el cultivo microbiano puede proporcionar una visión sesgada de la composición microbiana ya que se ha estimado que entre el 20% y el 60% de las bacterias no son cultivables en condiciones estándar de laboratorio (Peterson et al., 2009). La aparición de las tecnologías moleculares, especialmente la secuenciación masiva, ha permitido realizar una evaluación global de la composición bacteriana. Principalmente, se han establecido firmas bacterianas a través de la secuenciación del gen que codifica para la subunidad 16S del ARN ribosómico (ARNr 16S), el cual tiene secuencias que se han ido diversificando hasta ser específicas de especie. Actualmente, las nuevas técnicas de secuenciación de genomas completos (WMS, del inglés whole metagenome sequencing) se utilizan cada vez más para describir el microbioma humano. Esta tecnología analiza todos los genes presentes en la muestra y proporciona información sobre su función, las rutas metabólicas codificadas, la estructura y organización del genoma, y la estructura y función de la comunidad microbiana (Roumpeka et al., 2017).

Según se determinó en el proyecto del microbioma humano, el microbioma urogenital femenino representa aproximadamente el 9% de nuestro microbioma y en condiciones fisiológicas está dominado principalmente por *Lactobacillus* spp. (Peterson et al., 2009). La gran mayoría de los estudios que caracterizan el microbioma del tracto genital analizan muestras del epitelio cervico-vaginal ya que hasta principios del siglo XX se consideraba que el útero era un órgano estéril (Tissier, 1900). Gracias a los avances en las técnicas de secuenciación, se ha confirmado la presencia de bacterias más allá de la vagina, incluyendo el útero, las trompas de Falopio y los ovarios (Koedooderet al., 2019). Recientemente se ha descrito que el tracto genital femenino es un sistema abierto, con una microbiota continua que disminuye gradualmente en abundancia y aumenta en diversidad bacteriana cuando se avanza desde la vagina hacia el útero (Chen et al., 2017), dando lugar a microbiomas de baja biomasa (O'Callaghan et al., 2020).

Los estudios publicados hasta la fecha sugieren que las bacterias más abundantes de la microbiota endometrial pertenecen a los filos: Firmicutes, Bacteriodetes, Proteobacteria y Actinobacteria (Baker et al., 2018). Dentro de estos filos se han encontrado varios géneros reportados previamente en el tracto genital; por ejemplo, Firmicutes contiene géneros como *Lactobacillus*, *Streptococcus* y *Staphylococcus*; Bacteroidetes géneros como *Prevotella*; Proteobacteria contiene Enterobacterias como *Escherichia coli* y *Klebsiella pneumoniae*; y Actinobacteria contiene géneros como *Bifidobacterium* y *Gardnerella*. Como sucede en la vagina, la mayoría de los estudios han identificado a *Lactobacillus* como el género más prominente del microbioma uterino (Fang et al., 2016; Franasiak et al., 2016; Hashimoto & Kyono, 2019; Khan et al., 2016; Kitaya et al., 2019; Kyono et al., 2019; Kyono et al., 2018; Miles et al., 2017; Moreno et al., 2018; Moreno et al., 2016; Pelzer et al., 2018; Tao et al., 2017; Wee et al., 2018), aunque la composición de la microbiota identificada varía mucho entre los estudios publicados, por lo que no está claro qué bacterias son miembros genuinos del microbioma uterino o colonizadores transitorios.

En los últimos años, se ha analizado la microbiota uterina para evaluar el posible impacto de las bacterias endometriales en los resultados reproductivos. Durante el siglo XX varios grupos evaluaron por cultivo la flora endometrial de la punta del catéter de transferencia de embriones y relacionaron el aislamiento de *Lactobacillus* spp. con mejores posibilidades de éxito en los tratamientos de reproducción asistida (TRA) (Moore et al., 2000, Salim et al., 2002, Verstraelen & Senok, 2005). Por el contrario, el aislamiento de patógenos endometriales, como *E. coli*, *Streptococcus* spp., Enterobacteriaceae, *Enterococcus* spp., *Staphylococcus* spp. y bacterias Gram-negativas, se asoció con una disminución significativa de las tasas de implantación y embarazo (Egbase et al., 1996, Fanchin et al., 1998, Moore et al., 2000, Salim et al., 2002, Selman et al., 2007).

Recientemente, varios estudios han utilizado tecnologías de secuenciación para investigar el impacto del microbioma endometrial. Franasiak y colaboradores caracterizaron el microbioma endometrial de la punta del catéter de transferencia en 33 pacientes sometidas a fecundación *in vitro* (FIV) pero no encontraron una correlación estadísticamente significativa entre el perfil bacteriano y los resultados reproductivos (Franasiak et al., 2016). En el mismo año, Moreno y colaboradores analizaron el impacto del microbioma endometrial en una cohorte de 35 pacientes con fallo repetido de implantación que presentaban un endometrio receptivo analizado mediante la herramienta de análisis de receptividad endometrial ERA (del inglés Endometrial Receptivity Analysis). Las pacientes con un microbioma no dominado por *Lactobacillus* (< 90%) y por tanto una mayor presencia de disbióticos y/o patógenos presentaron peores tasas de implantación, embarazo, y nacidos vivos en comparación con pacientes con un microbioma dominado por *Lactobacillus* (≥ 90%) (Moreno et al., 2016). En la misma línea, Kyono y colaboradores analizaron el microbioma endometrial de 92 pacientes de FIV, encontrándose mejores tasas de embarazo en el grupo dominado por *Lactobacillus*. En este caso utilizaron como punto de corte el 80% de *Lactobacillus* y consideraron el género *Bifidobacterium*

como una bacteria favorable para la implantación (Kyono et al., 2018). Usando esta misma clasificación, Hashimoto y colaboradores analizaron la microbiota endometrial en el momento de la transferencia de embriones en 99 pacientes de FIV, pero en este estudio las tasas de embarazo y las tasas de aborto espontáneo fueron comparables entre pacientes con endometrio eubiótico y disbiótico (Hashimoto & Kyono, 2019).

Para sacar conclusiones y poder comparar con éxito los estudios publicados se deben considerar las diferencias en el diseño de los estudios: el tamaño de la cohorte y la demografía de las pacientes, el tipo y método de recolección de muestras, la extracción de ADN, los métodos de amplificación y secuenciación, y el análisis bioinformático de los datos (Baker et al., 2018; Benner et al., 2018). Además, el microbioma uterino es un microbioma de baja biomasa difícil de caracterizar, por lo que todavía quedan por determinar las bacterias comensales que se encuentran en condiciones fisiológicas de las bacterias del ruido de fondo (O'Callaghan et al., 2020).

Además de su implicación en los resultados reproductivos, también se está investigando el impacto de la microbiota endometrial en varias causas de infertilidad como la endometritis crónica (EC). La EC es un trastorno inflamatorio que aparece de forma asintomática en el 28% de pacientes infértiles con etiología desconocida, 14%-41% de pacientes con fallo repetido de implantación y 8%-28% de pacientes con abortos de repetición (Kitaya et al., 2018). La principal causa de EC es la infección microbiana de la cavidad uterina por bacterias comunes como: *Enterococcus faecalis*, Enterobacteriaceae, *Streptococcus spp.*, *Staphylococcus spp.*, *Gardnerella vaginalis* y *Mycoplasma spp.*, así como patógenos genitales asociados con infecciones de transmisión sexual como *Ureaplasma urealyticum*, *Chlamydia trachomatis*, y *Neisseria gonorrhoeae* (Cicinelli et al., 2008; Cicinelli et al., 2009; Kitaya et al., 2017).

El método de referencia para el diagnóstico de la EC es la histología, con la identificación de células plasmáticas en el estroma endometrial mediante inmunotinción del marcador plasmacitario CD138 (también conocido como Sindecan-1) (Bayer-Garner et al., 2004). La histeroscopia de la cavidad uterina también se considera una técnica diagnóstica fiable, con una precisión del 93.4% en comparación con los hallazgos histológicos. Los principales signos histeroscópicos incluyen micropólipos, edema estromal, e hiperemia focal o difusa (Cicinelli et al., 2005). Por último, el cultivo microbiano es otro de los métodos de diagnóstico clásico utilizados. En el 75% de las mujeres con EC confirmada histológicamente se obtienen cultivos endometriales positivos con patógenos comunes del tracto reproductivo (Cicinelli et al., 2014). Sin embargo, el diagnóstico de EC es bastante complejo ya que los métodos clásicos tienen algunas limitaciones que afectan a su diagnóstico (Moreno & Simon, 2019). El análisis microbiano basado en herramientas moleculares como la PCR en tiempo real (RT-PCR, del inglés real-time polymerase chain reaction) y/o la secuenciación del gen ARNr 16S podría permitir la identificación de microorganismos patógenos, tanto cultivables como no cultivables, y podría ayudar a personalizar los tratamientos y mejorar el manejo clínico de pacientes infértiles.

Existen evidencias de que la disbiosis endometrial puede ser una causa emergente del fallo de implantación y pérdida de embarazo, aunque el conocimiento de cómo las diferentes especies bacterianas pueden influir en la homeostasis del útero y afectar los resultados reproductivos todavía es limitado. Para tratar de evaluar el impacto de múltiples taxones bacterianos en el huésped y desarrollar intervenciones más efectivas, se han utilizado varios modelos *in vitro* e *in vivo* (Peric et al., 2019). Además, el estudio de casos clínicos también puede mejorar nuestra comprensión de la etiología, la patogénesis y el tratamiento de infecciones endometriales. En resumen, conocer los procesos subyacentes que impactan en los resultados de embarazo podría ayudar a desarrollar nuevas herramientas preventivas, diagnósticas y terapéuticas personalizadas para las mujeres que intentan concebir.

Antecedentes

Los resultados obtenidos en un estudio piloto de nuestro grupo de investigación desafiaron el dogma previo de esterilidad de la cavidad uterina y demostraron la existencia de una microbiota endometrial altamente estable durante la adquisición de la receptividad endometrial. Además, pacientes con una microbiota endometrial con < 90% de *Lactobacillus* presentaron peores tasas de implantación, embarazo, y nacidos vivos (Moreno et al., 2016). Este hallazgo, junto con otras publicaciones de la comunidad científica, han agregado una nueva dimensión microbiológica al proceso reproductivo.

Hipótesis

Siguiendo los resultados preliminares, planteamos la hipótesis de que la presencia de patógenos bacterianos en el útero tiene consecuencias negativas en la salud reproductiva. Por ello, consideramos que una mejor comprensión del microbioma endometrial puede ayudar en el manejo clínico de pacientes infértiles, a través de la mejora de las herramientas de diagnóstico y la personalización de los tratamientos.

Objetivos

El objetivo principal de esta tesis fue caracterizar en profundidad el microbioma endometrial y su impacto funcional en la salud reproductiva de mujeres sometidas a tratamientos de reproducción asistida. Con este fin, se plantearon los siguientes objetivos específicos:

1. Evaluar la presencia de patógenos de endometritis crónica en muestras de endometrio mediante métodos moleculares objetivos y comparar los resultados con técnicas diagnósticas clásicas (histología, histeroscopia y cultivo microbiano).

2. Caracterizar el perfil del microbioma endometrial en muestras de líquido y biopsia mediante secuenciación del gen ARN ribosómico 16S y su impacto potencial con metagenómica funcional.

3. Confirmar el impacto del microbioma endometrial sobre la implantación embrionaria y el embarazo mediante un estudio prospectivo observacional.

4. Estudiar el impacto funcional de las infecciones endometriales utilizando un modelo *in vitro* de disbiosis endometrial.

Materiales y métodos

Diagnóstico de infecciones endometriales por métodos moleculares

Muestras endometriales de 113 pacientes infértiles sometidas a diagnóstico clásico de EC mediante histología, histeroscopia y/o cultivo microbiano se evaluaron de forma ciega mediante RT-PCR para detectar la presencia de las bacterias más comunes de la enfermedad (*C. trachomatis*, *Enterococcus* spp., *E. coli*, *G. vaginalis*, *K. pneumoniae*, *M. hominis*, *N. gonorrhoeae*, *Staphylococcus* spp., y *Streptococcus* spp.). Posteriormente, se evaluó la sensibilidad, precisión, valor predictivo positivo y negativo, y tasas de falsos positivos y negativos del análisis molecular frente a las técnicas de diagnóstico clásico. Para evaluar la especificidad del método molecular también se analizaron por RT-PCR muestras de endometrio de 10 controles negativos. Finalmente, para confirmar los resultados obtenidos por RT-PCR, se analizó el microbioma endometrial de las pacientes con diagnóstico clásico concordante utilizando la secuenciación del gen ARNr 16S.

Caracterización del microbioma endometrial en mujeres infértiles

Se diseñó un estudio de cohortes, internacional, observacional, multicéntrico y competitivo. Para ello se reclutaron pacientes infértiles con indicación del test ERA que fueran a recibir una transferencia embrionaria personalizada con blastocistos

congelados (día 5/día 6). El mismo día que se indica la toma de la biopsia endometrial (BE) para la prueba ERA, en el día 5 de un ciclo de terapia de reemplazo hormonal, se aspiró una muestra de líquido endometrial (LE). En aquellas pacientes en las que se requirió una segunda BE para la recomendación de la transferencia personalizada, también se repitió la recolección de la muestra de LE. Ambos tipos de muestra se utilizaron para evaluar el microbioma endometrial mediante secuenciación del gen ARNr 16S.

Para estudiar el impacto de la microbiota endometrial en los resultados reproductivos, se compararon los perfiles bacterianos de pacientes que tuvieron nacidos vivos con los de mujeres sin embarazo, embarazo bioquímico y aborto clínico espontáneo. En concreto, se estableció la microbiota normal como aquella identificada en las mujeres con nacidos vivos (considerando un intervalo de confianza del 95%) y se evaluaron las bacterias que excedían este intervalo en cada uno de los grupos analizados. Además, para analizar en profundidad la microbiota del LE y BE, se realizaron análisis de asociación bacteriana para cada tipo de muestra y para cada resultado reproductivo.

Evaluación funcional de infecciones endometriales mediante un modelo in vitro

Para investigar el efecto de bacterias disbióticas o patógenas sobre el endometrio se diseñó un modelo *in vitro* de colonización bacteriana en células epiteliales endometriales humanas. Las células se colonizaron con bacterias previamente asociadas con infecciones del tracto reproductivo (*A. vaginae*, *G. vaginalis*, *P. acnes* y *S. agalactiae*) solas o en combinación con la cepa probiótica *Lactobacillus rhamnosus* BPL005 durante 18 h en condiciones anaeróbicas. El sobrenadante del cultivo se usó para medir el pH y la concentración de moléculas inflamatorias, mientras que las células bacterianas se usaron para evaluar por RT-PCR el potencial inhibidor de *Lactobacillus* sobre las poblaciones patógenas seleccionadas.

Evaluación funcional de la microbiota endometrial en pacientes infértiles

Para investigar el impacto funcional de la microbiota endometrial en la infertilidad se realizó un seguimiento microbiológico a dos pacientes infértiles con problemas reproductivos. En ambas pacientes, se usó la secuenciación de ARNr 16S para evaluar taxonómicamente la composición bacteriana, mientras que se utilizó WMS para comprender mejor la funcionalidad de las bacterias detectadas, la resistencia a los antibióticos y su impacto en la salud reproductiva.

El primer caso clínico describe la microbiota endometrial en un total de seis muestras de LE tomadas longitudinalmente durante 18 meses a una paciente con fallos reproductivos repetidos (un embarazo ectópico y dos abortos clínicos). Por otro lado, el segundo caso clínico describe la microbiota endometrial de dos muestras de LE de la misma paciente. La primera tomada antes de una transferencia de embriones que resultó en un aborto espontáneo y, la segunda, de manera incidental, en la cuarta semana de gestación que terminó en un recién nacido vivo.

Resultados

Diagnóstico de infecciones endometriales por métodos moleculares

De las 113 pacientes sometidas a diagnóstico de EC por métodos clásicos, 95 muestras endometriales fueron finalmente analizadas por RT-PCR para evaluar la presencia de patógenos asociados a la enfermedad (set 1) **(Figura 19)**. Usando este análisis molecular, 42 de las muestras analizadas fueron negativas para EC (44.21%), mientras que 53 fueron positivas (55.79%). De las 65 muestras utilizadas para la comparación con las técnicas clásicas (set 2), 27 fueron negativas para EC (41.54%) mientras que 38 fueron positivas (58.46%) **(Figura 23)**.

La microbiología molecular coincidió con el diagnóstico histológico en 30 muestras (precisión del 46,15%), con el diagnóstico histeroscópico en 38 muestras

(precisión del 58.46%) y con el cultivo microbiano en 37 muestras (precisión del 56,92%). Al considerar los microorganismos que no se incluyeron en el diagnóstico molecular o que no son cultivables, la precisión se incrementó al 66,15% **(Tabla 4 y 5)**. De este conjunto de 65 pacientes, solo 27 presentaron resultados consistentes de histología + histeroscopia (tasa de concordancia del 41,54%), con 25 resultados doblemente positivos y 2 doblemente negativos. De estos, la RT-PCR mostró resultados concordantes en 15 casos, con una precisión del 55,56%. Curiosamente, la detección de ADN bacteriano por RT-PCR siempre coincidió con la histeroscopia, mientras que el diagnóstico negativo de EC coincidió con la histología **(Tabla 4)**.

Finalmente, de las 65 pacientes analizadas solo 13 (20%) tuvieron resultados concordantes en los tres métodos clásicos. El análisis molecular de estas 13 muestras endometriales (set 3) mostró que 4 fueron negativas para EC (30,77%) mientras que 9 fueron positivas (69,23%) **(Figura 23)**. La única paciente diagnosticada como negativa según los métodos clásicos también fue negativa para EC según la evaluación molecular **(Figura 24)**. De las 12 pacientes restantes, 9 también fueron positivas por RT-PCR, y en 5 de estos casos se detectaron los mismos microorganismos que en el cultivo. Además, en 2 casos se detectó *G. vaginalis* junto con otros patógenos, proporcionando información adicional sobre bacterias difíciles de cultivar. En resumen, 10 de los 13 casos analizados presentaron resultados similares a la RT-PCR con una precisión del 76,92% **(Tabla 5)**. En todos los sets de datos examinados, el patógeno más detectado fue *Streptococcus spp.*, seguido de *Enterococcus spp.* y *G. vaginalis*, mientras que *C. trachomatis* y *N. gonorrhoeae* no se detectaron en las muestras analizadas **(Figura 23)**.

Para corroborar los resultados obtenidos en la evaluación molecular, se analizó por secuenciación ARNr 16S el microbioma de estas 13 pacientes. Los resultados confirmaron la detección de ADN bacteriano en 12 muestras, detectando bacterias previamente asociadas al tracto reproductivo **(Tabla 6)**. Por otro lado, se evaluó el

porcentaje de *Lactobacillus*, mostrando un bajo porcentaje en todas las muestras con un diagnóstico de EC positivo según los métodos clásicos, con la excepción de un caso, y un alto porcentaje de *Lactobacillus* en la muestra que fue negativa para el diagnóstico de EC **(Figura 25)**. Estos resultados confirmaron los datos obtenidos con en el análisis molecular propuesto, ya que coincidieron con el cultivo microbiano en 9 casos mientras que con la RT-PCR en 11 casos, mostrando una precisión del 75,00% y 91,67% respectivamente.

Por último, dado que de las 65 muestras analizadas solo una paciente presentó resultados negativos por los tres métodos clásicos, se quiso evaluar la eficacia de la RT-PCR para detectar casos negativos analizando muestras de 10 sujetos control. En todas las muestras los resultados fueron negativos para las nueve bacterias patógenas testadas, sin embargo, la RT-PCR pudo detectar cantidades muy pequeñas de ADN de *G. vaginalis y M. hominis*, aunque sus niveles estaban por debajo del umbral de los controles positivos **(Tabla 7)**. Estos datos ponen de manifiesto la eficacia de la microbiología molecular para discriminar entre casos positivos y negativos de EC, en función de la detección de los patógenos que comúnmente causan la enfermedad.

Caracterización del microbioma endometrial en mujeres infértiles

De las 342 pacientes reclutadas para evaluar el microbioma endometrial, 198 (57,89%) quedaron embarazadas, mientras que 144 (42,10%) no quedaron embarazadas. De las mujeres embarazadas, 141 (41,23%) tuvieron un nacido vivo, 27 (7,89%) un embarazo bioquímico y 28 (8,18%) un aborto espontáneo. Además, 2 pacientes sufrieron un embarazo ectópico, pero este resultado reproductivo no se consideró para el análisis final debido al pequeño tamaño muestral **(Figura 26)**.

Dado que el endometrio presenta una microbiota de baja abundancia, en primer lugar se analizó la concentración de los amplicones para clasificar las muestras en

detectables y no detectables. A pesar de partir de cantidades equivalentes de ADN extraído, las muestras con microbiota detectable mostraron una mayor concentración de amplicón, mientras que en las muestras con microbiota no detectable la concentración fue equivalente a la de los blancos **(Figura 27)**. Finalmente, 208 muestras de LE y 190 muestras de BE se clasificaron como detectables y se incluyeron en el análisis de asociación de la microbiota endometrial con los resultados reproductivos.

Lactobacillus fue el género mayoritario tanto en muestras de LE como de BE. Además, en ambos tipos de muestras se detectaron otros géneros bacterianos como *Anaerococcus*, *Atopobium*, *Bifidobacterium*, *Corynebacterium*, *Gardnerella*, *Haemophilus*, *Microbacterium*, *Prevotella*, *Propionibacterium*, *Staphylococcus* y *Streptococcus*. Tres taxones fueron exclusivos de muestras de LE (*Streptomyces*, *Clostridium* y *Chryseobacterium*), mientras que siete taxones fueron únicos de muestras de BE (*Cupriavidus*, *Escherichia*, *Klebsiella*, *Bacillus*, *Finegoldia*, *Micrococcus* y *Tepidimonas*) **(Figura 28)**. Al construir redes bacterianas de co-ocurrencia se observó que la microbiota de la BE era más dispersa que la del LE **(Figura 29)**. Del mismo modo, en las redes construidas para cada resultado reproductivo, se observó que las redes de pacientes con nacidos vivos eran más densas en comparación con las de pacientes con peores resultados reproductivos, donde el número de nodos no conectados fue mayor **(Figura 30-31)**.

En la microbiota del LE, en las pacientes con nacido vivos observamos que *Lactobacillus* se relacionó negativamente con bacterias patógenas como *Chryseobacterium*, *Staphylococcus* y *Haemophilus*, y positivamente con *Streptomyces*, que a su vez formaba parte de una comunidad compuesta principalmente por bacterias comensales (*Corynebacterium*, *Microbacterium*, *Propionibacterium* y *Clostridium*). En las pacientes que no quedaron embarazadas se observó un comportamiento similar, mientras que en el grupo de pacientes con

embarazo bioquímico y aborto espontáneo estas interacciones desaparecieron, y las redes resultantes quedaron desconectadas y formadas por una comunidad dispersa **(Figura 30)**. En las redes de co-ocurrencia de la BE las conexiones beneficiosas/perjudiciales entre taxones no fueron tan evidentes **(Figura 31)**.

Al analizar la diferencia entre *Lactobacillus* y otros taxones del tracto reproductivo se observó, tanto en muestras de LE como de BE, una mayor abundancia de *Lactobacillus* en el grupo de pacientes con nacidos vivos en comparación con los grupos con resultados reproductivos negativos **(Figura 32A)**. El análisis de probabilidad predictiva mostró una mayor probabilidad de tener una mayor abundancia de *Lactobacillus* en pacientes con un nacido vivo, especialmente para las muestras de LE **(Figura 32B)**.

Cuando se analizó la población bacteriana con relación a los resultados de TRA, en el LE, los taxones con una abundancia significativamente mayor al intervalo de confianza establecido para pacientes con nacidos vivos fueron *Atopobium*, *Bifidobacterium*, *Chryseobacterium*, *Gardnerella* y *Streptococcus* para mujeres no embarazadas, y *Haemophilus* y *Staphylococcus* para mujeres que sufrieron un aborto espontáneo. Por el contrario, los taxones con una distancia significativamente menor al intervalo de confianza fueron *Lactobacillus* y *Microbacterium* para mujeres no embarazadas, y *Lactobacillus* para pacientes con aborto espontáneo.

En las muestras de BE, para mujeres no embarazadas, los taxones con abundancia significativa por encima del intervalo de confianza fueron *Bifidobacterium*, *Gardnerella* y *Klebsiella*, y los taxones con abundancia significativa por debajo del intervalo de confianza fueron *Cupriavidus*, *Finegoldia*, *Lactobacillus* y *Tepidomonas*. Para el resto de las comparaciones, la diferencia observada no alcanzó la significación estadística, posiblemente debido al pequeño número de pacientes **(Figura 33 y 34)**.

Al considerar las principales bacterias patógenas que causan EC, en las muestras de LE se observó un aumento significativo de los géneros *Gardnerella*, *Klebsiella* y *Streptococcus* en mujeres no embarazadas, *Enterococcus* en pacientes con embarazo bioquímico, y *Klebsiella* y *Staphylococcus* en pacientes con aborto espontáneo. En la microbiota de la BE, los niveles de *Gardnerella*, *Neisseria* y *Klebsiella* aumentaron significativamente en mujeres no embarazadas, mientras que la abundancia de *Enterococcus* estuvo por debajo del intervalo de confianza **(Figura 35)**. En las otras categorías reproductivas no se detectaron taxones significativos en las muestras de BE. Curiosamente, *Gardnerella* y *Klebsiella* fueron los únicos patógenos significativamente enriquecidos tanto en muestras de LE como de BE de pacientes que no quedaron embarazadas después de una transferencia embrionaria personalizada.

Evaluación funcional de infecciones endometriales mediante un modelo in vitro

Tras testar varias cepas probióticas obtenidas de aislados vaginales, se seleccionó la cepa *L. rhamnosus* BPL005 para evaluar el impacto de bacterias disbióticas o patógenas en el endometrio y estudiar su potencial papel protector en las infecciones endometriales. Para ello, se usó un modelo de colonización *in vitro* en el que se midió el pH, la secreción de citocinas, quimiocinas y factores de crecimiento, y la evolución de los patógenos testados.

A excepción de las infecciones por *Streptococcus*, los niveles de pH no se redujeron tras la colonización con los patógenos, mientras que en el cocultivo con *L. rhamnosus* BPL005 los niveles de pH cayeron por debajo de pH 5 **(Tabla 10)**. Al analizar la secreción de citocinas, quimiocinas y factores de crecimiento, no se registraron en el medio niveles detectables de GM-CSF, HB-EGF, IFN gamma e IL-1 RI. La cantidad secretada de RANTES no mostró cambios tras la colonización con *L. rhamnosus* BPL005, bacterias patógenas o combinaciones de ellas. En cambio, la colonización de *L. rhamnosus* BPL005 produjo una disminución en IL-6, IL-8 y MCP-

1, que fue mucho más evidente en presencia de patógenos, y presentó valores significativos en el caso del cocultivo con *A. vaginae* (IL-6: disminución de 2 veces, $p < 0.01$; IL-8: disminución de 3.5 veces, $p < 0.001$; MCP-1: disminución de 16.5 veces, $p < 0.001$), *G. vaginalis* (IL- 8: disminución de 2.6 veces, $p < 0.05$; MCP-1: disminución de 16.5 veces, $p < 0.001$), *P. acnes* (MCP-1: disminución de 5 veces, $p < 0.001$) y *S. agalactiae* (IL- 6: disminución de 5 veces, $p < 0,001$). Por otro lado, la adición de *L. rhamnosus* BPL005 al cultivo produjo un aumento generalizado en IL-1RA e IL-1β, mostrando un aumento estadísticamente significativo en presencia de *A. vaginae*, *G. vaginalis* (IL-1RA: 8.9 y un aumento del cambio de 12.5 veces, respectivamente, $p < 0.05$) y *S. agalactiae* (IL-1β: cambio de 2.5 veces, $p < 0.05$) **(Figura 36)**. Finalmente, al cuantificar por RT-PCR la evolución de los patógenos en los ensayos de coinfección, se observó una reducción significativa de los niveles de *P. acnes* y *S. agalactiae* (log_{10} 1.36 células/mL y log_{10} 2.14 células/mL, respectivamente) y una tendencia, aunque no significativa, en *A. vaginae* (log_{10} 0.98 células/mL) y *G. vaginalis* (log_{10} 0.64 células/mL) **(Figura 37)**.

Evaluación funcional de infecciones endometriales en pacientes infértiles

Caso 1: microbiota endometrial en un contexto de fallo reproductivo recurrente

Una mujer de 37 años con infertilidad primaria de tres años de evolución y con antecedentes de fallo reproductivo asistió a la clínica en marzo de 2017 para realizarse un TRA. La paciente se sometió a un seguimiento microbiológico a lo largo de 18 meses donde se evaluó la microbiota endometrial en seis muestras de LE utilizando secuenciación de ARNr 16S **(Figura 38)**.

El primer LE recogido antes de la transferencia personalizada con un embrión euploide mostró una población bacteriana con *Gardnerella* (32.8%), *Pseudoalteromonas* (14.2%), *Bifidobacterium* (8.8%) y *Rhodanobacter* (5.8%), con solo 12.1% de *Lactobacillus*. Después de sufrir un embarazo ectópico con su

respectivo tratamiento antibiótico, se tomaron dos muestras más de LE separadas en el tiempo para analizar la presencia de bacterias patógenas. Los resultados de secuenciación del primer LE revelaron una composición microbiana con: *Lactobacillus* (18.8%), *Gardnerella* (16.5%), *Rhodanobacter* (15.2%) y *Pseudoalteromonas* (9.0%), y los resultados del segundo LE seguían mostrando la presencia *Gardnerella* (28.8%), *Pseudoalteromonas* (16.8%), *Atopobium* (13.4%), *Rhodanobacter* (13.0%) y *Lactobacillus* (0.8%). Posteriormente, la paciente se sometió a una nueva transferencia embrionaria personalizada pero sufrió un aborto clínico en la novena semana de gestación. Tras proporcionarle varios tratamientos antibióticos, se tomó una muestra de LE después de cada uno para evaluar los cambios en la composición microbiana. En ambas muestras, la secuenciación detectó una colonización persistente de *Gardnerella* (21.8% y 46.1%, respectivamente), *Atopobium* (19.7% y 4.1%) y *Bifidobacterium* (0.3% y 15.5%) con niveles subóptimos de *Lactobacillus* (50.2 % y 33. 4%). Finalmente, la paciente sufrió otro aborto clínico en la sexta semana de gestación y la secuenciación del LE analizado en el mismo ciclo de la última transferencia embrionaria confirmó de nuevo la presencia de *Lactobacillus* (48.1%), *Gardnerella* (32.8%), *Bifidobacterium* (6.2%) y *Atopobium* (5.4%) **(Figura 39)**.

Para comprender mejor la abundancia de *G. vaginalis*, su funcionalidad, resistencia a los antibióticos e impacto en la salud reproductiva, se realizó un análisis taxonómico y funcional más completo por WMS. Para este análisis se utilizó la última muestra de LE obtenida siete días antes de la transferencia de embriones que resultó en el tercer fallo reproductivo. El análisis por WMS verificó el perfil disbiótico dominado por *Gardnerella* (86.0%), *Lactobacillus* (8.2%) y *Atopobium* (5.1%) **(Figura 40)**. Las secuencias de *Gardnerella* se compararon con los genomas de un panel de 17 cepas de *G. vaginalis* pertenecientes a diferentes clados (Ahmed et al., 2012). Un total de 613 alineaciones de secuencia se asociaron con genes de estas cepas, la mayoría de ellas fueron coincidentes con el clado 1 (n = 452) y el clado 4 (n = 309)

(Figura 41). Al buscar genes específicos de clado (Balashov et al., 2014), se confirmó la presencia concomitante de múltiples clados de *G. vaginalis*, siendo el clado 4 el más representado, seguido del clado 3 y el clado 1. Los clados predichos en la muestra fueron confirmados por RT-PCR multiplex (Balashov et al., 2014), mostrando una detección positiva de los clados 1 y 4 **(Figura 42)**.

Por último, se realizó un análisis funcional utilizando la base de datos Kyoto Encyclopedia of Genes and Genomes (KEGG). La mayoría de las categorías funcionales detectadas pertenecían a los géneros *Gardnerella*, *Lactobacillus* y *Atopobium* (representando el 66%, 18% y 14% de las lecturas respectivamente). Nueve categorías fueron exclusivas de *Gardnerella*, encontrando vías relacionadas con el metabolismo, la motilidad celular, los procesos de señalización, y el sistema inmunitario **(Figura 43; Tabla 12)**. Además, se evaluó la presencia de genes previamente relacionados con el potencial patogénico de *G. vaginalis* (Yeoman et al., 2010), detectando varios genes relacionados con (i) el sistema de toxina-antitoxina y exclusión competitiva, (ii) la formación de biopelículas y adhesión epitelial, y (iii) factores de virulencia que incluyen citotoxicidad, resistencia antimicrobiana, adquisición de hierro y degradación de mucinas, entre otros **(Tabla 13)**.

<u>Caso 2: microbiota endometrial en el embarazo temprano</u>

Una mujer de 28 años con infertilidad primaria de dos años de evolución con un ciclo previo de FIV sin éxito asistió a la clínica en enero de 2017 para someterse a un TRA. Después de no conseguir un embarazo tras la transferencia embrionaria de dos blastocistos euploides, la paciente se incluyó en un estudio de investigación biomédica donde se analizó la microbiota endometrial en el LE recogido en el momento de la toma de muestra para el test ERA. En mayo, le volvieron a transferir dos blastocistos euploides, sin embargo, la paciente sufrió un aborto espontáneo en la octava semana de gestación. El análisis de los productos de la concepción confirmó que el embrión era cromosómicamente normal con un perfil 46, XX. Dos meses

después de la dilatación y el legrado, la paciente fue a una vista para comenzar un nuevo ciclo y se le tomó una nueva muestra de LE para investigar posibles cambios en la microbiota. Posteriormente, se hizo evidente que la paciente había concebido espontáneamente y tenía un embarazo de cuatro semanas cuando se obtuvo la segunda muestra de LE. El embarazo continuó sin incidentes, y la paciente dio a luz a un varón sano a las 40 semanas de gestación **(Figura 44)**.

La secuenciación ARNr 16S del LE obtenido el ciclo antes del aborto espontáneo mostró un perfil disbiótico con 5% de Actinobacterias, 19% de Firmicutes y 76% de Proteobacterias. Dentro de estos filos, se encontró el 15% de *Lactobacillus* junto con varios patógenos del tracto reproductivo como Enterobacteriaceae (3%), *Streptococcus* (2%), *Pseudomonas* (2%) y *Staphylococcus* (0.8%). Por el contrario, la microbiota de la muestra recolectada en la cuarta semana de embarazo tuvo un perfil con 91% de Firmicutes y 9% de Proteobacteria, siendo *Lactobacillus* la única bacteria presente bajo el filo Firmicutes **(Figura 45)**. El análisis taxonómico realizado por WMS también mostró un perfil disbiótico en el LE obtenido antes del aborto espontáneo y una mayor abundancia de *Lactobacillus* en el LE recolectado durante el embarazo **(Figura 46A)**. Aunque la asignación taxonómica derivada de WMS mostró una mayor diversidad que la secuenciación de ARNr 16S, cuando se combinaron los análisis taxonómicos y funcionales la diversidad microbiana presente en cada muestra se redujo.

El análisis metagenómico mostró que la muestra recolectada antes del aborto espontáneo contenía *Lactobacillus crispatus* (15%) junto con *Propionibacterium* (21%), *Pseudomonas* (10%) y *Streptococcus* (3.5%). En cambio, en la muestra recolectada durante el embarazo exitoso, *Lactobacillus iners* fue la única bacteria encontrada **(Figura 46B)**. El análisis de metagenómica funcional asoció la presencia de *L. iners* con mecanismos de defensa y funciones basales (como traducción, producción de energía y división celular), mientras que la población bacteriana

identificada en el ciclo previo al aborto clínico presentó un patrón funcional heterogéneo caracterizado por transposasas y elementos de inserción típicos de microbiomas genéticamente inestables **(Figure 48)**.

Discusión

La implantación es un proceso complejo que requiere la sincronización de varias funciones biológicas a nivel celular y molecular, siendo necesario el diálogo sincronizado entre un endometrio receptivo y un embrión en desarrollo en la etapa de blastocisto (Simón et al., 2000). Las causas del fallo de implantación y la pérdida de embarazo siguen sin estar claras en muchos casos. En los últimos años, gracias a los avances en las técnicas de secuenciación masiva, se han identificado microorganismos en el útero y se ha visto que pueden tener implicaciones importantes en los resultados reproductivos. La identificación de la disbiosis endometrial como una nueva causa de infertilidad, abre un nuevo campo microbiológico en la evaluación del factor endometrial para tratar de mejorar y personalizar la atención clínica de pacientes infértiles.

Las infecciones bacterianas intrauterinas a menudo pasan desapercibidas en la práctica clínica ya que generalmente son asintomáticas (Greenwood et al., 1981). Además, actualmente no hay estándares de diagnóstico aceptados y su diagnóstico parece depender del método utilizado (Kitaya et al, 2013). Las limitaciones del diagnóstico de EC utilizando técnicas clásicas se evidencian en el primer estudio presentado, en el que solo 13 de las 65 (20%) pacientes analizadas tuvieron resultados concordantes por histología, histeroscopia y cultivo microbiano. Esto se debe principalmente a la contradicción entre los métodos de histología e histeroscopia (58.46% de los casos), ya que se observó que la evaluación histopatológica usualmente infradiagnostica la enfermedad, mientras que la histeroscopia la sobrediagnostica. Ambas técnicas son altamente subjetivas, inespecíficas y dependen de las observaciones del patólogo o cirujano endoscópico.

Además, no pueden discriminar el agente causal de la infección, lo que lleva a la prescripción de terapias de amplio espectro que suelen provocar infecciones recurrentes (Kitaya et al., 2018). Por el contrario, el cultivo microbiano sí que permite identificar los microorganismos presentes en el tejido endometrial, pero los resultados pueden verse sesgados por contaminaciones con bacterias de la piel o del medio ambiente, y por la incapacidad para crecer y aislar bacterias no cultivables.

El estudio presentado evidencia la utilidad de testar mediante técnicas moleculares los microorganismos más comunes involucrados con la EC. La RT-PCR presentó un grado de concordancia del 77%, una sensibilidad del 75% y una especificidad del 100% en las muestras con resultados concordantes por los tres métodos clásicos. Además, la secuenciación ARNr 16S confirmó los diagnósticos obtenidos en la RT-PCR en el 92% de los casos. En general, los resultados indican que la microbiología molecular proporciona resultados similares al conjunto de los tres métodos clásicos, superando el sesgo de usar cualquiera de los métodos por separado.

Para abordar mejoras técnicas y clínicas de esta herramienta y aumentar el número de microorganismos incluidos en el panel, en los siguientes estudios se utilizó la secuenciación del gen bacteriano ARNr 16S. Esta metodología permite distinguir entre especies positivas y negativas para ciertos patógenos o incluso identificar firmas microbianas que puedan estar asociadas a un estado de enfermedad particular. Con este enfoque, se analizó el microbioma endometrial, tanto de muestras de LE como BE, de pacientes infértiles sometidas a TRA con el fin de evaluar su impacto en los resultados reproductivos de estas pacientes.

Los resultados de este estudio mostraron una estrecha relación entre la microbiota del LE y BE, aunque se encontraron algunas diferencias en los géneros detectados entre ambos tipos de muestra. Una posible explicación es que las

bacterias presentes en la superficie del epitelio luminal pueden ser diferentes de las que residen más profundamente en el tejido endometrial, incluido el estroma. Sin embargo, estas diferencias también pueden deberse al procesamiento de las muestras y a los protocolos de extracción de ADN. Anteriormente, solo un estudio había comparado la microbiota endometrial de ambos tipos de muestras, concluyendo también que la composición de la microbiota en el LE no refleja completamente la de la BE, y que el muestreo de ambas puede permitir una visión más completa de la colonización microbiana (Liu et al., 2018).

Los estudios informados hasta la fecha no han llegado a un consenso sobre el perfil de patógenos bacterianos que podría interferir con la implantación embrionaria y el éxito de embarazo. Los resultados de este estudio muestran que una microbiota endometrial desfavorable se asocia con una implantación y embarazo comprometidos. El estudio de asociación bacteriana mostró que las redes de pacientes con nacidos vivos eran más densas y tenían una mayor distribución de nodos en contraste con las redes de pacientes con malos resultados reproductivos. Además, las asociaciones revelaron que *Lactobacillus* generalmente se correlaciona negativamente con bacterias patógenas y positivamente con bacterias comensales, lo cual puede ser importante para la estabilidad del ecosistema. La reducción de *Lactobacillus* y la presencia de bacterias patógenas como *Atopobium*, *Bifidobacterium*, *Chryseobacterium*, *Gardnerella*, *Haemophilus*, *Klebsiella*, *Neisseria*, *Staphylococcus* y *Streptococcus* se asoció a pacientes con malos resultados reproductivos.

Se sabe que *A. vaginae* y *G. vaginalis* estimulan la respuesta inmune innata de las células epiteliales vaginales y contribuyen a la patogénesis de la vaginosis bacteriana (VB) (Libby et al., 2008). Sin embargo, el impacto de estos patógenos no se limita a la vagina, ya que se ha demostrado que mujeres con VB presentan un mayor riesgo de desarrollar biopelículas endometriales con *G. vaginalis* (Swidsinski

et al., 2013). *S. agalactiae*, *K. pneumoniae*, *Enterococcus*, *N. gonorrhoeae*, y en algunos estudios también *G. vaginalis*, se consideran los principales patógenos causantes de EC (Cicinelli et al., 2008; Kitaya et al., 2018). Se ha sugerido que esta enfermedad disminuye las tasas de éxito reproductivo y contribuye a las complicaciones obstétricas y neonatales (Kitaya et al., 2016). Además, *S. agalactiae* es una de las principales causas de infecciones neonatales por transmisión vertical (Patras & Nizet, 2018). Por último, nuestros resultados respaldan varios estudios que reportan especies de *Bifidobacterium* como patógenos en diversas condiciones infecciosas (Bhaskar et al., 2017; Chen et al., 2019; Pathak et al., 2014).

La relevancia de este estudio se manifiesta en que es el primer trabajo internacional y multicéntrico que analiza prospectivamente la microbiota endometrial en dos tipos de muestras tomadas simultáneamente de la misma paciente y evalúa su impacto en los resultados de embarazo en un gran grupo de pacientes (n = 342). Los datos presentados son sólidos porque ambos tipos de muestras se secuenciaron con las mismas regiones hipervariables del gen ARNr 16S y se analizaron utilizando las mismas herramientas bioinformáticas. Asimismo, para eliminar las lecturas contaminantes, las muestras se clasificaron como detectables y no detectables, excluyendo del análisis aquellas que agruparon con los blancos y tenían una concentración de amplicón equivalente. Otra fortaleza de este trabajo es que se analizó la receptividad endometrial y se realizó una transferencia embrionaria personalizada para evitar el desplazamiento de la ventana de implantación (Díaz-Gimeno et al., 2011; Ruiz-Alonso et al., 2013; Simón et al., 2020). Por último, el régimen hormonal y el día del muestreo fueron consistentes entre pacientes, evitando el sesgo potencial introducido por diferentes estados hormonales.

Estudiar las interacciones funcionales entre la comunidad de microorganismos y su huésped también es crucial para comprender el efecto de la microbiota endometrial sobre la infertilidad. Para ello, se realizó un modelo *in vitro* de

colonización bacteriana en cultivos primarios de epitelio endometrial. Usando este modelo, se confirmó la capacidad de la cepa *L. rhamnosus* BPL005 para bajar el pH a niveles inferiores de 5.0 en los cocultivos con los patógenos testados (a excepción de *S. agalactiae)*. Este potencial de acidificación se debe principalmente a la producción de ácido láctico, con efectos positivos ampliamente reportados entre los que se incluyen el control de las poblaciones de patógenos y la modulación inmune (Borges et al., 2014). La evaluación del crecimiento bacteriano por RT-PCR también confirmó que *L. rhamnosus* BPL005 reduce la colonización de la mayoría de los patógenos testados, lo que le confiere un potencial efecto positivo sobre la inhibición de biopelículas. Además, al medir las citoquinas y/o quimiocinas secretadas se observó que la adición de BPL005 a las células epiteliales colonizadas con patógenos restableció significativamente los niveles de algunas moléculas (IL-6, IL-8, MCP-1, IL-1RA e IL-1β), lo que apoya el papel de esta cepa probiótica para disminuir los niveles de moléculas proinflamatorias y mantener la homeostasis. Según la literatura, se ha demostrado que *Lactobacillus* disminuye la concentración de moléculas proinflamatorias como IL-6, IL-8, MIP-3α, RANTES y TNFα, al tiempo que aumenta la secreción de la citocina antiinflamatoria IL-1RA (Hearps et al., 2017; Tachedjian et al., 2017). Esto es clínicamente relevante ya que se sabe que las citocinas y las quimiocinas desempeñan un papel fundamental en la reproducción.

Finalmente, para estudiar el impacto funcional de la microbiota endometrial en la infertilidad, también se caracterizó taxonómica y funcionalmente a lo largo del tiempo el microbioma endometrial de dos pacientes infértiles con fallos reproductivos utilizando herramientas de secuenciación de ARNr 16S y WMS. En el primer caso clínico, se analizaron seis muestras de LE a lo largo de 18 meses, detectando una infección persistente con *Gardnerella* y otros taxones bacterianos como *Atopobium* y *Bifidobacterium*. *G. vaginalis* es un patógeno bacteriano que se adquiere sexualmente y produce condiciones infecciosas como la VB y la EC a través del establecimiento de biopelículas polimicrobianas a lo largo del tracto reproductivo

(Swidsinski et al., 2013). Las biopelículas de *G. vaginalis* pueden estar presentes durante largos períodos de tiempo sin causar síntomas (Swidsinski et al., 2008), además, suelen presentar recurrencias debido a la eficacia inconsistente de las terapias de rutina (Koumans et al., 2002: Bradshaw et al., 2006) (Bradshaw et al., 2006). La resistencia a los antimicrobianos podría deberse al hecho de que las cepas de *G. vaginalis* presentan una susceptibilidad variable al metronidazol, y algunas incluso muestran resistencia intrínseca (De Backer et al., 2006).

Para evaluar la resistencia de *G. vaginalis* al tratamiento, se analizaron marcadores genéticos específicos de los cuatro ecotipos por WMS. Los resultados mostraron la presencia concomitante de múltiples clados, lo que se ha identificado como un factor de riesgo en mujeres con VB (Balashov et al., 2014). Específicamente, se detectó una mayor abundancia del clado 4 en la muestra recolectada siete días antes de la transferencia embrionaria que terminó en un aborto clínico de seis semanas. Curiosamente, se ha informado que este clado confiere resistencia intrínseca al metronidazol (Schuyler et al., 2015). Este estudio muestra la relevancia de identificar las diferencias genéticas responsables de rasgos de patogenicidad para tratar de identificar aquellos casos que pueden tener un mayor riesgo de enfermedad recurrente.

El segundo caso clínico describió el microbioma endometrial de una paciente que sufrió un aborto espontáneo de ocho semanas con embriones euploides y, por primera vez, durante un embarazo exitoso a las cuatro semanas de gestación. El análisis de secuenciación reveló diferencias tanto taxonómicas como funcionales entre ambas de muestras, detectando *L. crispatus* en el endometrio antes del aborto espontáneo y *L. iners* durante el embarazo temprano. *L. crispatus* y *L. iners* son bacterias comunes del tracto reproductivo. Estas dos especies están estrechamente relacionadas y se cree que realizan funciones ecológicas similares. Sin embargo, las diferencias en sus genomas pueden explicar su especificidad para un nicho dado.

A diferencia de otras especies estudiadas, *L. crispatus* tiene el genoma más grande con genes únicos de ADN polimerasa, bacteriocina y toxina-antitoxina que codifican elementos genéticos móviles, especialmente transposasas (Petrova et al., 2015; France et al., 2016), lo que es consistente con las funciones observadas en la muestra recolectada antes del aborto espontáneo. Por otro lado, aunque se ha demostrado que el tracto reproductivo de mujeres sanas puede ser colonizado por *L. iners* (McMillan et al., 2013), se suele identificar en comunidades de transición entre VB y una microbiota normal (Gajer et al., 2012; Petrova et al., 2017). En este estudio, se observó la transición a una microbiota dominada por *L. iners* después de un período de inestabilidad (aborto clínico, seguido de dilatación, legrado y tratamiento con antibióticos). El genoma de *L. iners* contiene un grupo de hierro que puede suponer una ventaja competitiva contra otros patógenos bacterianos, o incluso puede desempeñar un papel en el suministro de nutrientes y la supervivencia en condiciones adversas como la menstruación (Macklaim et al., 2011). Se necesitan más estudios para determinar de manera más precisa el papel de estas especies bacterianas en la salud y la enfermedad endometrial y si estas cepas pueden servir como biomarcadores del éxito reproductivo.

Aunque todavía no se ha alcanzado un consenso sobre la composición del microbioma uterino, su impacto en la salud y la enfermedad, y en concreto su implicación en la reproducción humana, están siendo cada vez más evidentes. Los resultados presentados en esta tesis pueden ayudar a comprender las causas del fallo de implantación y el aborto espontáneo. Asimismo, facilitan el desarrollo de herramientas diagnósticas que podrían ser la base de procedimientos terapéuticos personalizados que intenten restaurar la microbiota endometrial. La investigación futura en este campo arrojará una mayor comprensión de los mecanismos traslacionales que explican la interacción de la microbiota uterina con la salud y la reproducción de las mujeres.

Conclusiones

- La microbiología molecular permite la caracterización fiable de patógenos de endometritis crónica clínica y subclínica. En comparación con la histeroscopia, la histología o el cultivo microbiano, la RT-PCR detecta eficazmente los patógenos causantes de endometritis crónica con una precisión del 77%, una sensibilidad del 75% y una especificidad del 100%.

- La composición de la microbiota en el líquido endometrial muestra una estrecha relación con la de la biopsia endometrial.

- *Lactobacillus,* junto con otras bacterias comensales, se encuentra enriquecido en muestras de endometrio de pacientes con un nacido vivo.

- La presencia de bacterias patógenas en el endometrio - *Atopobium*, *Bifidobacterium*, *Chryseobacterium*, *Gardnerella*, *Haemophilus*, *Klebsiella*, *Neisseria*, *Staphylococcus* y *Streptococcus* - se asocia con resultados reproductivos negativos y debe considerarse como una causa emergente del fallo de implantación y pérdida de embarazo.

- *L. rhamnosus* BPL005 (CECT 8800) puede tener una función protectora en las infecciones endometriales por *A. vaginae*, *G. vaginalis*, *P. acnes* y *S. agalactiae*. El cocultivo de BPL005 con los patógenos testados en un modelo *in vitro* de colonización bacteriana endometrial produce una reducción de los niveles de pH, la colonización de patógenos, así como modula la secreción de diferentes citocinas y quimiocinas (disminuye IL-6, IL-8, MCP-1 y aumenta IL-1RA e IL-1β).

- El estudio funcional del microbioma en una paciente infértil con fallos reproductivos recurrentes muestra una colonización persistente por *G. vaginalis* durante los 18 meses de seguimiento, expresando factores de virulencia y genes de resistencia antimicrobianos.

- La microbiota endometrial en un embarazo exitoso temprano y antes de un aborto espontáneo clínico con embriones euploides en la misma paciente revela una menor riqueza y diversidad de la comunidad bacteriana y una mayor abundancia de *Lactobacillus* durante el embarazo. La metagenómica funcional muestra distintas especies de *Lactobacillus* y perfiles funcionales, en los que el metabolismo basal y la regulación de la traducción asociada a *L. iners* son las principales funciones en un embarazo exitoso.

- Las implicaciones de estos resultados pueden contribuir a la conciencia médica del impacto potencial de los patógenos microbianos en el tratamiento de la infertilidad. Asimismo, nos lleva a proponer nuevas herramientas de diagnóstico y procedimientos terapéuticos personalizados a través de la evaluación microbiológica molecular.

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ABBREVIATONS

	16S rRNA	16S ribosomal RNA gene
A	ART	Assisted reproductive technology
B	β-HCG	Beta human chorionic gonadotropin
	BHI	Brain heart infusion
	BLAST	Basic Local Alignment Search Tool
	BMI	Body mass index
	BV	Bacterial vaginosis
	BWA	Burrows-Wheeler Aligner
C	CE	Chronic endometritis
	CFU	Colony-forming units
	CI	Confidence interval
	CLR	Centered log-ratio
	COG	Clusters of orthologous
	Cp	Crossing point-PCR-cycle
	CPM	Counts per million
	CST	Community state types
D	DNA	Deoxyribonucleic acid
	DSMZ	Deutsche Sammlung von Mikroorganismen und Zellkulturen
E	EB	Endometrial biopsy
	EF	Endometrial fluid
	EP	Endometrial polyps
	ERA	Endometrial Receptivity Analysis
F	FGT	Female genital tract
	FLASh	Fast Length Adjustment of Short reads
G	GNAT	Gcn5-related N-acetyltransferases
	GPCRs	G protein-coupled receptors
H	hEECs	Human endometrial epithelial cells
	HMP	Human Microbiome Project
	HPD	Highest posterior density interval
	HRT	Hormone replacement therapy
	HUMAnN2	HMP Unified Metabolic Analysis Network

I	ICSI	Intracytoplasmic sperm injection
	IDT	Integrated DNA Technologies
	IVF	*In vitro* fertilisation
K	KEGG	Kyoto Encyclopedia of Genes and Genomes
L	LB	Live birth
	LD	*Lactobacillus*-dominated
	LGT	Lower genital tract
M	MISC	Miscarriage
	MRS	Man, Rogosa and Sharpe
N	NCBI	National Center for Biotechnology Information
	NGS	Next-generation sequencing
	NLD	Non-*Lactobacillus*-dominated
	NP	No pregnancy
O	OTUs	Operational taxonomic units
P	PCR	Polymerase chain reaction
	pET	Personalised embryo transfer
	PGT-A	Preimplantation genetic test for aneuploidy
	PID	Pelvic inflammatory disease
	POC	Product of conception
R	RIF	Repeated implantation failure
	RNA	Ribonucleic acid
	ROS	Reactive oxygen species
	RPKM	Reads per kilobase million
	RPL	Recurrent pregnancy loss
	RT-PCR	Real-time polymerase chain reaction
S	SCFAs	Short-chain fatty acids
	STIs	Sexually transmitted infections
T	TLRs	Toll-like receptors
	Tm	Melting temperature
U	UGT	Upper genital tract
	UNG	Uracyl-N-glycosidase
W	WMS	Whole metagenome sequencing

I. INTRODUCTION

1. HUMAN MICROBIOME OVERVIEW

1.1. Definition

The concept of the human microbiome was first defined by Lederberg and McCray in 2001 as "the ecological community of commensal, symbiotic, and pathogenic microorganisms that literally share our body space" (Lederberg & McCray, 2001). This community includes microorganisms such as eukaryotes, archaea, bacteria, and viruses, wherein bacteria are the most abundant and better-studied members. Currently, the collection of microbes that live in association with the human body is called microbiota, while the collection of genes and genomes of these members is the microbiome (Marchesi & Ravel, 2015).

Humans have always lived in a symbiotic relationship with microbes, and should therefore be considered, more than a single organism containing a single nuclear genome, as holobiont organisms. Holobionts comprise a host and all its symbiotic microbes, which can be constant or transient, can be vertically or horizontally transmitted, and can act in a context-dependent manner as harmful, harmless, or helpful. Therefore, a holobiont phenotype can change over time as microbes come into and out of the holobiont. The host and microbial genomes of a holobiont are collectively defined as its hologenome **(Figure 1)** (Theis et al., 2016). The human genome contains about 20,000 genes, but its hologenome contains > 33 million genes due to contributions from its microbiome (Simon et al., 2019), which highlights the importance of microorganisms in human health.

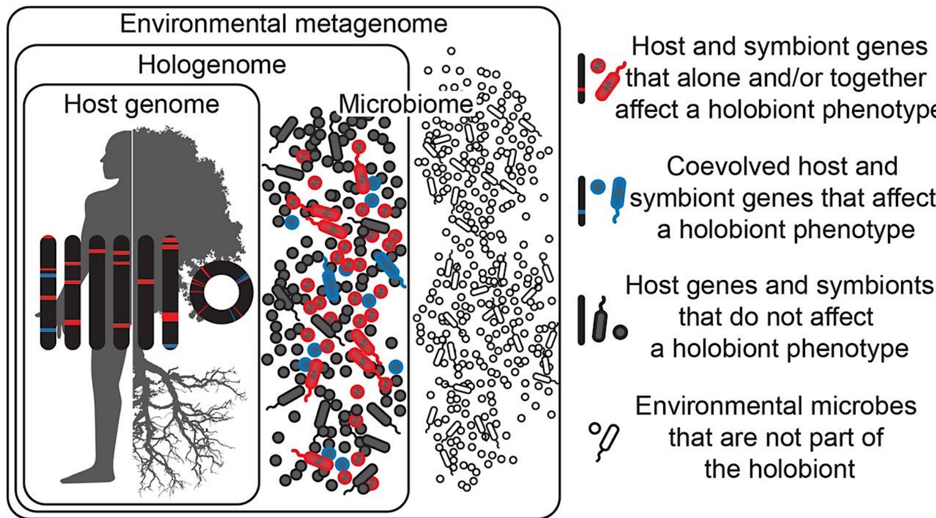


Figure 1 I Graphic definition of holobiont and hologenome.

Holobionts comprise a host organism and all its symbiotic microbes, including those that affect the holobiont's phenotype and have coevolved with the host (blue), those that affect its phenotype but have not coevolved with the host (red), and those that do not affect the holobiont's phenotype at all (gray). Microbes in the environment are not part of the holobiont (white). Reprinted with permission from Theis et al., (2016).

1.2. Microbiome in health and disease

Increasing knowledge of the microbiome is further solidifying its significant effect on human physiology. The majority of bacterial communities co-exist in synergistic relationships with humans, exerting functional roles and communicating with host cells by influencing metabolic function, training the immune system, and modulating drug interactions (Grice & Segre, 2012). By contrast, the colonisation by different bacteria may turn this mutualistic or commensal interaction into a parasitic relationship, predisposing the host to pathological conditions with variable severity of symptoms. Common examples such as dental caries and bacterial vaginosis (BV); chronic conditions, such as obesity, cardiovascular disease, inflammatory bowel disease, and malnutrition; and even diseases not traditionally suspected to be linked to the microbiome, such as Parkinson's disease, autism, and depression, have been related to alterations in the microbiome (Knight et al., 2017). Despite recognition of the importance of these interactions, there remain many unanswered questions regarding

how the microbial population varies within and among individuals in healthy and diseased states.

Most human microbiology studies have focused on disease-causing microorganisms, but the benefits of resident bacteria have long been underestimated. The important physiological role of commensal bacteria was evidenced in 2001, when the magnitude of the microbial genome was determined in the Human Genome Project (Venter et al., 2001). This project prompted scientists to study the synergistic activities between humans and microbes through the Second Genome Project, which investigated, using high-throughput sequencing, the human microbiome on a large scale (Davies, 2001; Relman & Falkow, 2001). The Human Microbiome Project (HMP) was launched in 2008 by the U.S. National Institutes of Health to characterise in healthy volunteers the microbial communities of different body sites (nasal passages, oral cavity, skin, gastrointestinal tract, and urogenital tract). Unsurprisingly, different body sites harboured diverse microbial communities in terms of both diversity and composition. While oral communities were the most diverse based on the number of different organisms present, the microbial communities of the vagina proved to be the least diverse, being composed mainly of *Lactobacillus* spp. (Consortium, 2012a). The HMP provided standardised data resources and new technological developments that allowed the scientific community to move forward in microbiome studies and raised the possibility of improving human health through monitoring or manipulating the human microbiome (Peterson et al., 2009).

1.3. Technical assessment of the human microbiome

Identification of microorganisms inhabiting the human body has been traditionally performed by microscopy, allowing the classification of bacteria based on characteristics like Gram staining and cell morphology. In the field of human reproduction, microscopy still has an important role in the detection of clue cells or determination of the Nugent Score for BV diagnosis (Nugent et al., 1991). However,

the role of microscopy in daily clinical practice has diminished due to its time-consuming nature and the introduction of techniques such as polymerase chain reaction (PCR).

Culture-dependent methods are another classic technique used to study the microbiota. Although these methods are simple and inexpensive, many microorganisms are not easily detected. Depending on the body site, it has been estimated that between 20% and 60% of bacteria are uncultivable under standard laboratory conditions (Peterson et al., 2009). Also, under *in vitro* conditions only microbiota whose metabolic and physiological needs are provided by the specific culture medium will proliferate, with high-abundance and fast-growing species suppressing others (Hiergeist et al., 2015). Therefore, culture-dependent methods do not reliably reproduce the global composition of the microbiota and may underestimate microorganisms potentially associated with a particular state (Ward et al., 1990).

The advent of next-generation sequencing (NGS) technologies has enabled a more global assessment of bacterial composition than culture-dependent methods. NGS establishes bacterial signatures by analysing the 16S ribosomal RNA (16S rRNA) gene, which is present in all bacteria but includes unique genetic features for each bacterial species owed to evolution. This gene is ~1500 base pairs (bp) long and contains nine hypervariable regions (V1–V9), located between highly conserved sequences, that serve as a molecular fingerprint for the identification of bacteria **(Figure 2)**. Comparing the hypervariable sequences with phylogenetic reference trees, bacterial taxonomy is assigned, according to its similarity, in operational taxonomic units (OTUs). These OTUs are classified in different ranks: domains, kingdoms, phyla, classes, orders, families, genera, and species (Pirih & Kunej, 2018; Yarza et al., 2014). Since the original 16S rRNA-based phylogeny was described from an initial group of 11 bacterial phyla in 1987 (Woese, 1987), reference databases have grown

exponentially to contain more than 3 million 16S rRNA sequences in 2020 [Ribosomal Database Project (RDP) Release 11].

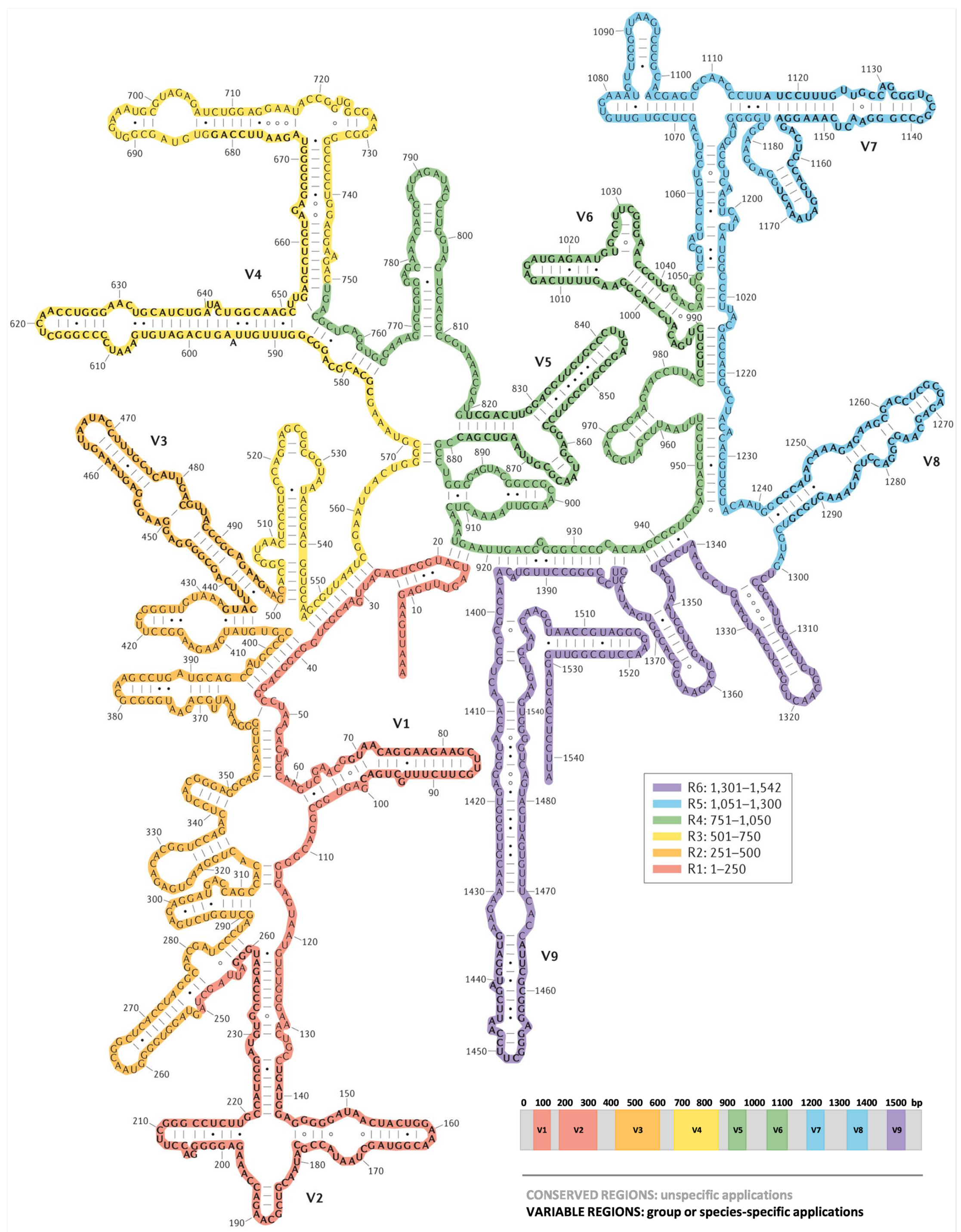


Figure 2 I Hypervariable regions of the 16S ribosomal RNA gene.

Secondary structure of the 16S rRNA gene of *Escherichia coli* representing the nine hypervariable regions used for taxonomic classification of bacteria. Adapted with permission from Yarza et al.,, (2014).

New techniques of shotgun metagenomic sequencing/whole metagenome sequencing (WMS) are increasingly being used to describe the human microbiome, as they provide another level of depth in bacterial identification. Whereas 16S rRNA-based analyses are limited to the examination of bacterial diversity based on a single gene, WMS techniques sequence DNA from the genomes of all organisms present in a particular sample. This approach yields information about the function of genes, metabolic pathways encoded, genome structure and organisation, and structure and function of the microbial community (Roumpeka et al., 2017) **(Figure 3)**.

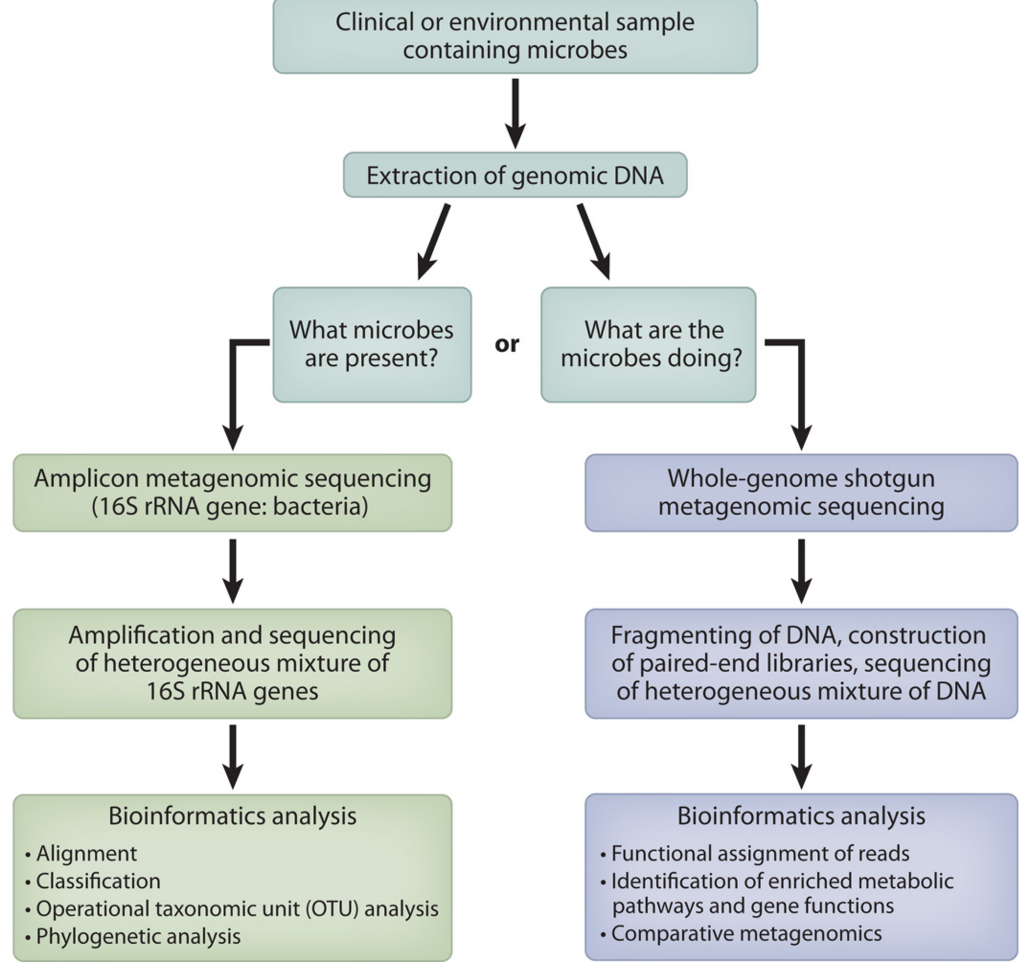


Figure 3 I Workflow for 16S rRNA and metagenomic sequencing.
While 16S rRNA gene sequencing is used to taxonomically classify bacteria and study the bacterial composition and relative abundance, WMS can analyse microbial communities from a functional point of view. Reprinted with permission from Grice et al., (2012).

Despite the multiple advantages of sequencing, an important consideration when interpreting its results is that the genetic material detected may be derived from organisms that are non-viable. The detected sequences may represent live bacteria, bacterial residues resulting from anti-microbial activities, or even DNA-containing vesicles (Einenkel et al., 2019). Bacterial DNA fragments can be detected by all cells expressing pattern recognition receptors, such as toll-like receptors (TLRs), and thereby activate intracellular signalling cascades that initiate host defense reactions (Gu et al., 2019).

The combined information from culture- and molecular-based methods may verify the presence of live bacteria in a given environment and clarify their role in human health (Green et al., 2015). However, this is one of the main limitations in microbiome analysis since few studies use this dual approach to verify the viability of bacteria.

2. FEMALE GENITAL TRACT MICROBIOTA

Advancements in culture-independent methods have revealed that fertilisation occurs within a nonsterile environment and that the presence of microorganisms in the female, and even male, reproductive organs can influence human reproduction. Particularly, the microbiome of the female genital tract (FGT) is implicated in different stages of the reproductive process, from gametogenesis to fertilisation, embryo migration, implantation, pregnancy establishment and maintenance, and even in the microbial colonisation of the newborn **(Figure 4)**. In fact, alterations in this microbiome have been related to pregnancy loss and poor obstetric outcomes during gestation and delivery (Franasiak & Scott, 2015). Therefore, knowledge of the microbial communities inhabiting the different niches of the FGT could help to understand its potential role in reproductive competence.

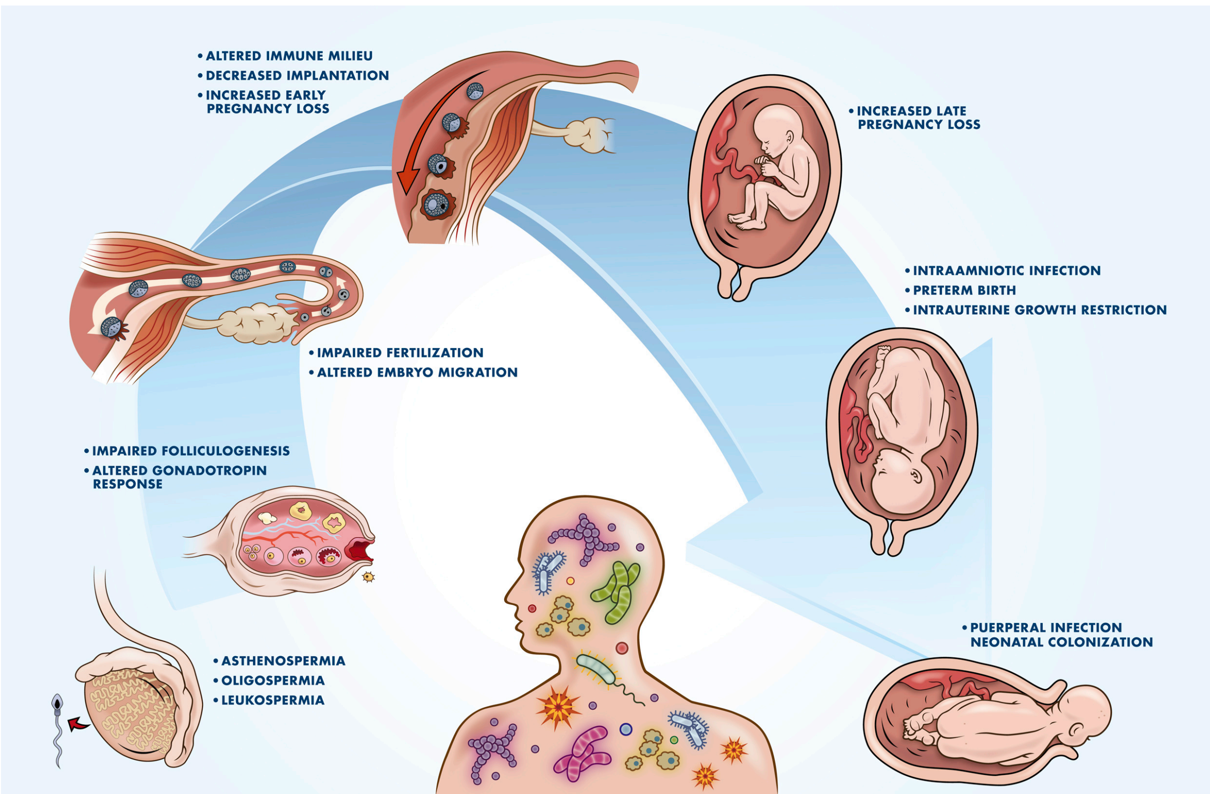


Figure 4 I The microbiome in human reproduction.
The female genital tract microbiome affects all facets of reproduction, from gametogenesis to fertilisation, embryo migration, implantation and pregnancy. Reprinted with permission from Franasiak & Scott (2015).

2.1. Lower genital tract microbiota

Most data characterising the FGT microbiome come from studies analysing the lower genital tract (LGT), specifically vaginal samples. The vaginal microbiome was characterised in depth in the HMP, where it was determined to represent approximately 9% of the total human microbiome (Peterson et al., 2009). Interestingly, the vaginal tract exhibited the lowest alpha (within samples from the same patient) and beta (comparison between patients) diversity compared to other body sites such as the mouth or the skin. Indeed, when samples were taken at the vaginal introitus, midpoint, and posterior fornix, the species variation was low, and *Lactobacillus* spp. dominated at all sites (Consortium, 2012b). Yet, the vaginal microbiota is not always dominated by *Lactobacillus.* It changes throughout a woman's lifetime. During childhood, the vaginal flora is a mixture of aerobic and

anaerobic bacterial populations (Hammerschlag et al., 1978), but following menarche, there is *Lactobacillus* dominance until menopause. During reproductive years, high circulating levels of oestrogen promotes deposition of glycogen in the vaginal epithelium. This process favours the growth of glucose-fermenting microorganisms that metabolise it to lactic acid creating an acidic environment (pH 3.5-4.5) suitable for protection against bacterial pathogens (Amabebe & Anumba, 2018). Finally, after menopause, the proportion of *Lactobacillus* spp. decreases again due to a decline in endogenous oestrogen (Muhleisen & Herbst-Kralovetz, 2016).

One of the earliest studies using 16S rRNA sequencing in vaginal samples from reproductive-age women revealed the existence of five major types of vaginal microbiota, termed community state types (CST). Four of these CSTs were dominated by different *Lactobacillus* spp.: *Lactobacillus crispatus* (CST I), *Lactobacillus gasseri* (CST II), *Lactobacillus iners* (CST III), or *Lactobacillus jensenii* (CST V). In contrast, CST IV was characterised by increased diversity due to polymicrobial colonisation with anaerobic bacteria including *Aerococcus*, *Atopobium*, *Dialister*, *Gardnerella*, *Megasphaera*, *Prevotella,* and *Sneathia* (Ravel et al., 2011) **(Figure 5A)**.

Transition through CST IV type is considered a risk factor for BV (Torcia, 2019). This common vaginal infection is associated with: gynaecological and obstetric complications – including preterm delivery and early or late miscarriage – (Anahtar et al., 2018; Solt, 2015), development of pelvic inflammatory disease (PID) (Ness et al., 2005), and acquisition of sexually transmitted infections (STIs) (Brotman, 2011). However, the CST IV profile is also common in asymptomatic reproductive-age women depending on their racial background. While the vaginal specimens of 90% of white women are dominated by *Lactobacillus*, 80% of Asian women and only 60% of Hispanic and Black women harbour *Lactobacillus*-dominant communities (Ravel et al., 2011) **(Figure 5B)**.

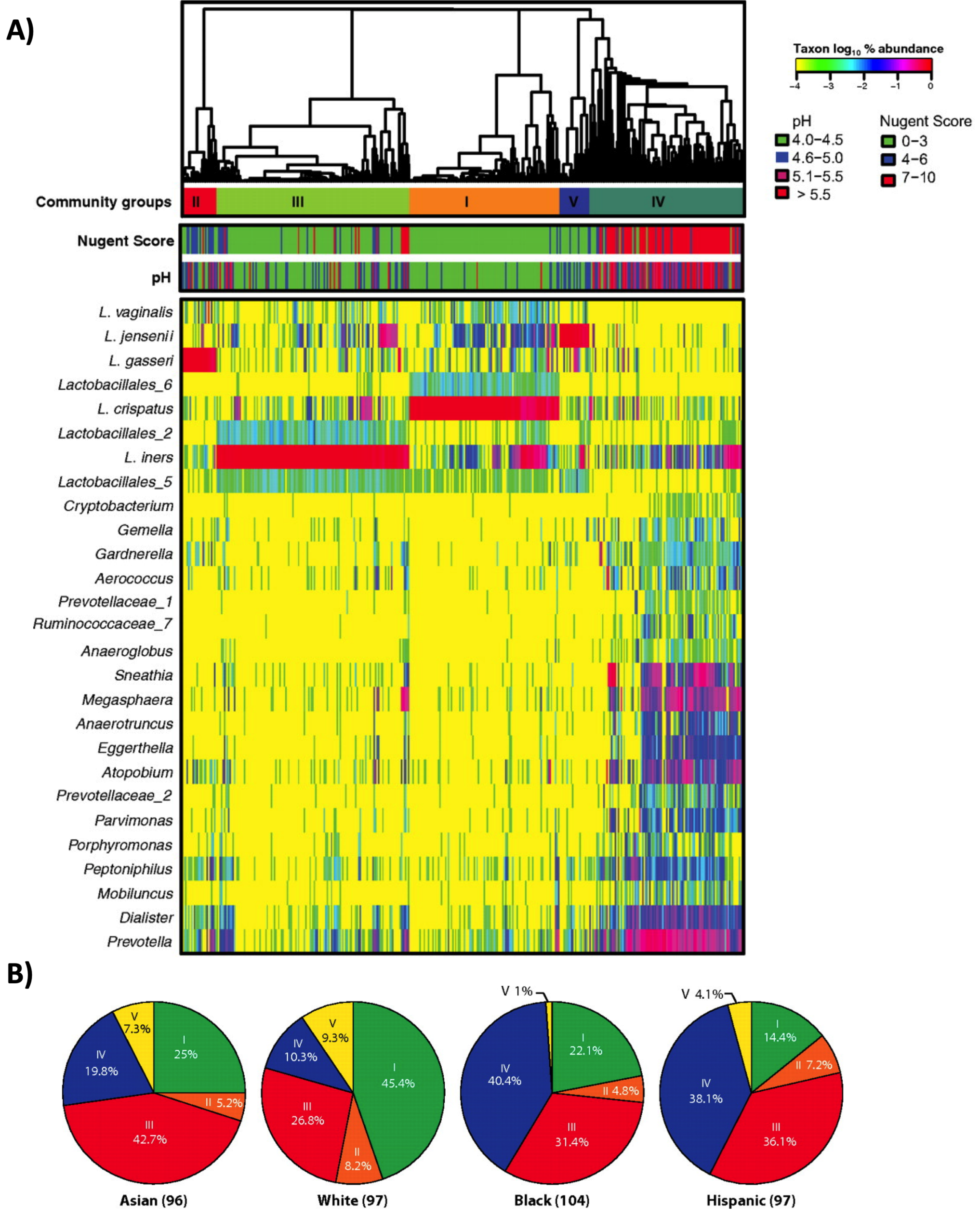


Figure 5 I Vaginal microbiome of reproductive-age women.

(A) Heatmap with proportions of microbial taxa found in the vaginal bacterial communities of 394 women of reproductive age, defining the clustering of community groups I to V. (B) Representation of vaginal bacterial community groups within each ethnic group. The number of women from each ethnic group is in parentheses. Adapted with permission from Ravel et al., (2011).

The variation of microbiota profiles may reflect not only racial or genetic predisposition, but also socioeconomic, behavioural, and environmental factors **(Figure 6)**. Longitudinal studies demonstrated that the vaginal microbiota can be dynamic and may undergo shifts between CSTs over time (Gajer et al., 2012).

Shifts in bacterial composition are observed in response to numerous endogenous and exogenous factors, such as hormonal changes during menstrual cycle, hygiene habits, sexual behaviours, or the use and type of contraceptives (Łaniewski et al., 2020). Nonetheless, fluctuations in bacterial composition do not necessarily lead to a change in community behaviour. Fluctuations may occur while maintaining community performance due to perseverance of the metabolic state or when there is functional redundancy among community members. A better understanding of vaginal community dynamics may lead to interventions that shift the vaginal microbiota towards more protective states.

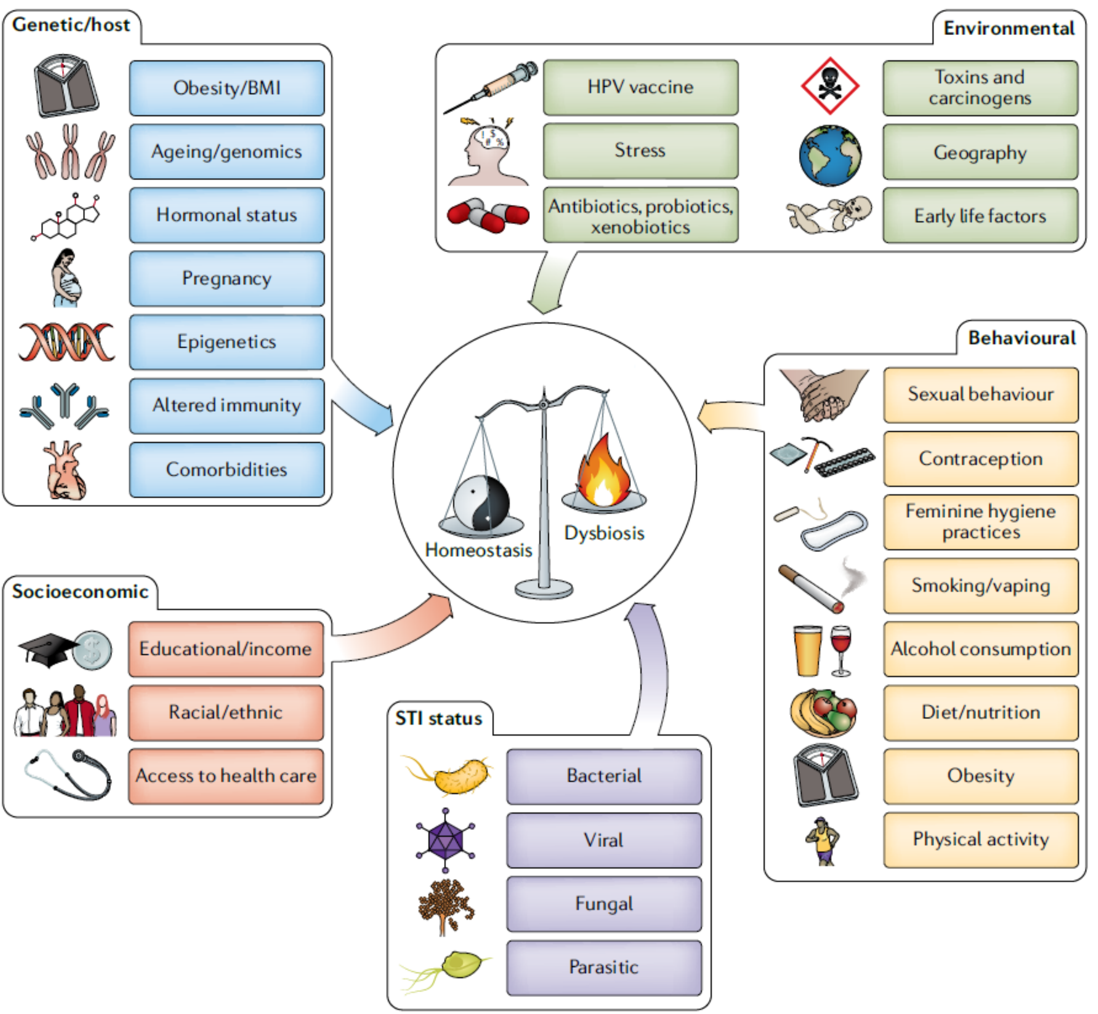


Figure 6 | Factors influencing the composition of vaginal microbiota.

Behavioural, socioeconomic, genetic, and environmental factors contributing to dysbiosis of vaginal microbiota in reproductive-age women. Reprinted with permission from Łaniewski et al., (2020).

2.2. Upper genital tract microbiota

Traditionally the upper genital tract (UGT) was considered physiologically sterile (Tissier, 1900). However, emerging evidence demonstrates the presence of bacteria beyond the vagina including in uterus, fallopian tubes, and ovaries (Koedooder, Mackens, et al., 2019).

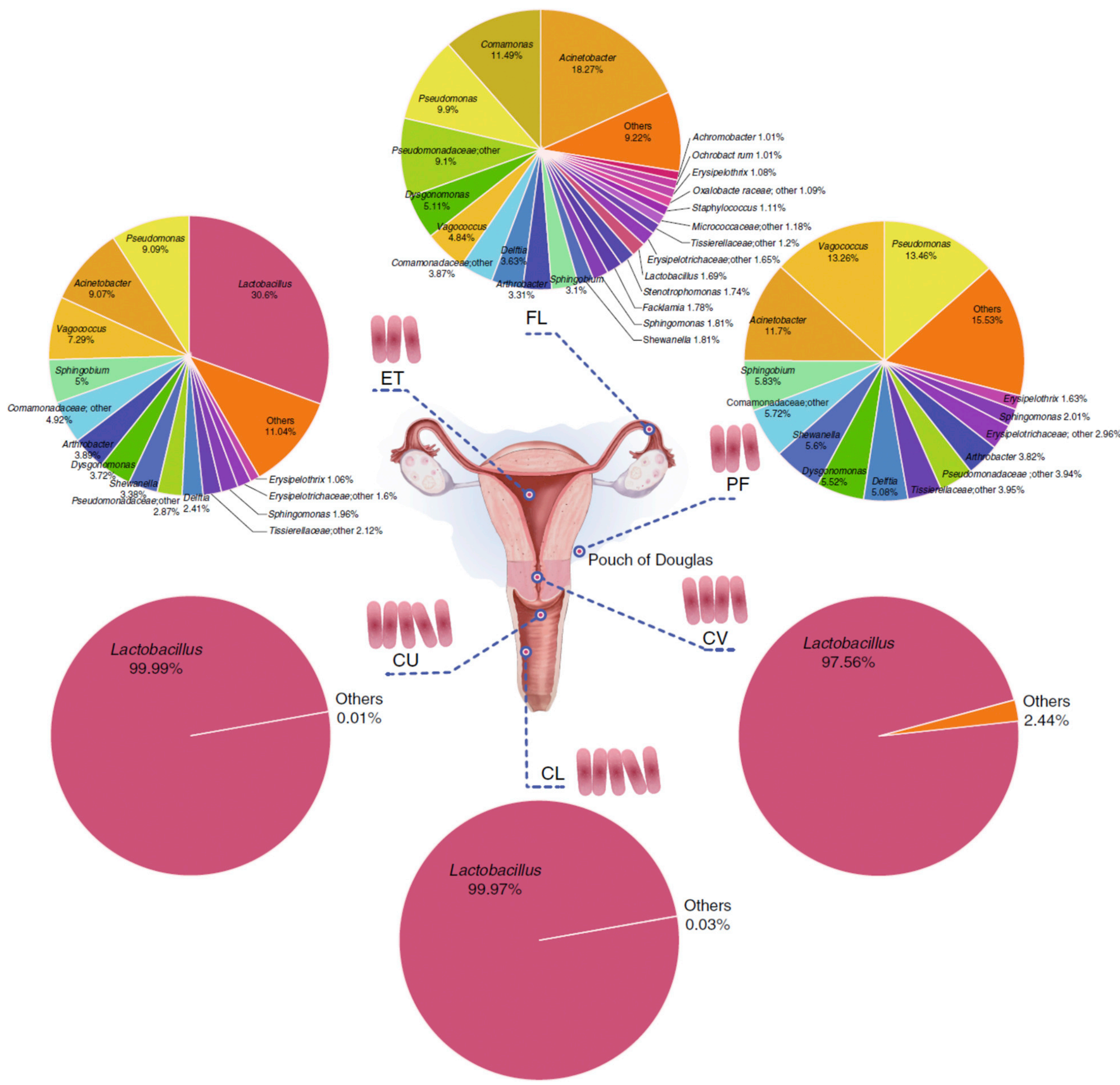


Figure 7 I Microbial composition of the female genital tract microbiota.
Pie chart with the relative abundance of the microbial taxa at each site of the female genital tract. CL, lower third of vagina; CU, posterior fornix; CV, cervical canal; ET, endometrium; FL, fallopian tubes; PF, peritoneal fluid. Reprinted with permission from Chen et al., (2017).

Recently, the FGT was described as an open system with a continuous microbiota that gradually changes from the vagina to the ovaries (Chen et al., 2017; F. Li et al., 2018) **(Figure 7)**. Despite the trafficking of microorganisms along the tract, there is evidence of site-specific microbial communities, with a decline in the abundance and greater bacterial diversity when moving from the vagina towards the UGT. Unlike the rich and well-characterised vaginal microbiota (estimated to harbour 10^{10}–10^{11} bacteria), the uterine microbiota is less-defined and has been estimated to contain 100–10,000 less bacterial load (Chen et al., 2017; Mitchell et al., 2015), resulting in a low-abundance flora also known as low-biomass microbiota (O'Callaghan et al., 2020).

Quantitative and qualitative variations observed between the microbiota in the LGT and UGT could be explained by the cervix, which secretes mucus and defensive substances (like mucins, protegrins, lysozyme, histatins, and nitric oxide) that serve as a partial barrier for ascending microbiota (Becher et al., 2009). However, the cervical canal is not impermeable to bacterial ascension (Hansen et al., 2014). Mucins of the cervix change their conformation depending on pH variations during the menstrual cycle (Brunelli et al., 2007), which may allow passage of bacteria during certain conditions.

Some bacteria can attach to human spermatozoa, allowing transport through the cervix into the intrauterine space (Gomez et al., 1979; Svenstrup et al., 2003; Wølner-Hanssen & Mårdh, 1984). Studies using small labelled particles also reported particle translocation into the uterine cavity by uterine peristaltic contractions within minutes after their administration (Suff et al., 2018; Zervomanolakis et al., 2007). The presence of polymicrobial biofilms attached to the endometrium and fallopian tubes of women with BV (Swidsinski et al., 2013) supports this hypothesis, and confirms that cervical mucus does not completely inhibit the ascension of bacteria into the uterus. Differences in bacterial composition can also be influenced by the microbial ability to

evade immune responses, or because some microbes may remain in the UGT after disappearing from the vagina, and/or have better growth due to specific physicochemical conditions (Mitchell et al., 2015).

Table 1 summarises the main studies that evaluate variations in the microbial composition between the UGT and LGT microbiota. Most scientific publications support a modest degree of independence between microbiota in both sites in some women/conditions, with different profiles and bacterial amounts. Notably, the differences observed between upper and lower sites were independent of the method of collecting uterine specimens — transcervically (Fang et al., 2016; Kitaya et al., 2019; Moreno et al., 2016; Pelzer et al., 2018; Wee et al., 2018) or at surgery (Chen et al., 2017; F. Li et al., 2018; Miles et al., 2017; Mitchell et al., 2015; Walther-António et al., 2016; Winters et al., 2019) — which confirms the existence of endogenous microbiota and supports the vaginal–cervical canal as a valid route for sampling the uterine cavity for microbiome analysis.

The reported differences between the LGT and UGT microbiomes show that, although the vaginal and endometrial communities are similar in most women, they are not identical. This highlights the relevance of analysing the uterine microbiota in patients undergoing assisted reproductive technology (ART), as it is in the endometrium and not in the vagina where the embryo must adhere, implant, and grow to develop a healthy pregnancy to term.

Table 1 | Main studies evaluating variations between the lower and upper genital tract microbiota.

Study	Cohort	Sampling	Conclusions
Mitchell et al. (2015)	Women undergoing hysterectomy for benign uterine conditions (n=58)	Vaginal swabs Endometrial and endocervical swabs collected from the excised uterus	95% of women had UGT colonization with vaginal species, but with different profile and bacterial amount to their vaginal counterparts
Fang et al. (2016)	Women with endometrial polyps (n=10), endometrial polyps and chronic endometritis (n=10) and healthy controls (n=10)	Vaginal swabs Endometrial swabs collected transcervically	Bacterial composition in vagina significantly differed from intrauterine microbiome
Walther-Antonio et al. (2016)	Women undergoing hysterectomy for benign uterine conditions (n=10), hyperplasia (n=4), and endometrial cancer (n=17)	Vaginal and cervical swabs Uterine, fallopian tubes and ovarian biopsies collected from the excised organs	All organs tested were significantly correlated, suggesting a microbial movement across organs
Moreno et al. (2016)	Asymptomatic fertile women in prereceptive and receptive phases of the same menstrual cycle (n=13)	Vaginal aspirates Endometrial fluid collected transcervically	Different bacterial communities were detected between the uterine cavity and the vagina in approximately 20% of the women tested
Miles et al. (2017)	Women undergoing a total hysterectomy with bilateral salpingo-oopherectomy (n=10)	Vaginal swabs Cervical, uterine, fallopian tubes and ovarian swabs collected from the excised organs	Although with some differences across the organs tested, microbial communities were highly related across samples and patients
Chen et al. (2017)	Women operated for conditions not know to involve infection hysteromyoma, adenomyosis, endometriosis and salpingemphraxis (n=110)	Vaginal and cervical swabs Uterine, fallopian tubes and peritoneal fluid swabs collected during laparoscopy or laparotomy	Distinct bacterial communities were detected along the FGT forming a continuum microbiota gradually changing from the vagina to the ovaries

Table 1 | Continued.

Study	Cohort	Sampling	Conclusions
Wee et al. (2018)	Women with a history of infertility (n=15) and women with a history of fertility (n=16)	Vaginal and cervical swabs Endometrial biopsies collected transcervically	Bacterial profiles of the endometrium differed with the vagina and cervix, especially in the proportions of the most abundant taxa
Pelzer et al. (2018)	Women undergoing hysteroscopy and/or laparoscopy (n=60)	Endocervical swabs Endometrial curettings collected transcervically	Endocervix and endometrium share a minor microbial community, but each site harbours significantly different microbial populations
Kyono et al. (2018)	Women undergoing (n=79), or not (n=23), IVF, and healthy volunteers (n=7)	Vaginal aspirates Endometrial fluid collected transcervically	The endometrial microbiota was not suspected to be carried over from the vaginal microbiota because there was a difference in the pattern of the bacterial community between paired samples
Li et al. (2018)	Women operated for conditions not known to involve infection (n=6) Patients from Chen et al. (2017)	Vaginal and cervical swabs Uterine, fallopian tubes and peritoneal fluid swabs collected during laparoscopy or laparotomy	The different reproductive tract samples analysed using the BGISEQ-500 metagenomic platform showed that in the same individuals, microorganisms were largely shared throughout the reproductive tract
Kitaya et al. (2019)	Infertile patients with a history of RIF (n=28), and women undergoing the first IVF (n=18)	Vaginal aspirates Endometrial fluid collected transcervically	Bacterial profiles of the endometrium were highly correlated with vagina, but had higher diversity and broader bacterial species
Winters et al. (2019)	Women undergoing hysterectomy (n=25)	Vaginal, cervical, rectal and oral swabs Endometrial swabs collected from the excised uterus	Bacterial profiles of the endometrium differed from those of the vagina but not of the cervix

FGT, female genital tract; IVF, in vitro fertilisation; RIF, repeated implantation failure; UGT, upper genital tract.

3. STUDY OF THE UTERINE MICROBIOME

Due to limitations in microbial characterisation and challenges in the acquisition of endometrial samples, the importance of bacteria present in the UGT has been overlooked for years (Viniker, 1999). However, increasing evidence supports the presence of an endometrial microbiome, prompting a paradigm shift.

3.1. Evolution of the concept

In contrast to the long-standing beliefs that microbes colonise the cervico–vaginal epithelium, at the beginning of the 20th century there was a consensus regarding the uterus as a sterile cavity (Tissier, 1900) **(Figure 8)**. The presence of bacteria in the UGT was associated with infections such as endometritis or PID (Gardner, 1935; Goplerud et al., 1976). This notion was questioned by multiple reports in the 1980s, when the results of culture-based studies documented recovery of microorganisms from the endometrium of healthy and asymptomatic women (Eschenbach et al., 1986; Heinonen et al., 1985; Hemsell et al., 1989). Contamination with bacteria from the LGT, particularly when using a transcervical technique for specimen collection, was the major criticism for these findings (Duff et al., 1983). Nevertheless, similar results were obtained in samples from patients subjected to hysterectomy for benign conditions (Møller et al., 1995). Remarkably, all studies during this period used culture-dependent methods to evaluate the uterine microbiota, but data derived from culture-based approaches should be interpreted with caution due to limitations of the technique.

In the 21st century, especially since 2007, the development of culture-independent molecular methods enhanced our understanding of the uterine microbiome and enabled a more global assessment of bacterial populations **(Figure 8)**. Many studies using NGS techniques demonstrated the presence of commensal bacteria in the uterus, in both humans and animal models, changing the old adage that the foetus

develops in a sterile environment. At present, ongoing research aims to define the endometrial microbial profile and elucidate the potential mechanisms by which bacteria can influence on reproductive health and other diseases.

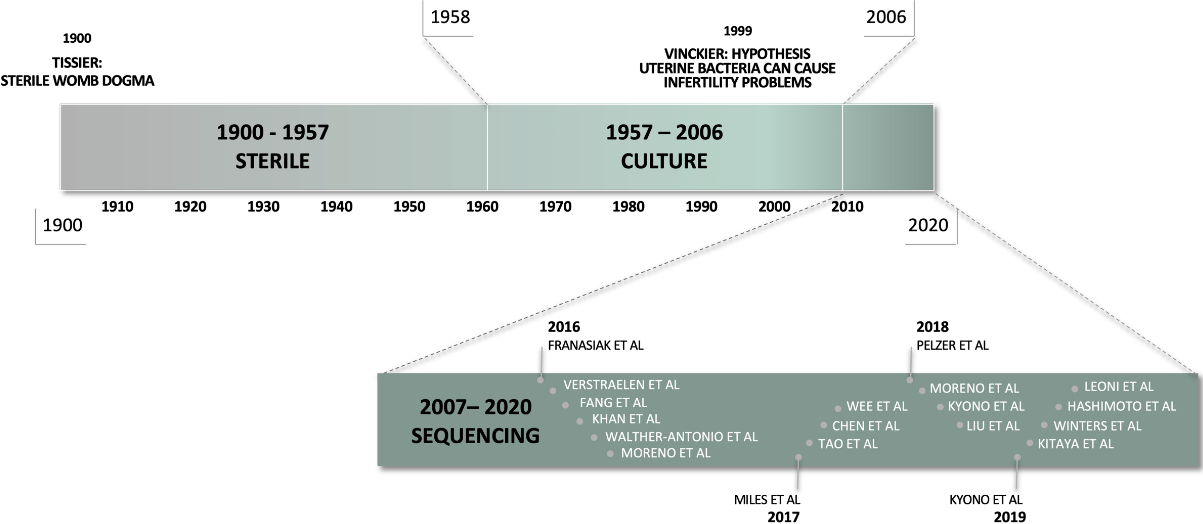


Figure 8 l Overview of uterine microbiome studies in the literature.
Overview from 1900 to 2020, from the assumption of sterility of the uterine microbiome to culture-based analysis and the detailed quantification of bacterial species through sequencing. Adapted with permission from Baker et al., (2018).

3.2. Routes of uterine bacterial colonisation

Discussions about the origin of the uterine microbiome have suggested different putative bacterial transmission routes: ascension of bacteria from the vagina, hematogenous dissemination from distal sites, sexual activity, and the interaction with the gut and the urinary tract microbiota **(Figure 9)** (Łaniewski et al., 2020).

The most plausible mode of UGT colonisation is ascension of microorganisms from the vagina. The FGT contains a continuous microbiota that gradually changes from the outer to the inner part (Chen et al., 2017), which would explain that some bacterial genera are residents of both sites and would justify the presence of polymicrobial biofilms attached to the endometrium of women with BV (Swidsinski et al., 2013). Moreover, gynaecological procedures whereby bacteria from the vaginal microbiome are inadvertently introduced into the uterus (i.e., transcervical procedures

such as hysteroscopy, embryo transfer, and intrauterine device placement) may be other sources of uterine microbiota seeding (Baker et al., 2018; Pereira et al., 2016; Sparks et al., 1981).

While bacterial ascent from the vaginal tract is recognised as the primary route of intrauterine colonisation, the microbiomes of the gastrointestinal, oral, and respiratory tracts are also involved by means of hematogenous spread (Aagaard et al., 2014; Solt, 2015). Bacterial DNA has been identified in peripheral blood mononuclear cells, suggesting that bacteria may be transported to distal sites through the blood (Chen & Gur, 2019; Jeon et al., 2017; Perez et al., 2007). Nevertheless, more research is needed to determine whether bacteria reach the UGT by themselves or whether immune cells could serve as a transporter for microbes.

Another possible route of introduction of bacteria into the uterus is through sexual activity. Recent analyses showed the presence of a seminal microbiome (Altmäe et al., 2019; Koedooder, Mackens, et al., 2019), and demonstrated that bacteria are shared among partners, influencing the species composition of each other's reproductive tract microbiota (Mändar et al., 2015; Mändar et al., 2018; Vodstrcil et al., 2017). Further, partners share and/or exchange members of the urogenital microbiota through sexual intercourse (Zozaya et al., 2016) since there is an interconnectivity within the urogenital tract (bladder–vagina axis in women and urethra–prostate axis in men) (Thomas-White et al., 2018).

Finally, changes in the vaginal microbiota, and ultimately possibly in the uterus, are associated with the colonisation of microorganisms present in the rectum. The rectum is postulated to be a key reservoir for lactobacilli that contribute to the maintenance of healthy vaginal microbiota (Łaniewski et al., 2020). Moreover, gut microbiota can also indirectly influence the genital microbiota composition (particularly *Lactobacillus* dominance) through oestrogen-mediated mechanisms. The 'oestrobolome' is the collection of enteric bacterial genes whose products are

capable of deconjugate oestrogens and promote their reabsorption to circulation. The free oestrogens are transported to distal sites (including the FGT). There, oestrogens bind to their receptors and trigger intracellular signalling such as glycogen production and other physiological changes such as mucus production and thickening of the epithelium. Thus, an alteration of oestrogen-metabolizing gut bacteria could also influence the FGT microbiome composition via the oestrobolome (Łaniewski et al., 2020).

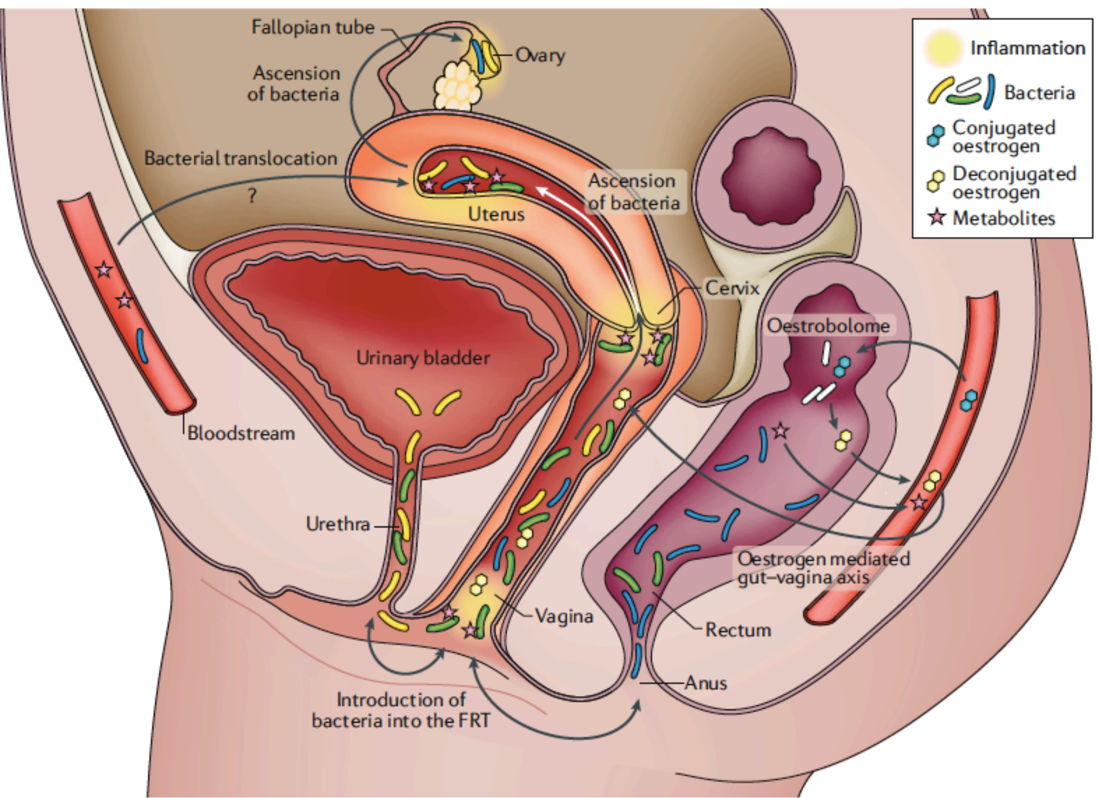


Figure 9 I Putative bacterial colonisation routes of the uterine microbiome.
The bacteria residing in the uterus ascend mainly from vagina; however, hematogenous spread of bacteria might also be a putative seeding route for the uterine microbiome. In addition, female genital tract microbiota interacts with other sites, as urinary tract or gut, through direct or oestrogen-mediated mechanisms. Reprinted with permission from Łaniewski et al., (2020).

3.3. Microbial fluctuations over time

It is unclear if the uterine microbiome changes over time or during the menstrual cycle. Longitudinal studies highlight the dynamic nature of vaginal microbial communities during the menstrual cycle, achieving a greater stability during periods

with high levels of oestrogen or progesterone **(Figure 10)** (Gajer et al., 2012). Therefore, it is reasonable to expect that uterine microbiota may also undergo fluctuations. However, since endometrial sampling is an invasive technique, there are almost no published longitudinal studies that describe the stability of the endometrial microbiota.

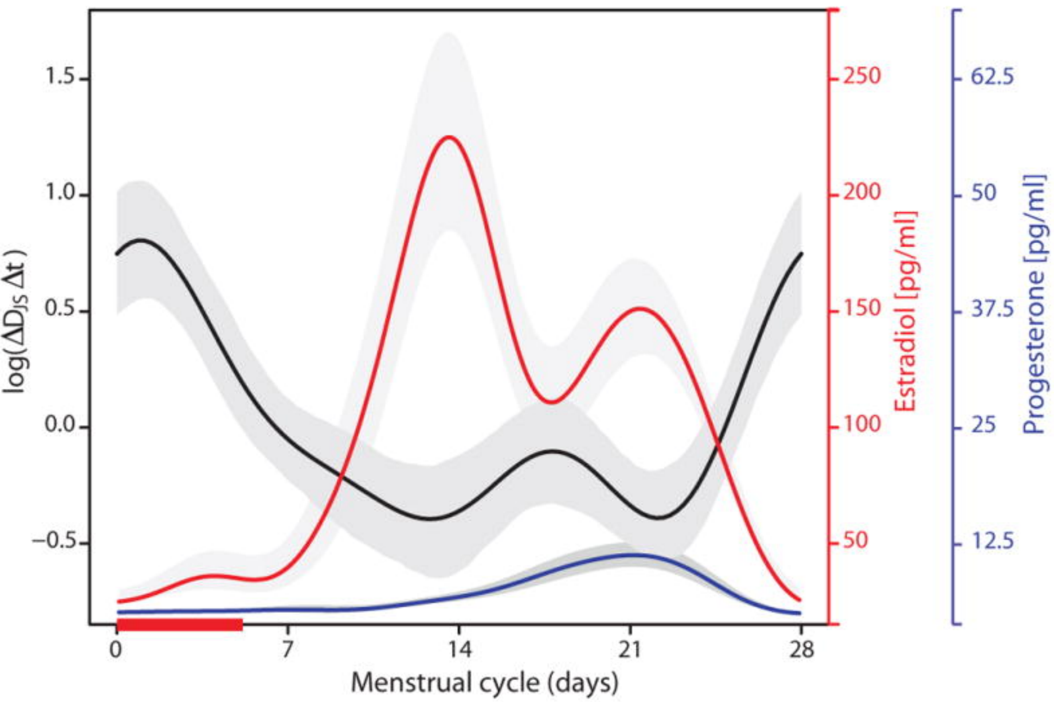


Figure 10 I Vaginal microbiota stability and sex hormones levels during the menstrual cycle. The composition of the vaginal community changes throughout the menstrual cycle, with menses representing the phase in which the microbiome is more diverse. The red bar corresponds to menses. The red line shows concentrations of oestradiol and the blue line shows concentrations of progesterone. The shaded areas around each curve show 95% point-wise confidence bands. Reprinted with permission from Gajer et al., (2012).

One longitudinal study evaluated endometrial samples from 22 fertile women at two different timepoints of the secretory phase of the menstrual cycle (prereceptive, and receptive, 2 and 7 days after the LH surge, respectively) to monitor possible changes in the microbiota at preconception. The uterine microbiome showed stable microbiota profiles during these timepoints in > 80% of patients analysed, suggesting that the endometrial microbiota is not hormonally regulated during acquisition of endometrial receptivity (Moreno et al., 2016). Later studies suggested that

proliferative and secretory phases of the menstrual cycle differ and may have their own microbiological signature. One study including endometrial samples from 95 women of reproductive age (Chen et al., 2017), and another that included 60 patients with dysmenorrhea and menorrhagia (Pelzer et al., 2018), found microbial changes in the endometrium between these two phases. The differences observed could be justified by the time elapsed between sampling. Moreno et al. took samples only five days apart within the secretory phase, where the action of oestradiol and progesterone favour a more stable microbiome (Gajer et al., 2012), while the latter studies compared proliferative and secretory phases of the cycle that are hormonally different.

Given the significant impact of menarche and menopause on the vaginal microbiome profile (Gajer et al., 2012; Muhleisen & Herbst-Kralovetz, 2016) the effects of hormonal fluctuations on the uterine microbiota should also be evaluated. In addition, the effects of environmental and behavioural factors that influence bacterial composition **(Figure 6)** should be considered. Longitudinal studies assessing the stability of uterine microbiota, during the lifecycle and in response to different stimuli, could help to anticipate dysbiotic shifts and allow the design of novel interventional strategies to restore the microbial profile.

3.4. Characterisation of the uterine microbiome profile

In recent years the uterine microbiota profile has been analysed to assess any possible role of endometrial bacteria on specific outcomes or pathologies. However, there is no clear consensus on what the physiological composition of the endometrial microbiota is. Therefore, it is difficult to define potential deviations leading to specific disorders. **Table 2** summarises the main studies performed by 16S rRNA sequencing on bacterial colonisation of the endometrium.

Table 2 | Studies characterising the uterine microbiome by 16S rRNA sequencing.

Study	Population & Objective	Ethnicity	Method & Sample type	Sequencing platform & Target 16S regions	Conclusions
Franasiak et al. (2016)	Women undergoing single embryo transfer with a euploid blastocyst (N=33) To characterise the endometrial microbiome by reproductive outcome	Caucasian: 79% Asian: 15% African-American: 3% Hispanic: 3%	Transcervical: embryo transfer catheter tips	Ion PGM™ (Thermofisher) V2,3,4,6,7,8,9	There was no difference in the endometrial microbiome between ongoing and non-ongoing pregnancy *Flavobacterium* and *Lactobacillus* were the most prevalent genus in both groups
Verstraelen et al. (2016)	Women with RIF, RPL, or both (N=19) To investigate the presence of uterine microbiota in non-pregnant women	Caucasian: 100%	Transcervical (TAO Brush™): endometrial biopsy	MiSeq (Illumina) V1-V2	In 90% of subjects the community was fairly similar, with different species of *Bacteroides* and *Pelomonas* taxa. In six cases, additional high abundance of *L. crispatus* and *L. iners* was found in the presence of *Bacteroides*
Fang et al. (2016)	Women with EP, EP+CE, and healthy asymptomatic women (N=30) To determine differences in uterine microbiota between women with EP and healthy donors	Non-reported; conducted in China	Transcervical: endometrial swabs	MiSeq (Illumina) V4	Subjects with EP and EP+CE had uterine microbiomes with higher proportions of Firmicutes (mainly *Lactobacillus*) and lower proportions of Proteobacteria (*Enterobacter* and *Pseudomonas*) than healthy subjects
Khan et al. (2016)	Asymptomatic women operated for different conditions and women with endometriosis. Both groups were further divided into GnRHa-treated and untreated (N=64) To assess impact of endometriosis on the uterine microbiome	Non-reported; conducted in Japan	Transcervical: endometrial swabs	MiSeq (Illumina) Custom primers	Microbial accumulation was detected in endometriosis patients compared to controls GnRHa treatment significantly impacted bacterial proportions. Lactobacillaceae decreased and Streptococcaceae, Staphyloccocaceae, and Enterobacteriaceae increased in GnRHa-treated versus untreated women with endometriosis
Walther-António et al. (2016)	Women undergoing hysterectomy for either benign uterine conditions, endometrial hyperplasia, or endometrial cancer (N=31) To determine the composition of the uterine microbiome and its role in endometrial cancer	Caucasian: 100%	Hysterectomy: endometrial biopsy	MiSeq (Illumina) V3-V5	The dominant bacteria in the uterine microbiome were *Shigella* and *Barnesiella* The diversity in the cancer cohort was significantly higher than in the benign cohort. Simultaneous presence of *Atopobium vaginae* and *Porphyromonas* spp. were statistically associated with endometrial cancer

Table 2 | Continued.

Study	Population & Objective	Ethnicity	Method & Sample type	Sequencing platform & Target 16S regions	Conclusions
Moreno et al. (2016)	Fertile women in prereceptive and receptive phases of the same natural cycle (N=22). Women with RIF undergoing IVF with receptive endometrium (N=35) To investigate the hormonal regulation of the endometrial microbiota and to analyse its impact on reproductive outcomes	Non-reported; conducted in Spain	Transcervical: endometrial fluid	454 Pyrosequencing (Roche) V3-V5	No variation in bacterial composition was observed in prereceptive vs receptive phase in most subjects The presence of a non-*Lactobacillus* dominated microbiota was associated with significantly decreased implantation, pregnancy, ongoing pregnancy, and live birth rates per embryo transfer
Miles et al. (2017)	Women undergoing a total hysterectomy with bilateral salpingo-oopherectomy (N=10). (5/10 premenopausal, 1/10 perimenopausal, and 4/10 postmenopausal) To determine microbial composition along the FGT	Non-reported; conducted in USA	Hysterectomy: endometrial and myometrial swabs	454 Pyrosequencing (Roche) V1-V3	The Firmicutes phylum and *Lactobacillus* genus were highly abundant along the FGT, although the composition of the microbial communities present in endometrium was quite varied
Tao et al. (2017)	Women undergoing IVF (N=70) To study the endometrial microbiome and to assess its limit of accurate quantification	Caucasian: 61% Asian: 17% African-American: 1.4% Hispanic: 5.6% Unknown: 15%	Transcervical: embryo transfer catheter tips	MiSeq (Illumina) V4	The metagenomics workflow used provides a rapid and sensitive method to identify ultra-low bacteria from embryo transfer catheter tips At genus level, *Lactobacillus* were highly abundant in the distal tip of the catheter after embryo transfer
Chen et al. (2017)	Women receiving surgery for conditions not known to involve infection (N=110) To determine microbiota along the FGT and its association with menstrual cycle, adenomyosis, and endometriosis	Asian: 100%	Laparoscopy or laparotomy: endometrial swabs	Ion PGM™ (Thermo Fisher) V4-V5	Distinct bacterial communities were found along the FGT, forming a continuum microbiota. *Pseudomonas*, *Acinetobacter*, *Vagococcus*, and *Sphingobium* constituted a notable fraction of the uterine microbiota Microbiota correlated with the menstrual cycle and were overrepresented in subjects with adenomyosis or infertility due to endometriosis
Wee et al. (2018)	Women with a history of infertility, and women with a history of fertility (N=31) To determine whether the reproductive tract microbiota differs between infertile and fertile women	Non-reported; conducted in Australia	Hysteroscopy: endometrial biopsy	MiSeq (Illumina) V1-V3	Endometrial microbiota was generally lower in relative abundance than vagina and cervix, and did not have any associated taxa for infertile women Testing for the expression of selected genes in the endometrium did not show correlation with case-control status or with microbial community composition

Table 2 | Continued.

Study	Population & Objective	Ethnicity	Method & Sample type	Sequencing platform & Target 16S regions	Conclusions
Pelzer et al. (2018)	Women undergoing hysteroscopy and/or laparoscopy (N=60) To characterise microbial community within the endometrial cavity and endocervix in women with menorrhagia or dysmenorrhea	Non-reported; conducted in Australia	Hysteroscopy or laparoscopy: endometrial curettings	454 Pyrosequencing (Roche) V5-V8	Microbial community profiling revealed differences in the endometrium compared to the endocervix, and women with menorrhagia vs dysmenorrhea Menstrual cycle dependent changes were identified within the endometrial microbiome during the proliferative and secretory phases of the cycle, and in association with exogenous progestin treatment
Moreno et al. (2018)	Women assessed for CE using histology, hysteroscopy, and/or microbial culture (N=113) To develop a molecular diagnostic tool for CE based on RT-PCR	Non-reported; conducted in Italy	Transcervical: endometrial biopsy	RT-PCR specific primers for 9 CE pathogens Ion S5 XL (ThermoFisher) V2,3,4,6,7,8,9	The molecular method proposed was similar to all 3 classic diagnostic methods together, with a degree of concordance of 77% Microbiome results using NGS were concordant with RT-PCR, and both provided additional detection of non-culturable bacteria
Kyono et al. (2018)	Women undergoing, or not, IVF, and healthy volunteers (N=109) To analyse the endometrial and vaginal microbiome among an infertile population and their impact on implantation	Asian: 100%	Transcervical: endometrial fluid	MiSeq (Illumina) V4	Endometrial lactobacilli in the IVF patients was significantly lower than that of the non-IVF patients and healthy volunteers, thus revealing a considerable percentage of NLD microbiome in IVF patients The median percentage of *Lactobacillus* in the 15 pregnant women was 96.45 ± 33.61%
Liu et al. (2018)	Women with unexplained recurrent miscarriage undergoing IVF (N=25) To compare the microbial composition of endometrial fluid and tissue samples	Non-reported; conducted in China	Transcervical: endometrial fluid and biopsy	MiSeq (Illumina) V4	The microbiota composition in endometrial fluid does not fully reflect that in endometrial biopsy The numbers of taxa identified, diversity, and evenness in fluid samples were smaller than those in tissue samples
Kyono et al. (2019)	Women undergoing IVF (N=92) To analyse the pregnancy outcomes of IVF patients, and to report cases treated for NLD microbiota	Asian: 100%	Transcervical: endometrial fluid	Non-reported	Pregnancy rates were higher in the LD group (>80% of *Lactobacillus)* compared to NLD group. *Bifidobacterium* dominancy may not have an adverse effect for implantation Nine NLD patients were treated with antibiotics and pre/probiotics and successfully became LD

Table 2 | Continued.

Study	Population & Objective	Ethnicity	Method & Sample type	Sequencing platform & Target 16S regions	Conclusions
Kitaya et al. (2019)	Infertile patients with a history of RIF, and women undergoing the first IVF (N=46) To characterise the microbiota in the endometrium and vagina in women with RIF	Asian: 100%	Transcervical: endometrial fluid	MiSeq (Illumina) V4	The endometrial microbiota had higher α-diversity and broader bacterial species than the vaginal microbiota both in the RIF and control groups *Burkholderia* species were not detected in the endometrial microbiota of any samples in the control group but were detectable in a quarter of the RIF group
Winters et al. (2019)	Women undergoing hysterectomy (N=25) To characterise the bacterial profile of endometrium and determine if it is different from that of cervix, vagina, rectum, and oral cavity	Non-reported; conducted in Italy	Hysterectomy: endometrial swabs	RT-PCR V1-V2 MiSeq (Illumina) V4	Bacterial profiles of the endometrium differed from those of the oral cavity, rectum, and vagina, but not of the cervix The bacterial profiles of the endometrium and cervix were dominated by *Acinetobacter* , *Pseudomonas* , *Cloacibacterium* , and *Comamonadaceae*
Hashimoto et al. (2019)	IVF patients undergoing vitrified-warmed blastocyst transfer (N=99) To analyse the pregnancy outcomes of IVF patients presenting eubiotic or dysbiotic endometrium at the time of embryo transfer	Asian: 100%	Transcervical: endometrial fluid	MiSeq (Illumina) V4	The pregnancy rates per transfer and miscarriage rates were comparable between patients with eubiotic or dysbiotic endometrium The endometrial bacterial profiles of pregnant cases with dysbiotic endometrium were comparable with those of non-pregnant cases
Leoni et al. (2019)	Patients with elective caesarean at the end of single physiological pregnancies (N=19) To study the uterine microbial composition during pregnancy and evaluate any possible correlation with its evolution	Caucasian: 100%	Cesarean section: endometrial biopsy	MiSeq (Illumina) V5-V6	Six common bacterial genera (*Cutibacterium* , *Escherichia* , *Staphylococcus* , *Acinetobacter* , *Streptococcus* , *Corynebacterium*) were found in the endometrial microbiota under the specific conditions examined

EC, chronic endometritis; EP, endometrial polyps; FGT, female genital tract; IVF, in vitro fertilisation; LD, Lactobacillus-dominated; NGS, next-generation sequencing; NLD, non-Lactobacillus-dominated; RIF, repeated implantation failure; RPL, recurrent pregnancy loss; RT-PCR, real-time polymerase chain reaction.

The most abundant bacteria reported in the uterine cavity belong to the phyla Firmicutes, Bacteroidetes, Proteobacteria, and Actinobacteria (Baker et al., 2018). These phyla contain several genera previously reported in the FGT. For example, Firmicutes contains genera such as *Lactobacillus*, *Streptococcus,* and *Staphylococcus*; Bacteroidetes contains genera such as *Prevotella*; Proteobacteria contains Enterobacteriaceae family members such as *Escherichia coli* and *Klebsiella pneumoniae*; and Actinobacteria contains genera such as *Bifidobacterium* and *Gardnerella*.

Like in the vaginal microbiota, the majority of studies identified *Lactobacillus* as the most prominent genus of the uterine microbiota (Fang et al., 2016; Franasiak et al., 2016; Hashimoto & Kyono, 2019; Khan et al., 2016; Kitaya et al., 2019; Kyono et al., 2019; Kyono et al., 2018; Miles et al., 2017; Moreno et al., 2018; Moreno et al., 2016; Pelzer et al., 2018; Tao et al., 2017; Wee et al., 2018). *Lactobacillus* spp. seem to be an important part of the endometrial microbiota because: (i) they were identified from the endometrium of women undergoing total hysterectomy, thus eliminating the contamination of the sample with cervicovaginal species during sample collection; (ii) they were identified in both fluid and biopsies samples; and (iii) *Lactobacillus* was the major genus detected in endometrial samples using different sequencing techniques and diverse variable regions of the 16S rRNA gene **(Table 2)**. However, the overall microbiota composition varies greatly across published studies, so whether these bacterial species are genuine members of the uterine microbiome or transient colonisers is unclear.

3.5. Challenges of analysing the uterine microbiota

Drawing conclusions or comparing results on the uterine microbiome requires consideration of the design of published studies **(Table 2)**. These considerations include: sample cohort and patient demographics, sample collection, DNA

extraction, microbial DNA amplification and sequencing, data trimming, and downstream bioinformatic analysis (Baker et al., 2018; Benner et al., 2018).

Cohort size is one of the most important limitations. Typically, sample size ranges between 10 and 40 samples per group. This may be due in part to the difficulty of recruiting patients, since endometrial sampling involves invasive techniques usually performed during surgical and exploratory procedures. A related issue is the lack of ethnic diversity registration across studies. The vaginal microbiome varies among ethnic groups (Ravel et al., 2011); therefore, larger and more inclusive studies are still needed. Another important limitation in this field is lack of healthy controls in the study design. Most studies use patients undergoing hysterectomy for benign or non-neoplastic conditions as controls, or evaluate the microbiota of infertile patients with reproductive dysfunction. The absence of exclusion criteria, such as the use of antibiotics, hormonal contraceptives, or intrauterine devices is another factor that limits the characterisation of healthy controls (Baker et al., 2018). To date, only two publications assessed the endometrial microbiome of 10 and 22 healthy asymptomatic women (Fang et al., 2016; Moreno et al., 2016), reporting differences in the identified taxa.

Apart from patient characteristics, method of sample collection is quite variable between studies since different endometrial samples (swabs, biopsies, or aspirates) can be obtained transcervically or by surgical procedures such as hysterectomy or laparoscopy. DNA extraction is another critical component in all microbiome studies because of the structural composition of microbial cell walls and the co-extraction with host cell DNA. Further, the DNA extraction process can be easily contaminated with environmental DNA of the surrounding microbiota and contaminants from reagents (named as kitome), which can bias the bacterial composition of low-biomass sites such as the uterus (Glassing et al., 2016; Kim et al., 2017; O'Callaghan et al., 2020; Salter et al., 2014). **Figure 11** represents the main taxa consistently reported as

kitome contaminants in the literature (Barton et al., 2006; Glassing et al., 2016; Grahn et al., 2003; Hashimoto & Kyono, 2019; Kitaya et al., 2019; Kyono et al., 2018; Lauder et al., 2016; Laurence et al., 2014; Stinson et al., 2019; Tanner et al., 1998; Weyrich et al., 2019).

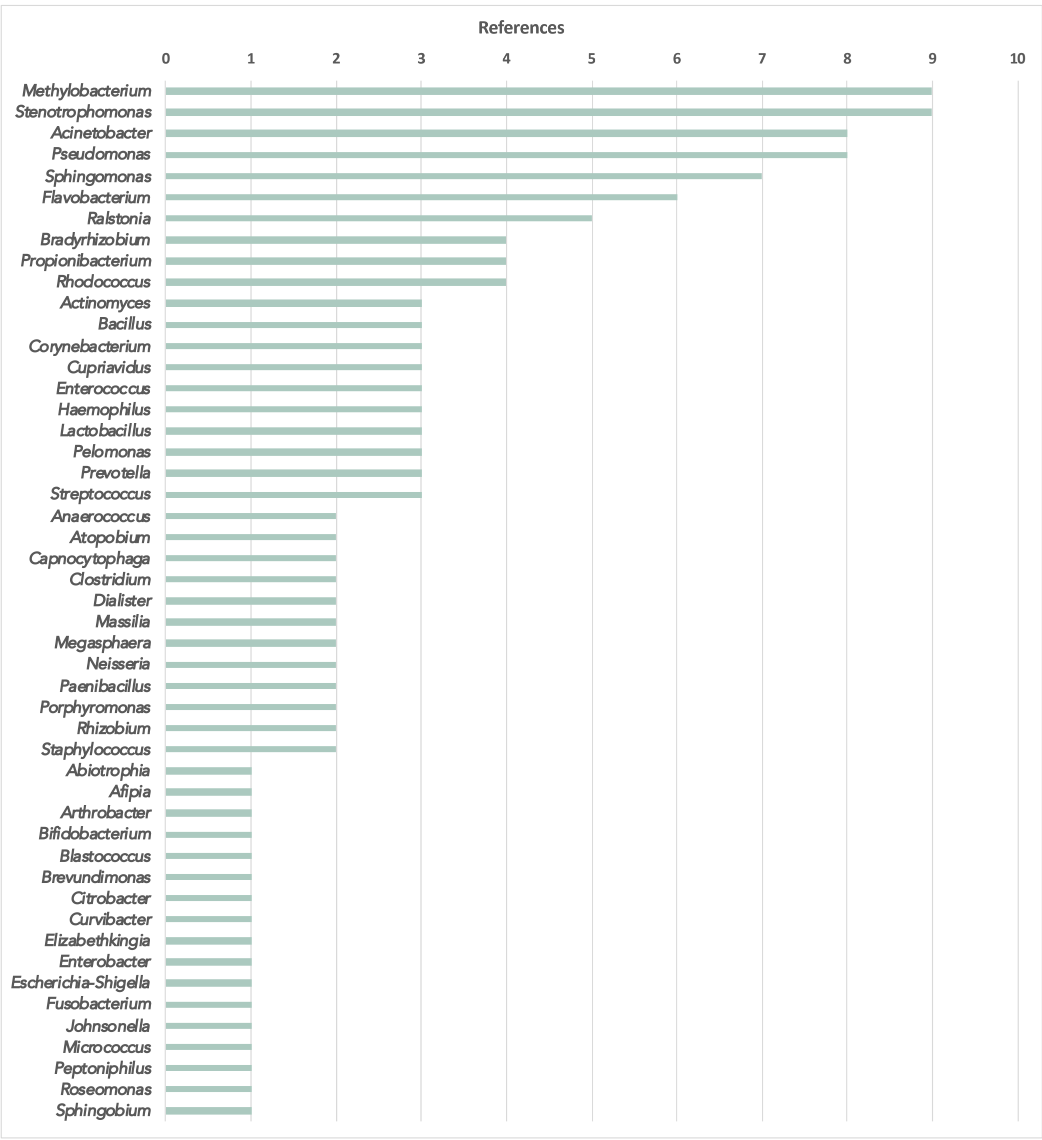


Figure 11 | Taxa previously identified as kitome contaminants from multiple studies.
Taxa identified in the negative controls of more than one study are listed. Taxa listed in this table should be treated with care to ensure that findings are not due to contamination.

For this reason, it is essential to follow strict protocols to avoid misleading conclusions. These measures include the prevention of contamination before sequencing by making best use of the protective equipment, during extraction and

sequencing by including enough negative and blank controls, and after sequencing by developing bioinformatic pipelines to identify and subtract potential contaminants from the sample (Eisenhofer et al., 2019; Weiss et al., 2014).

Another potential cause of discordance is the specific primers used for the 16S rRNA gene sequencing. The targeted hypervariable regions of the 16S rRNA gene are critical for ensuring that key genera associated with a specific anatomical niche are not accidentally excluded (Chakravorty et al., 2007; Tremblay et al., 2015). Interrogation of different hypervariable regions can generate vastly different microbial profiles, complicating comparisons between studies (Graspeuntner et al., 2018; Group, 2012). In addition, published studies to date use different sequencing procedures, platforms, bacterial databases, and downstream bioinformatic analyses that generate markedly different taxonomic profiles.

In short, there remains a need to clearly discriminate between the commensal bacteria present in the uterine cavity in physiological conditions and those coming from background noise. Moreover, to successfully compare published studies, the study design and the adoption of standardised approaches throughout sample extraction, processing, and data-analysis should be considered.

4. ROLE OF UTERINE MICROBIOME IN HUMAN REPRODUCTION

During implantation, the ability of a blastocyst to adhere and invade into uterine wall requires an adequate endometrial milieu. There is increasing evidence that endometrial microbiota could have an impact on embryo-maternal interactions at the time of implantation. For this reason, the intrauterine microbial environment makes an attractive target for investigating mechanisms by which changes in the mother can impact pregnancy success.

4.1. Uterine microbiome in assisted reproduction

During the 20th century, several groups using microbial culture of the distal tip of the embryo transfer catheter associated the isolation of *Lactobacillus* spp. with better chances of *in vitro* fertilisation (IVF) success (Moore et al., 2000; Salim et al., 2002; Verstraelen & Senok, 2005). On the other hand the isolation of endometrial pathogens, such as Enterobacteriaceae, *Streptococcus* spp., *Staphylococcus* spp., *E. coli*, and Gram-negative bacteria was associated with significantly decreased implantation and pregnancy rates (Egbase et al., 1996; Fanchin et al., 1998; Moore et al., 2000; Salim et al., 2002; Selman et al., 2007). A comprehensive study conducted in 1999 by microbial culture of the embryo transfer catheter showed that prophylactic antibiotics administered at the time of oocyte retrieval reduced the isolation of pathogens at the time of embryo transfer in 78.4% of patients. Patients who still had endometrial pathogens at the time of embryo transfer presented decreased pregnancy rates compared with those women with negative cultures or those who had responded to antibiotic therapy (18.7% vs 41.3% and 38.1%, respectively; $p < 0.01$) (Egbase et al., 1999). These results support the concept that reversibility of endometrial infections could improve reproductive outcomes in IVF patients.

More recently, several studies used sequencing technology to investigate the relationship between endometrial microbiome and ART outcomes. Franasiak and colleagues characterised the endometrial microbiome from 33 patients of different ethnicities at the time of embryo transfer with a single euploid embryo. Using this approach, *Lactobacillus* and *Flavobacterium* were the most prevalent genera identified; however, no statistically significant correlation was found between the bacterial profile and the reproductive outcome of these patients (Franasiak et al., 2016). Another publication showed differences in microbiome profiles between successful and unsuccessful IVF treatments using 16S rRNA sequencing. In this study,

Moreno and colleagues enrolled a cohort of 35 infertile Caucasian patients presenting receptive endometrium analysed with the Endometrial Receptivity Analysis (ERA) diagnostic tool. Their results showed that endometrial microbiota can be classified according to the structure and relative abundance of the bacteria identified as *Lactobacillus*-dominated microbiota (LD) (≥90%) or non-*Lactobacillus*-dominated microbiota (NLD) (<90%). Women who had a live birth had higher levels of *Lactobacillus* compared to those who had a miscarriage or who did not become pregnant, where the levels of reproductive tract pathogens (*Gardnerella*, *Streptococcus*, *Veillonella*, etc.) were higher (Moreno et al., 2016) **(Figure 12)**.

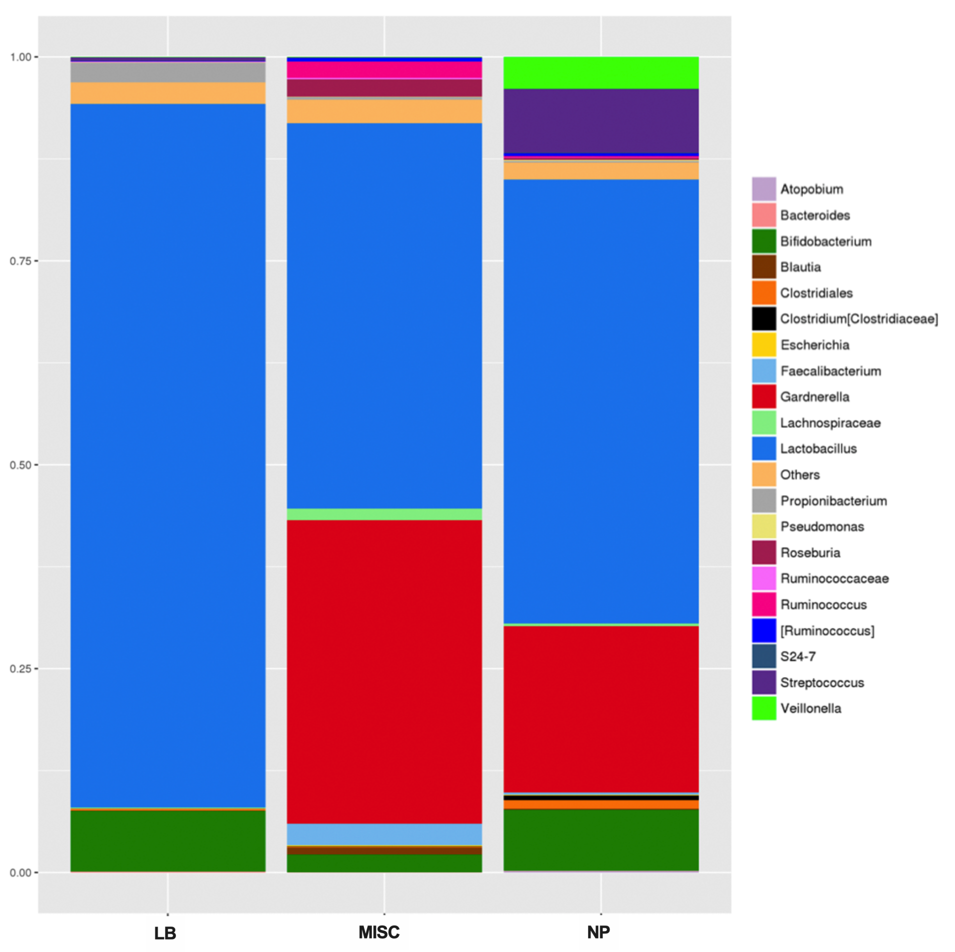


Figure 12 I Endometrial microbiome profile based on reproductive outcomes of ART patients. Bar charts showing mean values of 20 most abundant OTUs in receptive participants grouped by their reproductive outcomes: live births (LB) correspond to patients who became pregnant and successfully delivered; miscarriage (MISC) applied for those patients who became pregnant but experienced either biochemical or clinical pregnancy, and nonpregnant (NP) are patients who did not conceive. Reprinted with permission from Moreno et al., (2016).

Finally, Kyono and collaborators analysed the impact of the endometrial microbiome on the reproductive results of 92 patients undergoing IVF. Their results showed significantly higher pregnancy rates in the group dominated by *Lactobacillus* (61.30 % vs 40.00 %; p = 0.05), but they used the 80% of *Lactobacillus* as a cut-off value and proposed *Bifidobacterium*-dominant endometrium as a favourable environment for implantation (Kyono et al., 2018). In a separate study, the endometrial microbiome at the time of embryo transfer was analysed with the same bacterial classification in 99 patients undergoing IVF. Surprisingly, pregnancy rates and miscarriage rates per transfer were comparable between patients with eubiotic or dysbiotic endometrium (52.9% vs 54.8% and 11.1% vs 5.9%, respectively), and *Lactobacillus spp.* were not able to predict the reproductive outcomes of these patients (Hashimoto & Kyono, 2019).

The differences observed among the published studies may be due to the lack of standardised approaches (as described in the previous section) or even differences in the policies of each country. For example, in Japan, preimplantation genetic testing for aneuploidy (PGT-A) and oocyte donations are not allowed, and/or more than one embryo transfer is not permitted, which may affect reproductive outcomes in these studies (Hashimoto & Kyono, 2019). In addition, pathogenicity may differ according to the bacterial species detected, for example, *Staphylococcus aureus* and *Staphylococcus epidermidis* belong to the same bacterial genus but may act differently in the endometrium due to their different clinical relevance. Similarly, the level of protection against infection varies according to the species or strain of *Lactobacillus* (Kroon et al., 2018) since they differ in their ability to produce antimicrobial factors (e.g., lactic acid and hydrogen peroxide) (France et al., 2016; Tachedjian et al., 2018; Witkin et al., 2013). In that sense, analysing uterine microbiota at species-level resolution may be beneficial for identifying the true pathogenicity of bacteria and avoiding over-intervention in patients. Furthermore, not only the existence of pathogens, but also the immune status of the host itself, may be a critical

factor for presenting clinical manifestations (Campisciano et al., 2018; Nakagawa et al., 2017).

4.2. Gynaecological diseases and obstetrical complications: chronic endometritis

The impact of endometrial microbiota on several causes of infertility is under investigation to improve the clinical management of infertile patients with altered uterine microbiota. Table 2 includes some studies that characterise by 16S rRNA sequencing the endometrial microbiome of women with certain gynaecological diseases and obstetrical complications such as endometrial polyps (Fang et al., 2016), chronic endometritis (CE) (Fang et al., 2016; Moreno et al., 2018), endometriosis (Chen et al., 2017; Khan et al., 2016), adenomyosis, uterine fibroids (Chen et al., 2017), endometrial hyperplasia or cancer (Walther-António et al., 2016), and even menstrual cycle irregularities like menorrhagia or dysmenorrhea (Pelzer et al., 2018).

Among these conditions, CE is the best example of an altered endometrial microbiota, since the main cause of chronic endometrial inflammation is the presence of bacterial pathogens in the uterine cavity. The most common infectious agents responsible for CE are *Enterococcus faecalis*, Enterobacteriaceae — including *E. coli* and *K. pneumoniae* — *Streptococcus* spp., *Staphylococcus* spp., *Gardnerella vaginalis*, and *Mycoplasma* spp., as well as genital pathogens associated with STIs, such as *Ureaplasma urealyticum*, *Chlamydia trachomatis*, and *Neisseria gonorrhoeae* (Cicinelli et al., 2008; Cicinelli et al., 2009; Kitaya et al., 2017). Yet, the mere presence of infectious agents in the uterine cavity does not necessarily lead to CE; thus, it is necessary to clarify the identity and pathogenicity of microorganisms prone to establish an endometrial infection.

CE is an asymptomatic endometrial disorder characterised by superficial oedematous change, high stromal cell density, dissociated maturation between epithelium and stroma, and infiltration of plasma cells (Greenwood & Moran, 1981).

The condition occurs in 28% of infertile patients with unknown aetiology, 14%–41% of patients with repeated implantation failure (RIF), and 8%–28% of patients with unexplained recurrent pregnancy loss (RPL) (Kitaya et al., 2018). Infertile women with CE and a history of RIF have significantly lower implantation rates than those with RIF but without CE (15% vs 46%) (Johnston-MacAnanny et al., 2010). Likewise, the live birth rate in women with a history of RPL and untreated CE is poor (7%) (McQueen et al., 2014).

Mechanisms underlying poor obstetric outcomes associated with CE are not clearly understood. One hypothesis is that abnormal expression of cytokines and leukocytes may affect the immune tolerance of the endometrium to the embryo and alter endometrial vascular permeability, potentially damaging the embryo viability and the invasion of the trophoblast. Furthermore, abnormal uterine contractility during the luteal phase may inhibit *in vivo* fertilisation and affect the embryo migration before implantation. Finally, altered autophagy may impair the decidualisation of the endometrium in women with CE (Buzzaccarini et al., 2020).

There are currently no universally established diagnostic guidelines for CE. The gold standard for diagnosis relies on histology with the identification of plasma cells in the endometrial stroma using immunostaining for the plasmacyte marker CD138 (also known as Syndecan-1) (Bayer-Garner et al., 2004). Hysteroscopy of uterine cavity may also represent a reliable diagnostic technique, with 93.4% accuracy compared to histological findings (Cicinelli et al., 2005). Major hysteroscopic signs for CE include stromal oedema, focal or diffuse hyperaemia, and micropolyps (Cicinelli et al., 2005). Microbial culture is also classically used to diagnose CE. A reported 75% of women with histologically confirmed CE had positive endometrial cultures with common pathogens of the reproductive tract (Cicinelli et al., 2014). Nevertheless, the diagnosis of CE is quite challenging since the classic diagnostic methods have some limitations and discrepancies that affect the diagnosis of the disease (Moreno & Simon, 2019).

To treat those patients with abnormal endometrial microbiota, efforts are being made to restore the microbial composition by promoting *Lactobacillus* colonisation with probiotics, modulating pH, providing hormones, and eradicating pathogenic bacteria with antibiotics (Anahtar et al., 2018). Antibiotic therapies are efficient in reducing UGT infection in the majority of patients (cure rate up to 80% after a single antibiotic cycle) (Vitagliano et al., 2017). However, in some cases there is bacterial resistance to treatment (Cicinelli et al., 2015).

The benefits of antibiotic treatments on pregnancy outcomes are controversial (Kroon et al., 2012). This can be due to the lack of specificity of broad-spectrum antibiotics, which may also impair the growth of protective lactobacilli. A recent systematic review and meta-analysis evaluated the efficacy of antimicrobial therapy in patients with RIF. The results showed that antibiotic therapy is effective at eliminating the pathogens causing the infection, but also improving implantation, clinical pregnancy, ongoing pregnancy, and live birth rates. Moreover, there were no statistically significant differences in these outcomes between patients with resolved CE and those without CE (Vitagliano et al., 2018).

To further explore the causality between endometritis and reproductive failure, standardisation of the classic diagnostic methods and evaluation of microorganisms prone to establish an endometrial infection are considered a priority in clinical practice. Microbial analysis based on molecular tools could allow for the identification of both culturable and non-culturable pathogenic microorganisms associated with CE and could help to personalise treatments and improve the clinical management of infertile patients. However, more studies are needed to address the true impact of these strategies on reproductive health.

5. INTERACTIONS BETWEEN THE BACTERIAL COMMUNITY AND THE HOST

Although endometrial dysbiosis is an emerging cause of implantation failure and pregnancy loss, there is still a limited understanding of how different bacterial species could influence homeostasis of the UGT and affect reproductive outcomes. In this regard, not only are the microbial profiles leading to reproductive success or failure under investigation, but also the functional interactions between the community of microorganisms and its host are crucial to understand the effect of endometrial microbiota on human reproduction.

5.1. Mechanisms for bacterial–host interaction

Several mechanisms have been proposed to explain how bacterial cells communicate with its host **(Figure 13)** (Baker et al., 2018). On one hand, the presence of bacteria may affect host genomic stability through modulation of transcription factors and other genomic and epigenetic alterations. Also, downregulation of cell–cell junction expression is a key method of epithelial barrier breach and allows for the movement of bacteria between epithelial cells. Similarly, the degradation of the extracellular matrix by metalloproteinases also disrupts epithelial barrier integrity. On the other hand, bacteria can synthesise small molecules [i.e., short-chain fatty acids (SCFAs), reactive oxygen species (ROS), proteins, oligosaccharides, vitamins, short non-coding RNAs, neurotransmitters] that may interact with host cells in several ways. For example, due to chemical and structural similarity to human ligands, bacterial N-acyl amides may effectively bind to G protein-coupled receptors (GPCRs) and activate intracellular signalling cascades in the host (Cohen et al., 2017). GPCRs comprise the largest family of membrane receptors in humans and participate in the response to signals such as neuropeptides and essential hormones (i.e., gonadotropin-releasing hormone, luteinizing hormone, follicle-stimulating hormone, prostanoids) in the hypothalamus–pituitary–gonadal axis (Satake et al., 2013). GPCRs are pharmacologic targets of 35% of approved drugs, so the composition of the microbiota and its

derived products could interfere with drug efficacy (Sriram & Insel, 2018). Finally, inflammation triggered by TLRs activation and subsequent pro-inflammatory pathways can recruit immune cells and lead to the secretion of antimicrobial peptides and mucins that may impact bacterial colonisation (Baker et al., 2018).

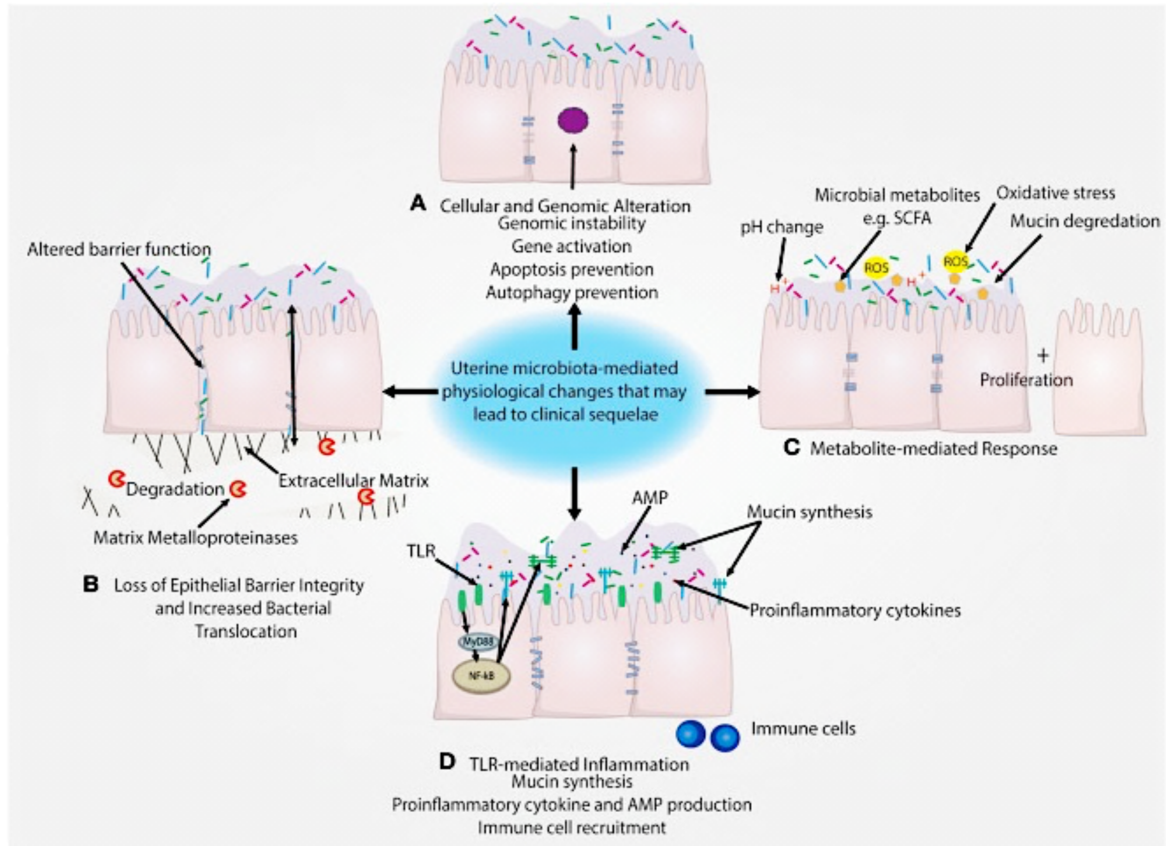


Figure 13 I Pathophysiological impact of uterine microbiome on the endometrial epithelium. Uterine microbiome may impact on endometrial epithelial health through the alteration of genomic stability, loss of epithelial barrier integrity, secretion of microbial molecules, and activation of the immune system. Reprinted with permission from Baker et al., (2018).

5.2. Role of *Lactobacillus* in the genital tract microbiota

The FGT microbiota is mainly populated by different species of *Lactobacillus* [*L. crispatus*, *L. gasseri*, *L. iners,* and *L. jensenii* (Ravel et al., 2011)]. However, there are no known genomic explanations why other *Lactobacillus* spp. with access to the urogenital tract do not colonise it. Resident species may have genetically adapted to this niche and have acquired a host-dependent lifestyle. The genomes of the

reproductive tract *Lactobacillus* spp. are significantly smaller and have lower GC content than closely-related lactobacilli residing in the gastrointestinal tract (Mendes-Soares et al., 2014). In addition, genes involved in intracellular trafficking, secretion, vesicular transport, translation, ribosomal structure and biogenesis, cell division, and chromosome repair are overexpressed in vaginal lactobacilli; while genes involved in the transport and metabolism of nucleotides, lipids, amino acids, coenzymes, and secondary metabolites are downregulated in these species (Mendes-Soares et al., 2014).

The activity of lactobacilli helps to maintain the homeostasis of the genital tract through different defensive mechanisms **(Figure 14)**. On one hand, lactobacilli can inhibit pathogens directly by co-aggregation or producing active compounds such as lactic acid, bacteriocins, and hydrogen peroxide (Younes et al., 2018). Specially, *Lactobacillus* spp. produce lactic acid and other compounds, which may acidify the vaginal pH below 4.5 and inhibit the growth of bacterial pathogens. *Lactobacillus* can also indirectly inhibit viral and bacterial pathogens by modulating the host's immune response and competitively excluding other bacteria. In the latter case, they prevent the adhesion and invasion of pathogens by adhering to epithelial cells, stimulating mucus secretion, and using the available nutrients competitively (Younes et al., 2018).

Interestingly, other mammals do not harbour *Lactobacillus* spp. in their reproductive tract microbiota, and consequentially their vaginal pH is not acidic (Miller et al., 2016). In the case of the human endometrium, despite the presence of *Lactobacillus*, no differences were found between the pH values and the bacterial composition (Moreno et al., 2016), possibly due to its low bacterial load. The uterine cavity displays different characteristics from the vagina, and it is not clear how these bacteria could promote endometrial homeostasis and influence embryo implantation. A better understanding of genetic and molecular pathways of genital tract

Lactobacillus could hold the key to fight the pathogenesis of dysbiosis and guide therapeutic interventions for patients with an altered microbiota.

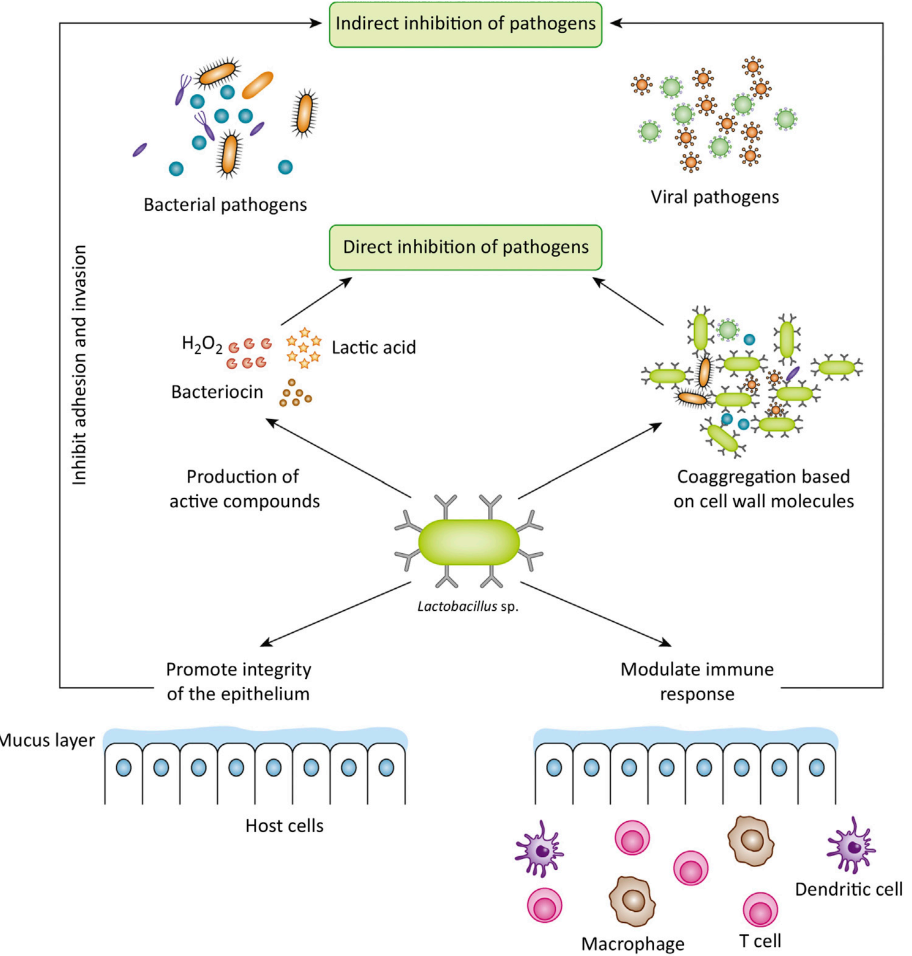


Figure 14 I Mechanism of action of *Lactobacillus* species.
Lactobacilli are able to inhibit pathogens in a direct manner by co-aggregation or production of active compounds such as bacteriocins, lactic acid, and hydrogen peroxide (H_2O_2). In addition, they can indirectly inhibit pathogens by promoting the integrity of the epithelium and modulating the immune response. Reprinted with permission from Younes et al., (2018).

5.3. Experimental assessment of the role of uterine microbiota

To elucidate host immune mechanisms in response to microbes and to develop new and more effective interventions, it is also important to recapitulate the complex environment of the FGT. In this regard, various *in vitro* and *in vivo* models have been used to assess the impact of multiple bacterial taxa on the host using synthetic combinations or patient-derived samples (Herbst-Kralovetz et al., 2016).

In vitro models represent an interesting approach to initially explore the microbiota. While a cellular layer consisting of endometrium-derived cells might be too simplistic to analyse complex interactions, this model is easy-to-use and economical. Novel models of air–liquid interface cultures and endometrial organoids are now available to assess the impact of bacterial colonisation in the UGT (Deane et al., 2017; D. Li et al., 2018; Weimar et al., 2013; Łaniewski et al., 2017). Fitzgerald and collaborators used a three-dimensional culture system to establish and generate endometrial epithelial organoids, which showed long-term expandability, responded to the reproductive hormones, and was able to be cryopreserved (Fitzgerald et al., 2019). Moreover, recent work developed multiple organoid models for a variety of endometrial diseases, providing powerful research models (Boretto et al., 2019). These systems are physiologically related to human organs/tissues and allow tight regulation of many parameters (such as pH, CO_2 partial pressure, and nutrients); however, they are complex, relatively expensive, and are still under development.

Animal models have also been used to study bacteria colonizing the genital tract. The most commonly used to study human pathogens like *C. trachomatis*, *Mycoplasma genitalium*, or *N. gonorrhoeae* are murine models of infection (Francis et al., 2018; Gondek et al., 2012; McGowin et al., 2010). Interestingly, embryo transfer in germ-free mice results in a lower rate of success compared to wild-type mice, demonstrating the relevance of the microbiota for embryo implantation and pregnancy success (Inzunza et al., 2005). Other experimental animals such as cows have provided helpful models to evaluate the impact of bacteria on fertility and reproductive outcomes, since bovine infertility due to CE results in great economic losses (Galvão et al., 2019; Peric et al., 2019; Wang et al., 2018). While this type of experimental setting is useful to assess the infectious process, these models have important physiological differences compared to the human reproductive tract. Therefore, extrapolation of the model data to the human system should be carefully considered. Ideally, combinations of *in vitro* and *in vivo* approaches may help in

understanding the role of specific bacteria colonizing the UGT. A more direct alternative to assess the role of the uterine microbiome is through the study of metadata generated from samples from women/patients, which may contribute to improving our understanding of aetiology, pathogenesis, and treatment of endometrial bacterial infections.

In short, a deeper investigation is crucial to understand the underlying processes that control the immune homeostasis and impact on pregnancy outcomes. Specifically, further studies are needed to clarify: (i) the physiological endometrial microbial composition; (ii) the distinction between harmless and pathological microbial amounts; (iii) the mechanisms of bacterial colonisation and survival in the endometrial environment; (iv) the origins and mechanisms of dysbiosis; (v) the metabolites they may synthesise and the nutrients do they need from the mucosal surface; (vi) the commensal or pathogenic relationships between microbes and endometrial cells; (vii) the signalling pathways potentially activated by these microbes; and (viii) the impact of reproductive tract dysbiosis on fertility. All this information could be important to improve the diagnosis of infectious subclinical complications and could help to develop new preventive and personalised therapeutic tools for women trying to conceive, through both spontaneous conception and ART.

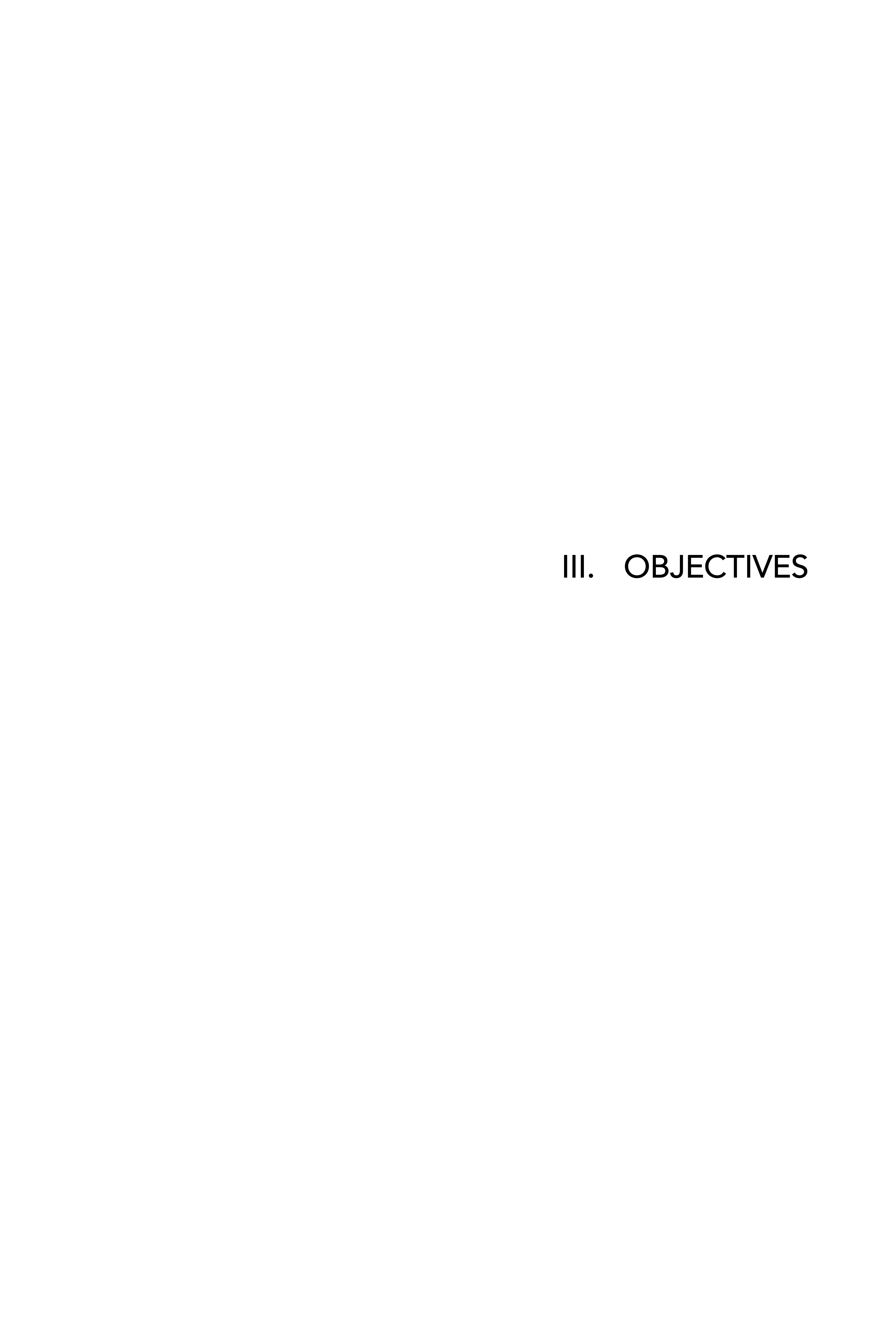
II. BACKGROUND AND HYPOTHESIS

1. BACKGROUND

Preliminary results obtained in a pilot study by our research group showed that endometrial and vaginal microbiota could differ in structure and composition in some women. These results challenged the previous dogma of the sterility of the uterine cavity and demonstrated the existence of an endogenous endometrial microbiota that was highly stable during the acquisition of endometrial receptivity. Based on its composition, the endometrial microbiota was classified as *Lactobacillus*-dominated (≥ 90% *Lactobacillus* spp.) or non-*Lactobacillus*-dominated (< 90% *Lactobacillus* spp. with > 10% of other bacteria), which was associated with reproductive outcomes in *in vitro* fertilisation patients. Specifically, the dysbiotic profile was associated with significant decreases in implantation [60.7% vs 23.1% ($p = 0.02$)], pregnancy [70.6% vs 33.3% ($p = 0.03$)], ongoing pregnancy [58.8% vs 13.3% ($p = 0.02$)], and live birth [58.8% vs 6.7% ($p = 0.002$)] rates per transfer (Moreno et al., 2016). This finding, along with other publications of the scientific community, added a novel microbiological dimension to the reproductive process.

2. HYPOTHESIS

Based on our preliminary results, we hypothesise that the presence of bacterial pathogens in the uterine cavity has a negative consequence on reproductive health. Therefore, we propose that a better understanding of the endometrial microbiome can help the clinical management of infertile patients, through the improvement of diagnostic tools and the personalisation of treatments.



III. OBJECTIVES

The main objective of the work described in this thesis was to characterise in depth the endometrial microbiome and its functional impact on the reproductive health of women undergoing assisted reproductive treatments.

To this end, the following specific objectives were addressed:

1. To assess the presence of chronic endometritis pathogens in endometrial samples using objective molecular methods and compare the results with classic diagnostic techniques (histology, hysteroscopy, and microbial culture).
2. To characterise the endometrial microbiome profile in fluid and biopsy samples using sequencing of the 16S ribosomal RNA gene and its potential impact with functional metagenomics.
3. To confirm the impact of the endometrial microbiome on embryo implantation and pregnancy through a prospective observational study.
4. To study the functional impact of the endometrial infections using an *in vitro* model of endometrial dysbiosis.

IV. MATERIALS AND METHODS

1. MATERIALS

1.1. Primers

Primers used to assess the presence of bacterial DNA by real-time polymerase chain reaction (RT-PCR) are described in **Table 3** according to the purpose of the studies performed. A literature search was conducted to identify species- and/or genus-specific primers for the bacteria of interest. Selected primers were reevaluated and subjected to Basic Local Alignment Search Tool (BLAST) using the National Center for Biotechnology Information (NCBI) genomic database. All primers were purchased from Integrated DNA Technologies (IDT, Coralville, IA, USA).

Table 3 | Primers used for RT-PCR assays.

	Bacterial taxa	Target gene	Primer sequences	Amplicon length (bp)	Reference
Molecular diagnosis of chronic endometritis	*Chlamydia trachomatis*	16S rRNA	F: GGATCCGTAAGTTAGACGAAATTTTG R: TTTAATGCGAAAGGAAATCTGATTG	83	Westhet al., 2008
	Enterobacteriaceae	rpoB	F: CAGGTCGTCACGGTAACAAG R: GTGGTTCAGTTTCAGCATGTAC	512	Arabestani et al., 2014
	Enterococcus spp.	rpoB	F: AGAGAGTAAGGTCCGATTGAAC R: GGTTGTTTCCCGTATTATGC	370	Arabestani et al., 2014
	Escherichia coli	16S rRNA	F: AGAAGCTTGCTCTTTGCTGA R: CTTTGGTCTTGCGACGTTAT	120	Lee et al., 2010
	Gardnerella vaginalis	16S rRNA	F: TTACTGGTGTATCACTGTAAGG R: CCGTCACAGGCTGAACAGT	320	Zariffard et al., 2002
	Klebsiella pneumoniae	gltA	F: ACGGCCGAATATGACGAATTC R: AGAGTGATCTGCTCATGAA	68	Clifford et al., 2012
	Mycoplasma hominis	16S rRNA	F: CATGCATGTCGAGCGAGGTT R: CCATGCGGTTCCATGCGT	129	Datcu et al., 2013
	Neisseria gonorrhoeae	16S rRNA	F: GTTTCAGCGGCAGCATTCA R: CCGGAACTGGTTTCATCTGATT	102	Hjelmevoll et al., 2006
	Staphylococcus spp.	rpoB	F: CAGGAGAAGTTAAAGAACAAGAAG R: GTGAACGAACTAATTGAGATACG	118	Arabestani et al., 2014
	Streptococcus spp.	tuf	F: GTACAGTTGCTTCAGGACGTATC R: ACGTTCGATTTCATCACGTTG	197	Picard et al., 2004

Table 3 I Continued

	Bacterial taxa	Target gene	Primer sequences	Amplicon length (bp)	Reference
In vitro dysbiosis model	*Gardnerella vaginalis*	3'16S rRNA - 5' 23S rRNA	F: TTACTGGTGTATCACTGTAAGG R: CCGTCACAGGCTGAACAGT	332	De Backer et al., 2007
	Atopobium vaginae	16S rRNA	F: CCCTATCCGCTCCTGATACC R: CAAATATCTGCGCATTTCA	86	Menard et al., 2008
	Streptococcus agalactiae	cfb gene	F: TTCACCAGCTGTATTAGAAGTACATGC R: CCCTGAACATTATCTTTGATATTTCTCA	150	Van den Brand et al., 2014
	Propionibacterium acnes	16S rRNA	F: GCGTGAGTGACGGTAATGGGTA R: TTCCGACGCGATCAACCA FAM-AGCGTTGTCCGGATTTATTGGGCG-TAMRA	131	Miura et al., 2010
Assessment of *G. vaginalis* clades	*Gardnerella vaginalis clade 1*	α-L-fucosidase	F: CCAGTCATAAGTTTGCGTTTTACC R: TGGCACTGGCAAAGTTTACAAC FAM-CTCGCCGCAAGCACCATCAAGCCA-BHQ1	139	Balashov et al., 2014
	Gardnerella vaginalis clade 2	Hypothetical protein	F: GCAAAGCAGACTGAGCGTATTAG R: GTAATAATCAGGCTCCTCATCGC 5MAXN-CGCAGGCGCTCGCATAACAGTGCA-BHQ1	124	Balashov et al., 2014
	Gardnerella vaginalis clade 3	Thioredoxin	F: TTCTGCTTCTTCTGCTATTTGCTG R: TTCGTTGACTTTTGGGCAACATG ROXN-CGGTCCGTGCCGTTCATTTGGTCC-3BHQ2	142	Balashov et al., 2014
	Gardnerella vaginalis clade 4	Chloride transporter, ClC family	F: CCTACGCAAGCTCCAGACGAC R: ACAAGTTGCACTCTTCGAGCTGG Cy5-ACTCGGCTGAAGCACACCACCACT-BHQ2	74	Balashov et al., 2014

1.2. DNA templates

A panel of DNA templates of bacteria causing CE was selected to evaluate the specificity of the RT-PCR assays. DNA templates were obtained from Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ, Braunschweig, Germany), including templates of common causal microorganisms, *E. faecalis* (DSMZ 2570), *E. coli* (DSMZ 1116), *G. vaginalis* (DSMZ 4944), *K. pneumoniae* (DSMZ 30104), *Mycoplasma hominis* (DSMZ 25592), *S. epidermidis* (DSMZ 1798), *Staphylococcus hominis* (DSMZ 20328), *Streptococcus agalactiae* (DSMZ 2134), and *Streptococcus bovis* (DSMZ 20065), as well as templates of STIs pathogens, *C. trachomatis* (DSMZ 19411) and *N. gonorrhoeae* (DSMZ 15130).

1.3. Bacterial strains and growth conditions

Different *Lactobacillus* strains (*Lactobacillus casei* BPL013; *L. jensenii* BPL016, BPL017, BPL035, BPL044; *L. rhamnosus* BPL005, *L. crispatus* CECT 4840, and *L. iners*

DSM 13335) were tested to assess their potential capacity to reduce pH levels. In collaboration with the microbiology laboratory of ADM Biopolis, standardised cultures of these strains were grown in Man, Rogosa, and Sharpe (MRS) medium anaerobically for 17 h at 37°C.

To characterise endometrial dysbiosis, an *in vitro* infection model was used, in which primary human endometrial epithelial cells (hEECs) were colonised with *Atopobium vaginae* (DSM 15829T), *G. vaginalis* (DSM 4944), *Propionibacterium acnes* (CECT 5684), and *S. agalactiae* (CECT 183) in presence/absence of *Lactobacillus rhamnosus BPL005*. In collaboration also with ADM biopolis, *L. rhamnosus BPL005* was grown using MRS medium supplemented with 0.05% (w/v) of cysteine, and incubated anaerobically at 37°C for 48 h. Pathogens were cultivated anaerobically at 37°C for 17h in brain heart infusion (BHI) medium (Oxoid, Basingstoke, Hampshire, United Kingdom), supplemented with bovine serum (10% vol/vol; Sigma-Aldrich, St. Louis, MO, USA) and cysteine (0.5% w/vol; Sigma-Aldrich, St. Louis, MO, USA) in the case of *A. vaginae* and *G. vaginalis*.

2. METHODS

2.1. Sample collection

2.1.1. Aspiration of endometrial fluid

With the patient in the lithotomy position, the vagina and cervix were cleaned. After the introduction of the disinfected speculum, a sterile and flexible catheter (the same used for embryo transfer) (Gynétics, Lommel, Belgium) was introduced into the uterine cavity avoiding any contact with vaginal walls. Endometrial fluid (EF) was aspirated with an average volume between 20 and 80 µL with a syringe of 10 mL. To prevent contamination, suction was stopped at the entrance of the internal cervical os during catheter removal. Finally, specimens were collected in sterile tubes containing 50 µL of RNAlater solution (Qiagen, Hilden, Germany), which were stored

at 4°C for 4 h, subsequently sent to Igenomix at room temperature and stored at −80°C until use.

EF aspiration is a painless and minimally invasive method that rarely generates discomfort to the patient because it is not a biopsy of endometrial tissue, but a slight suction of EF with painless cannulas that are used daily in performing embryo transfers. EF can be aspirated in the same cycle as embryo transfer is performed without negatively affecting implantation rates (van der Gaast et al., 2003).

2.1.2. Collection of endometrial biopsy

Endometrial biopsies (EB) were obtained by scraping the endometrium from the bottom of the uterus downwards with the help of a Pipelle cannula of Cornier Devices (CCD Laboratories; Paris, France) or a 3-mm Novak curette (Cooper surgical, Trumbull, CT, USA), both of them with transverse holes and chamfers in the tip. To minimise the risk that endometrial cultures might be contaminated by the vaginal flora, after placing a vaginal speculum, the cannula was inserted under visual control into the uterine cavity avoiding any contact with the vaginal walls. Once the sample was obtained (about 50-70 mg of tissue), it was transferred to a cryotube containing 1.5 mL of RNAlater solution (Qiagen, Hilden, Germany), which was stored at 4°C for 4 h, subsequently sent to Igenomix at room temperature and stored at −80°C until use. For the primary culture of hEECs in the *in vitro* colonisation model, endometrial samples were shipped fresh in transport medium.

EB was performed without anaesthesia. Risks and complications associated with this technique are rare, with the most frequent being a slight pain after the introduction of the cannula or a scant spotting after the biopsy. Nonetheless, in several cases the following complications have been described: uterine or cervical perforation, genital infection, or prolonged bleeding (Will & Sanchack, 2020).

2.2. Genomic DNA isolation

2.2.1. DNA extraction from endometrial fluid

EF samples were enzymatically pre-digested at 37°C for 30 min with 25 µg/µL lysozyme, 0.12 U/µL lysostaphin, 0.4 U/µL mutanolysin, and 1.8% Triton X-100, to degrade the cell walls of bacteria. DNA extraction was then performed with QIAamp DNA Blood Mini kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. Briefly, 20 µL of proteinase K and 200 µL Buffer AL were added to the samples, which were incubated for 30 min at 56°C and immediately for 15 min at 95°C. DNA was precipitated with ethanol, samples were passed through elution columns and washed with AW1 and AW2 buffers. Finally, the DNA was eluted with 35 µL of nuclease-free water, and quantified using a photometric technology (Nanodrop, Waltham, MA, USA).

2.2.2. DNA extraction from endometrial biopsy

Total DNA was isolated from the EB samples by performing a pre-digestion step for difficult-to-lyse bacteria. For this digestion, 25 mg of tissue were cut into small pieces and treated with proteinase K at 56°C for 3 h under agitation. Then, samples were mixed with ATL buffer and disrupted mechanically in a TissueLyser LT for 5 min at 50 Hz, using stainless-steel beads of 5-mm diameter (all acquired from Qiagen, Hilden, Germany). After these pretreatments, bacterial nucleic acids were purified using the QIAamp cador pathogen mini kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. Briefly, 20 µL of proteinase K and 100 µL Buffer VXL were added to the samples, which were incubated for 15 min at 25°C and immediately treated with 350 µL of ACB buffer. Finally, samples were passed through elution columns and washed with AW1 and AW2 buffers before the final elution with 50 µL of nuclease-free water. The eluted DNA was quantified using a photometric technology (Nanodrop, Waltham, MA, USA).

In the prospective study, to extract bacterial DNA from EB samples, the DNA tissue program V7-200-LC of the QIAsymphony equipment (Qiagen, Hilden, Germany) was used following the manufacturer's instructions. In this case, the DNA was eluted with 50 µL of nuclease-free water and was quantified using MultiskanGO (Thermo Scientific, Waltham, MA, USA).

2.3. Classic diagnostic techniques of chronic endometritis

2.3.1. Histological diagnosis

Histological examination of endometrial tissue was performed by two pathologists from the University of Bari (Italy). Paraffin-embedded endometrial specimens were used for the study. Samples were fixed overnight in 4% formaldehyde (in phosphate buffer, pH 7.3; Nacalai Tesque, Kyoto, Japan) and embedded in paraffin (Nacalai Tesque, Kyoto, Japan). Microsections of 5 µm were stained with hematoxylin-eosin. Inflammatory status of the endometrium was graded based on the presence of stromal infiltrate dominated by lymphocytes and plasma cells and a spindle cell change of stromal cells (Resta et al., 2012). Infiltration showing aggressive behaviour against glands, inflammatory cell-gathering inside glands, or a more structured infiltration was also relevant. Micropolypoid vegetations formed in the endometrium are macroscopic evidence of an inflammation-related process and can be used as markers for this pathology. All cases diagnosed with CE by histological criteria were confirmed by antisyndecan-1 (CD138) immunohistochemistry as previously described (Resta et al., 2012). This immunohistochemistry method of plasma cells detection is globally accepted as the gold-standard diagnostic method of CE.

2.3.2. Hysteroscopic diagnosis

All hysteroscopies were performed by two physicians from University of Bari (Italy) using a lens-based 2.7-mm outer diameter mini-telescope with a 105-degree angle

of visual field equipped with a 4.5-mm outer diameter double-flow operative sheath (Karl Storz, Tuttlingen, Germany). Hysteroscopies were performed in the follicular phase (days 7–12 of a natural cycle). Saline was employed to distend the uterine cavity at a pressure generated by a simple drip from a bag suspended 1 m above the patient. A 300-W light source with a xenon bulb and a high-definition digital camera (Karl Storz, Tuttlingen, Germany) were used. During hysteroscopy, both the anterior and posterior uterine walls were thoroughly examined by passing the hysteroscope parallel to the endometrial surface to identify any surface irregularity. The following criteria were used for the hysteroscopic diagnosis of CE: the presence of stromal oedema, focal or diffuse periglandular hyperaemia, and micropolyps of <1 mm in size (Cicinelli et al., 2005; Cicinelli et al., 2010). Finally, the hysteroscopic images were reviewed and diagnosed in the patient database at the Second Unit of Obstetrics and Gynaecology, Department of Biomedical and Human Oncological Science, University of Bari (Italy).

2.3.3. Microbial culture diagnosis

Endometrial tissue samples were processed according to the current standard for microbiological culture, using a separate test to detect the presence of the most prevalent CE pathogens. To detect the presence of culturable microorganisms (*E. faecalis*, *E. coli*, *K. pneumoniae, S. aureus*, *S. agalactiae*, *Streptococcus mitis*, and yeasts), the EB samples were inoculated onto culture media either directly or following enrichment in BHI medium. These media consisted of columbia-colistin-nalidix acid agar with 5% sheep blood, which is a selective and differential medium commonly used in the isolation of gram-positive organisms from mixed clinical specimens, and MacConkey agar and mannitol salt agar for the isolation of Gram-negative bacteria and *S. aureus*. Bacterial growth was considered positive when > 10,000 colony-forming units (CFU) were isolated from the culture. In the case of a positive culture, antibiotic sensitivity and resistance were tested by Vitek 2

(BioMérieux, Marcy-l'Étoile, France). According to microbiology guidelines, the presence of *S. epidermidis* was considered to indicate contamination. On the other hand, to detect non-cultivable strains recoverable from the genital tract such as *C. trachomatis*, *N. gonorrhoeae*, *U. urealyticum*, *Ureaplasma parvum*, and *M. hominis*, a multiplex RT method (AnyplexTM II STI-7 Detection [V1.1]; Seegene, Seoul, Republic of Korea) was used according to the manufacturer's instructions.

2.4. Molecular detection of endometrial microbiota

2.4.1. Real-time polymerase chain reaction

2.4.1.1. Molecular diagnosis of chronic endometritis

For the identification of CE pathogens by RT-PCR, specific primers for the nine most common bacteria responsible for causing CE were used (*C. trachomatis*, *Enterococcus* spp., *E. coli*, *G. vaginalis*, *K. pneumoniae*, *M. hominis*, *N. gonorrhoeae*, *Staphylococcus* spp., and *Streptococcus* spp.) **(Table 3)**. All RT-PCR reactions were performed on a LightCycler 480 II (Roche Diagnostics, Almere, Netherlands). Reaction mixtures contained 200 ng of DNA isolated from EB or commercial purified bacterial DNA as a template, 2.5 mmol/L $MgCl_2$, 0.25 µmol/L forward and reverse primers, and 1 µL of the LightCycler FastStar DNA Master SYBR Green I 10x (Roche Applied Science, Mannheim, Germany) resulting in a final reaction volume of 20 µL. The cycling program was as follows: an initial denaturation at 95°C for 10 minutes; amplification for 45 cycles of 10 seconds at 95°C, 10 seconds at 57°C, and 50 seconds at 72°C; melting curve analysis for 5 seconds at 95°C, 15 seconds at 65°C, and a temperature continuous acquisition up to 95°C (ramp rate of 0.11°C/s); and cooling for 30 seconds at 40°C.

Each experiment included negative and positive controls to determine any possible contamination and unspecific amplification. Positive controls included a mix of all the bacterial DNA templates at a concentration of 10,000 genomes each (using

as a reference 10,000 CFU from the culture, and considering that each genome has one single copy of the 16S rRNA gene). Negative controls consisted of pools of all DNA templates except the microorganism to be evaluated for each assay. A melting curve analysis was also made to distinguish the targeted PCR products from non-specific PCR products. The concentration of microorganism in each sample was calculated comparing the crossing point-PCR-cycle (Cp) values obtained from the sample with the Cp values of the positive control. The genome size of each bacterium was used for the calculation of genomes/ng of DNA (Zariffard et al., 2002).

2.4.1.2. Evaluation of bacterial growth in an in vitro colonisation assay

To quantify the inhibitory potential of *L. rhamnosus BPL005* over selected populations (*A. vaginae*, *G. vaginalis*, *P. acnes*, and *S. agalactiae*), the growth of pathogens in the infection assays was quantified by RT-PCR using primers described in **Table 3**. PCR amplification and detection were performed in a StepOne Real-Time PCR System with the aid of SYBR Green PCR Master Mix and Taqman Master Mix in the case of *P. acnes* quantification (All from Applied Biosystems, Foster City, CA, USA). Data were analysed with StepOne software. In all cases, standard curves were constructed with DNA coming from 10-fold diluted cell-standardised reference cultures.

2.4.1.3. Molecular assessment of Gardnerella clades

For the identification of clade-specific genes of *Gardnerella* (clade 1: α-L-fucosidase, clade 2: hypothetical protein, clade 3: thioredoxin and clade 4: chloride transporter, CIC family) **(Table 3)** a Multiplex TaqMan RT-PCR was performed as previously reported (Balashov et al., 2014). This was done in 20 μL reactions containing 1×PrimeTime Gene Expression Master Mix protocol (IDT, Coralville, IA, USA), 800 nM each DNA primer, 100 nM each TaqMan probe, and 3.2 μL DNA extracted from EF samples. Cycling parameters were 50°C for 2 min for uracyl-N-

glycosidase (UNG) treatment and 95ºC for 3 min for initial denaturation, followed by 40 cycles of denaturation at 95ºC for 15 s and annealing plus extension at 60ºC for 1 min with fluorescence acquisition at the end of each cycle. QuantStudio 5 Real-TimePCR System (Thermo Fisher Scientific, Waltham, MA, USA) was used for the RT-PCR. As a positive control, DNA from *G. vaginalis* strain ATCC 14018 (Clade 1) was included at concentrations ranging from 0 to 10^6 genome copies.

2.4.2. 16S rRNA gene sequencing

Endometrial microbiota profiles were obtained by NGS using the Ion 16S metagenomics kit (Thermo Fisher Scientific, Waltham, MA, USA). This kit includes two primer sets (V2-4-8 and V3-6, 7-9) that selectively amplify seven of the nine hypervariable regions of the bacterial gene encoding for the 16S ribosomal subunit, allowing the identification of a broad range of bacteria within a mixed population.

After amplification of the hypervariable regions with 10 µL of extracted DNA (per set of primers) and 30 PCR cycles, the library was prepared starting from 50 ng of the pooled short amplicons using the Ion Plus Fragment Library kit and Ion Xpress Barcode Adaptors following the manufacturer's instructions. The library concentration was adjusted using Ion Universal Library Quantitation Kit and QuantStudio 5 Real-Time PCR System. Diluted individual libraries were then pooled for amplification by emersion PCR in Ion OneTouch 2 System (10 pM) or Ion Chef System (30 pM). Finally, libraries were sequenced on Ion S5 XL system using the Ion 530 Chip (all acquired from Thermo Fisher Scientific, Waltham, MA, USA) **(Figure 15)**.

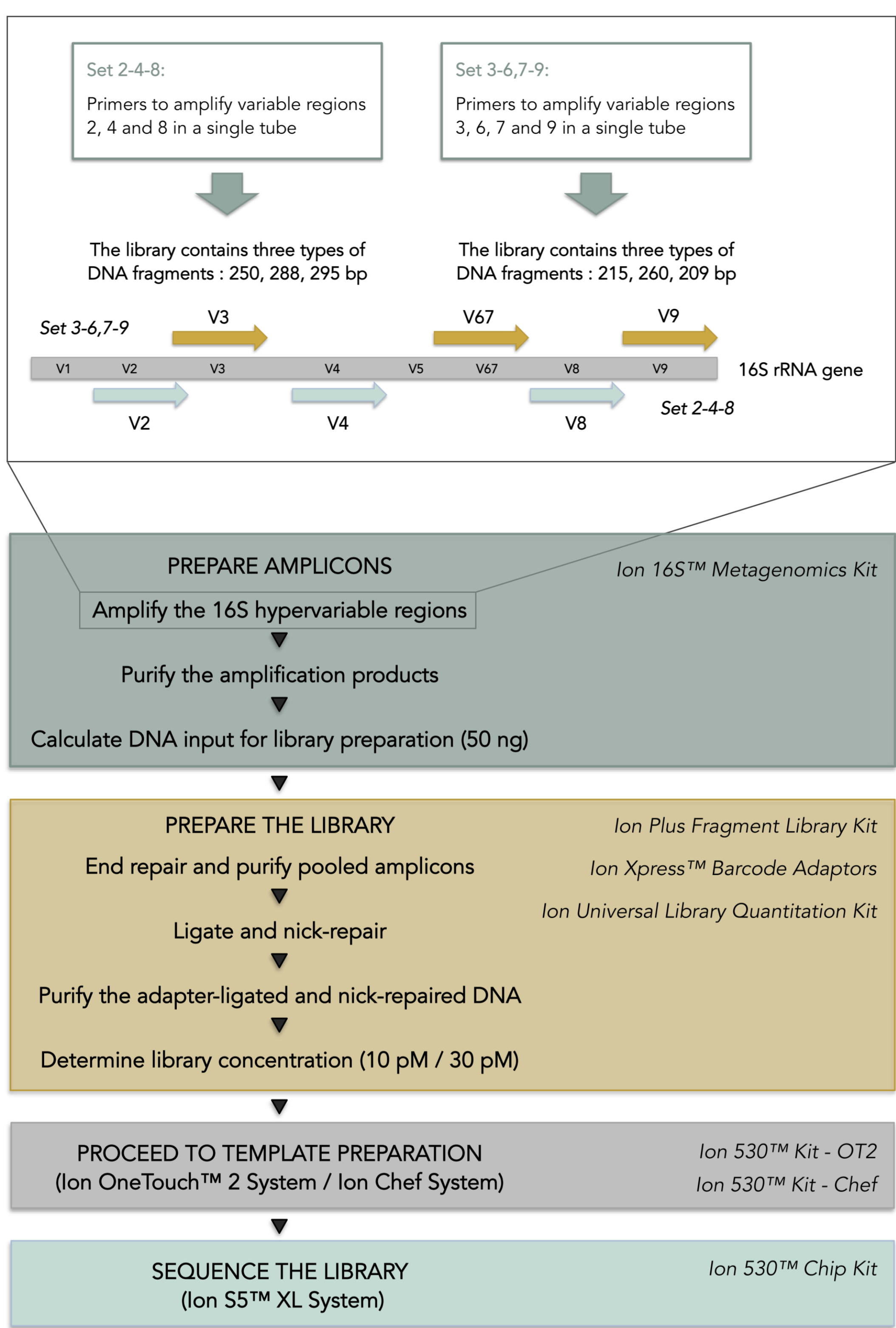


Figure 15 | Ion 16S™ Metagenomics Kit workflow.

The 16S metagenomic kit includes two primer sets that simultaneously amplify seven of the nine hypervariable regions of the 16S rRNA gene. Then, the library is prepared starting from 50 ng of amplicons using Ion Plus Fragment Library kit. Finally, after the template amplification and enrichment with the OT2/Chef System, the library is sequenced on the Ion S5 XL system.

To detect contamination, each sequencing run included between two and four blank samples, and negative and positive PCR controls. The blank samples consisted of an aliquot of the sample preserving solution (RNA later; Qiagen, Hilden, Germany), while positive and negative PCR controls were pure microbial DNA of E. coli (3 ng) and nuclease-free water, respectively (ThermoFisher Scientific, Waltham, MA, USA).

2.4.3. Whole metagenome sequencing

Endometrial microbiome functional composition was assessed by WMS using the Nextera DNA Flex Library Preparation kit (Illumina, San Diego, CA, USA) following the manufacturer's instructions. This kit is characterised by on-bead tagmentation chemistry that achieves a uniform tagmentation reaction and combines DNA fragmentation and adapter ligation steps into a single reaction, reducing the library preparation time **(Figure 16)**.

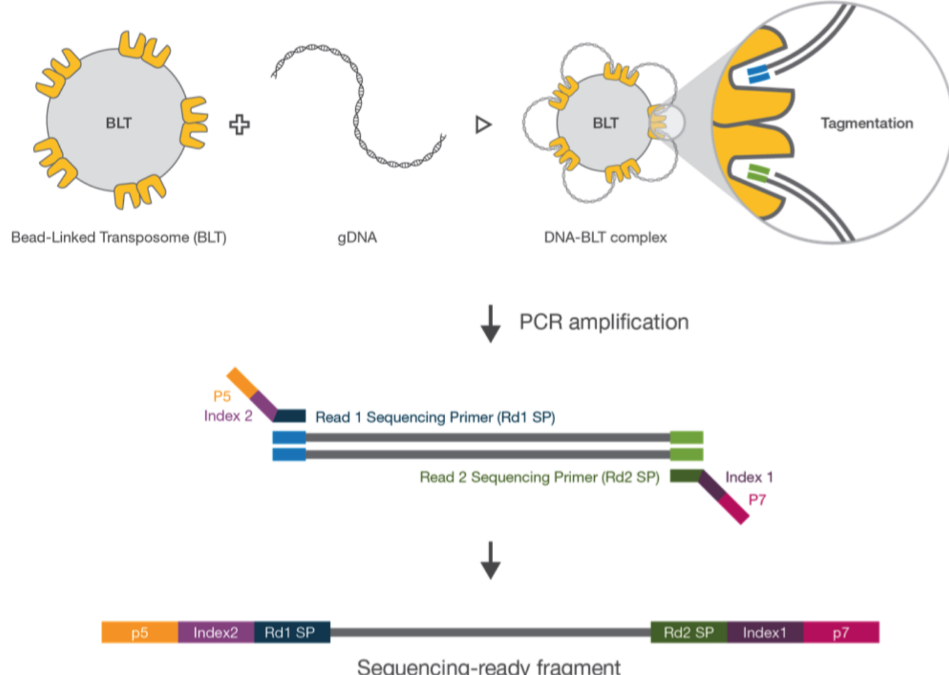


Figure 16 I Nextera bead-linked transposome chemistry.
Bead-linked transposomes mediate the simultaneous fragmentation of DNA and the addition of Illumina sequencing primers. Then, PCR amplifies sequencing ready DNA fragments and adds indexes and adapters. Reproduced from https://emea.illumina.com/.

The recommended cycles for the bead-linked transposome PCR were adjusted based on sample input concentration and quality, resulting in dual-indexed final libraries. Libraries were analysed for concentration, pooled, and denatured for loading onto a flow cell for cluster generation. Finally, denatured libraries were sequenced on the NextSeq 500 system (Illumina, San Diego, CA, USA).

2.5. Bioinformatic sequencing analysis

2.5.1. 16S rRNA gene sequencing analysis

The 16S rRNA sequencing results were analysed using the QIIME 2.0 package (https://qiime2.org) and RDP classifier 2.2 for taxonomic assignment along with the Greengenes database version 13.8 (http://greengenes.second.genome.com), an update of version 13.5 released to address missing genus and species names. For all the processed samples, the RDP classifier is run with the default minimum confidence estimate of 0.5 to record an assignment (i.e., an OTU). The results were generated using the default parameters as in the original QIIME 2 (Bolyen et al., 2019) and RDP (Wang et al., 2007) methods, as documented in the respective scientific publication.

In the prospective study, the obtained data were transformed in log-ratio data using the centered log-ratio (clr) transformation (Aitchison, 1986) making them symmetric and linearly related, and thus potentially avoiding spurious correlations and sub-compositional incoherencies (Calle, 2019; Gloor et al., 2017; Pawlowsky-Glahn et al., 2015). Then, identification and removal of contaminant sequences were done using decontam R Bioconductor package (Davis et al., 2018), using prevalence in blank samples as the method used for statistical identification of contaminant taxa. Additionally, in this work, quality parameters such as the percentage of empty reads, the dispersion index, and the ratio between filtered and mapped reads were considered to classify the samples as detectable or non-detectable biomass based

on the filtered versus mapped reads ratio threshold of 0.65 in EF and 0.7 in EB samples. Only samples with a detectable microbiota were included in the study.

For the prospective study, in each dataset of endometrial 16S rRNA profiles, the abundance of each taxon was also analysed, and low-abundant species were removed from further consideration. We retained taxa that either (1) exhibited an abundance of at least 1% in 5% of the samples or (2) exhibited an abundance of at least 0.1% in at least 15% of samples (Fettweis et al., 2019). Taxa that failed to meet both criteria were removed. Genera not colonizing humans or associated with kitome contaminants were also removed from the analysis. Specifically, those taxa consistently reported as contaminants in more than 5 of the 11 published references were excluded **(Figure 11)** (Barton et al., 2006; Glassing et al., 2016; Grahn et al., 2003; Hashimoto & Kyono, 2019; Kitaya et al., 2019; Kyono et al., 2018; Lauder et al., 2016; Laurence et al., 2014; Stinson et al., 2019; Tanner et al., 1998; Weyrich et al., 2019).

2.5.2. Correlation network analysis

In the prospective study, to further analyse the EF and the EB microbiota composition, co-occurrence bacterial networks were built for these two microbiota data sets (i.e., EF and EB) and for each outcome condition (i.e., live birth, no pregnancy, biochemical pregnancy, and clinical miscarriage). Pearson matrices for network analysis were generated using the Scipy Stats package (version 1.4.1) and visualised with Networkx (version 2.4) considering taxa with significant Pearson correlation coefficient (Li et al., 2008). To account for both the correlation and significance, each edge was assigned a weight w computed as: abs (corr) – pval, where abs (corr) was the absolute correlation value between each pair of taxa and pval the corresponding p-value. Negative correlations were represented as red edges.

2.5.3. Bayesian inference for reproductive outcome differences

Differences between *Lactobacillus* and other reproductive tract taxa were modelled using Bayesian inference following a normal distribution with mean μ_outcome and dispersion **σ**_outcome for each reproductive outcome.

$$Y_{ij} \sim N\ (\mu_outcome, \boldsymbol{\sigma}_outcome)$$

Priors selected to estimate parameters were normal distribution for μ_outcome centered on *Lactobacillus* and others mean difference from dataset and a dispersion of 10 (μotucome~N($\bar{x}$,10)) and, for **σ**_outcome, vague continuous distribution from 0 to 10 (**σ**_outcome~Uniform (0,10). Markov chain Monte Carlo (MCMC) sampling for posterior distribution parameter inference and predictive probability analysis were implemented using PyMC3 Python library.

2.5.4. Taxa reference ranges

To evaluate the potential reproductive impact of the whole bacterial community, confidence intervals (CI) established for patients with live birth were used in the prospective study. The log-ratio transformed 16S rRNA abundances of each taxon in live birth samples were used to define reference ranges for each taxon, since we consider that the detected levels of bacteria found in the samples from women having a live birth should not be a problem for a successful pregnancy. A 95% CI was calculated to establish reference ranges for Student's t-distribution taxon distribution model (Almonacid et al., 2017). Scipy Stats package (version 1.4.1) was used for calculations (Virtanen et al., 2020).

Statistical inference between taxonomic contributions to different outcome conditions were calculated using two-sided Mann-Whitney U-test. The distance between samples outside range and upper/lower bounds reference values was used to assess taxonomic association to each outcome. Taxa were reported when

significant difference was observed between upper and lower distance distributions. Scipy Stats package (version 1.4.1) (Virtanen et al., 2020) was used for statistical methods and Plotly to visualise significant taxonomic distributions.

2.5.5. Whole metagenome sequencing analysis

Reads generated in WMS sequencing were quality-trimmed and length-filtered using PRINSEQ (Schmieder & Edwards, 2011). Then, paired-end reads were merged using Fast Length Adjustment of Short reads (FLASh) software tool (Magoč & Salzberg, 2011), and finally, host-reads were removed using Burrows-Wheeler Aligner (BWA) mapper against human genome reference (Li & Durbin, 2010).

For the first clinical case, functional and taxonomic profiling was obtained using the co-assembly procedure from SqueezeMeta automatic pipeline (Tamames & Puente-Sánchez, 2018). This method pools reads from all samples, and a single shared assembly is defined. ORF prediction and annotation are retrieved by homology searching onco-assembly contigs using Kyoto Encyclopedia of Genes and Genomes (KEGG) (Kanehisa & Goto, 2000) reference databases. Gene abundance was estimated mapping individual reads to the reference co-assembly contigs annotation.

For the second clinical case, functional and taxonomic joint profiling was performed using the HMP Unified Metabolic Analysis Network (HUMAnN2) pipeline (Abubucker et al., 2012). This method combines taxonomic profiling of samples using MetaPhlAn2 (Truong et al., 2015), which provides a panmicrobial annotation, using a combination of clade-specific markers and functional annotation inferred by the pangenomic database resulting from MetaPhlAn2 taxonomical classification. Another annotation to assess taxonomical classification robustness was obtained using the KRAKEN software with complete bacterial, archaeal and viral NCBI Reference Sequence (RefSeq) genomes database MiniKraken DB_4GB (Wood & Salzberg,

2014). The presence of biomedical interest protein families, such as GPCRs ligand producers, was assessed with InterProScan 5 and PFAM reference protein database (Jones et al., 2014; Punta et al., 2012). Finally, pipeline outputs were processed using the R statistical software (R Development Core Team, 2008) for statistical description and graphical representation of the sample's taxonomic and functional profile.

2.6. *In vitro* model of endometrial bacterial colonisation

2.6.1. Selection of *Lactobacillus* strains

To select a potential probiotic strain to improve FGT based on its capacity to initially lower pH and promote the reduction of pathogenic bacteria, we studied the capacity of different lactobacilli strains to reduce pH levels. To do that, standardised cultures of the selected strains were grown (see section 1.3), and final pH was measured with the aid of a pH meter. Assays were performed in triplicate.

2.6.2. Primary cultures of human endometrial epithelial cells

EB samples were mechanically disaggregated, digested with collagenase type A1 (Sigma-Aldrich, St. Louis, MO, USA) and subjected to gravity sedimentation to separate the epithelial and stromal fractions as previously described (Simón et al., 1997). Epithelial fraction was plated in 24-well plates and cultured in hEEC medium (75% DMEM, 25% MCDB-105, 10% FBS, 5 pg/mL insulin, and 0.1% fungizone and gentamicin) until they reached a confluence of 80–90%. Then, cells were washed twice with DMEM basal media (Sigma-Aldrich, St. Louis, MO, USA) to deplete from residual antibiotic and cultured in antibiotic-free media for an additional 8 h before colonisation assays.

2.6.3. Colonisation Assays

Bacterial cells were cultured individually (see section 1.3) and collected by centrifugation at 4,000 rpm for 15 min. Supernatant was discarded, and pellet washed

with saline solution (2 mL). Bacteria concentration was adjusted based on optical density measurements to 10^6 CFU/mL in the case of *Lactobacillus* and 10^4 CFU/mL for pathogens. One mL of bacterial suspensions was added to each well containing confluent hECCs and incubated at 37ºC anaerobically for 18 h. Tested conditions included individual colonisation of hEECs with *L. rhamnosus* BPL005 or pathogens (*A. vaginae*, *G. vaginalis*, *P. acnes*, and *S. agalactiae*) and all the different combinations of *Lactobacillus* with each pathogen. Purity of the *in vitro* co-culture was checked microscopically. Aliquots of supernatant were recovered and centrifuged (13,000 rpm, 15 min), and both pellet and supernatant were stored at −20°C until use. Each condition was tested in experimental duplicates and in three biological replicates.

2.6.4. Evaluation of pH-reducing capacity

After the colonisation assay, the pH levels were measured with an MI-170 pH probe (Microelectrodes Inc, Bedford, NH, USA) in all the conditions tested. In addition, negative controls without bacteria and controls without cells were included in each experiment to better measure the pH variations induced by the bacteria tested.

2.6.5. Quantification of secreted inflammatory molecules

Twenty-five µL of the spent culture media from *in vitro* colonisation of hEECs with bacteria were analysed for the secretion of cytokines, chemokines, and growth factors (GM-CSF, HB-EGF, IFN gamma, IL-1β, IL-1 RI, IL-1RA, IL-6, IL-8, MCP-1, and RANTES) using Luminex R Screening Assays Catalog# LxSAH (R&D SystemsTM, Minneapolis, MN, USA) following manufacturer's instructions. Briefly, the filter-bottomed microplate was first wet with 100 µL of Wash Buffer. Then, 50 µL of the microparticle cocktail and 50 µL of sample (diluted 1:1) were added to each well incubating for 2 h at room temperature under agitation. Immediately, 50 µL of diluted Biotin Antibody Cocktail was added to all wells incubating for 1 h and then 50 µL of diluted

Streptavidin-PE was incubated for another 30 min. Between incubations wash procedures were performed three times. Finally, 100 µL of wash buffer were used to resuspend the microparticles. Concentration of each cytokine was calculated by interpolation with the standard curves generated for each analyte. Each sample was measured in duplicate for the three biological replicates. Fluorescence was read on a Luminex 200 Multiplexing Instrument.

3. EXPERIMENTAL DESIGNS

3.1. Study 1: Diagnosis of endometrial infections by molecular methods

3.1.1. Study design

Endometrial tissue samples from 113 women with infertility undergoing classic CE diagnosis using endometrial histology, hysteroscopy, and/or microbial culture were blindly evaluated for the presence of CE pathogens by RT-PCR. Patients were diagnosed for CE using the three classic diagnostic techniques following the usual routine of the clinic. In parallel, frozen EB specimens were analysed by RT-PCR to detect the presence of the nine most common bacteria responsible for causing CE (*C. trachomatis*, *Enterococcus* spp., *E. coli*, *G. vaginalis*, *K. pneumoniae*, *M. hominis*, *N. gonorrhoeae*, *Staphylococcus* spp., and *Streptococcus* spp.). Sensitivity, accuracy, positive and negative predictive values, and false-positive and -negative rates of the molecular analysis vs the classic diagnostic techniques were assessed. Endometrial samples of 10 negative controls (based on histology and microbial culture diagnosis) were also evaluated to assess the diagnostic specificity of the proposed molecular method. Finally, to confirm the molecular results obtained by RT-PCR, the endometrial microbiome was evaluated using 16S rRNA sequencing in patients with concordant diagnosis by the three classical techniques.

3.1.2. Study participants and ethical committee

Participants involved in this study were 21- to 53-year-old patients with infertility recruited at the Second Unit of Obstetrics and Gynaecology, Department of Biomedical and Human Oncological Science, University of Bari (Italy) to undergo a diagnosis of CE. The group of negative controls consisted of women undergoing surgery for benign ovarian conditions (oophorectomy for dermoid cyst with exclusion of endometriosis) or for myomas (myomectomy) (n = 6), and women treated with antibiotics for previous CE with no current signs of the disease at the time of sample collection by either histology or microbial culture (n = 4).

The ethical committee of the Second Unit of Obstetrics and Gynaecology, Department of Biomedical and Human Oncological Science, University of Bari (Italy), approved the study (register number 4880). All women signed an informed consent form agreeing to the comparative analysis of the techniques performed for the routine diagnosis of CE vs the molecular diagnosis.

3.1.3. Statistical analysis

Comparative assessment of the molecular test vs the classic diagnostic methods (considered the gold standard) were calculated as follows (Pewsner et al., 2004):

- Sensitivity: percentage of true-positive cases among the total positives in the gold standard test.
- Specificity: percentage of true-negative cases among the total negatives in the gold standard test.
- Accuracy: percentage of correct assessments on the total assessments.
- Positive predictive value: percentage of true-positive cases among the total positives in the molecular test.
- Negative predictive value: percentage of true-negative cases among the total negatives in the molecular test.

- False-positive rate: percentage of false-positive cases among the total negatives in the gold standard test.
- False-negative rate: percentage of negative cases among the total positives in the gold standard test.

3.2. Study 2: Characterisation of the endometrial microbiome in infertile women and its impact on reproductive outcomes

3.2.1. Study design

This was an international, observational, multi-center, and competitive cohort study. Patients recruited were women with infertility undergoing ART with indication of ERA test, available from Igenomix (https://www.igenomix.es/servicios/era-analisis-receptividad-endometrial/), to diagnose the state of the endometrium during the window of implantation and determine the optimal time frame for embryo transfer. Therefore, in all patients a personalised embryo transfer (pET) with frozen blastocyst-stage embryos (day 5/day 6) was performed according to clinical standard of care (Díaz-Gimeno et al., 2011; Ruiz-Alonso et al., 2013; Simón et al., 2020).

Before the EB for the ERA test, in a hormone replacement therapy (HRT) cycle after 120 h of progesterone exposure, an EF sample was aspirated for the analysis of the endometrial microbiota using 16S rRNA sequencing. A small portion of the EB obtained for the ERA test was also used for the study of the bacterial communities. Patients continued their ART standard protocol and received the pET on the day recommended by the ERA test result. For those patients in which a second biopsy was required for the recommendation of the pET, the collection of EF sample was also repeated. Then, association studies were performed to evaluate the potential impact of the bacterial composition in EF and EB on the reproductive outcomes of these patients. Finally, the results obtained for both types of samples were compared **(Figure 17)**.

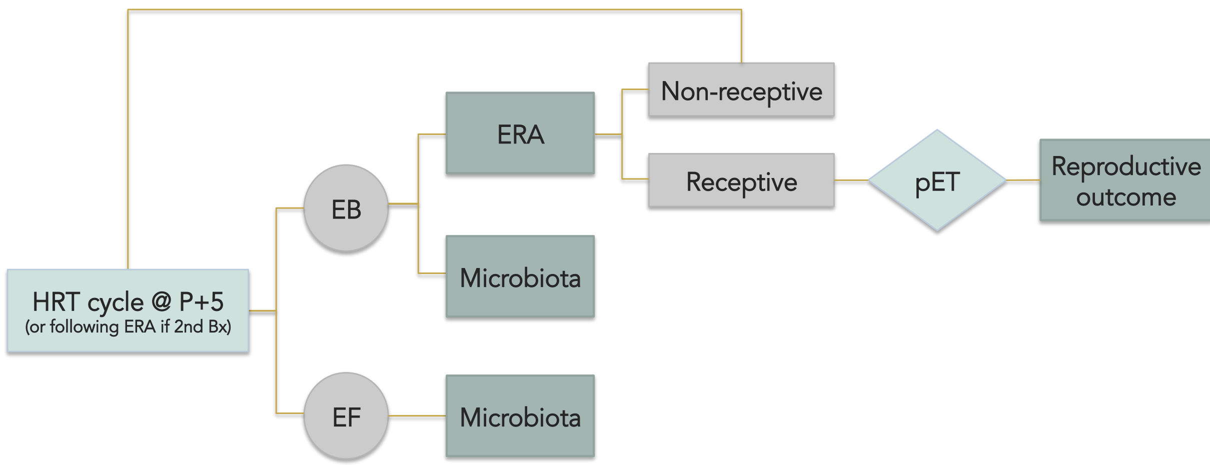


Figure 17 I Workflow of samples and variables analysed.

EF was obtained by aspiration on the day of EB for ERA test, after five days of progesterone treatment during HRT cycle. If the patient was non-receptive, the collection of samples was repeated on the day indicated by the ERA result in a new substituted cycle. Samples were used for the study of endometrial microbiome by 16S rRNA sequencing and their impact on the reproductive outcomes. EB, endometrial biopsy; EF, endometrial fluid; ERA, endometrial receptivity analysis; HRT, hormone replacement therapy; P, progesterone; pET, personalised embryo transfer.

3.2.2. Study participants and ethical committee

Infertility patients in intracytoplasmic sperm injection (ICSI) or ovum donation treatments with indication of ERA test, who received pET with frozen blastocyst-stage embryos (day 5/day 6) on an HRT cycle were included in the study. The inclusion criteria were: maternal age ≤40 years for IVF/ICSI patients or ≤50 years for ovum donation patients; body-mass index (BMI) of 18.5-30 kg/m^2 (both inclusive); negative serological tests for human immunodeficiency virus, hepatitis B virus, hepatitis C virus, and syphilis; regular menstrual cycles (3-4 / 26-35 days); and sperm concentration > 2 million sperm/mL. Exclusion criteria were: carriers of intrauterine devices or patients who have taken prescribed antibiotics in the last three months previous to the sample collection (patients that received prophylactic antibiotics for egg retrieval one month previous to sample collection were accepted to participate in the study); presence of uncorrected adnexal or uterine pathologies as uterine malformations; patients with severe or uncontrolled bacterial, fungal or viral infections, or any illness

or medical condition that is unstable or can put patient's safety or study compliance at risk.

From August 2017 to February 2019, 452 participants were recruited from 13 reproductive clinics in Europe, America, and Asia. As this was a competitive study, each center was recruiting patients and sending their samples to Igenomix until the estimated sample size of the study was completed. Ethical approval was given by the corresponding local Ethics Committees to the protocol with reference IGX1-MIC-CS-17-05 as follows: Western Institutional Review Board (IVF-Florida, USA, study code 1176556, July 17, 2017; RMA Connecticut, USA, study code 1177331, July 31, 2017; Dominion Fertility, USA, study code 1176555, July 22, 2017; Missouri Center for Reproductive Medicine, USA, study code 1179405, October 10, 2017; Pacific Centre for Reproductive Medicine, Canada, study code 1179667, December 13, 2017); Comite de Etica de la Investigación con Medicamentos del Hospital Universitario de la Princesa (ProcreaTec, Spain, register number 3133, July 13, 2017); Comite de Etica de la Investigacion Costa del Sol (Clinica Fertia, Spain, code 006_jun17_PI-ERA-Microbioma, June 29, 2017); Comite de Bioetica del Instituto de Investigaciones Clinicas Rosario (Gestanza Medicina Reproductiva, Argentina, March 27, 2018); Comite de Etica en Investigación Centro de Educacion Medica e Investigaciones Clinicas "Norberto Quirno" (Pregna Medicina Reproductiva, Argentina, October 13, 2017); Human Medical Research Ethics Committee (HMREC) for Private Healthcare and Research Centres Malaysia (Alpha Fertility Center, Malaysia, October 3, 2017); Oak Clinic Group's Ethics Committee (Oak Clinic Sumiyoshi, Japan, May 29, 2017); Comite de Etica en Investigación New Hope Fertility Center (New Hope Fertility Center, Mexico, Register number RA-2017-04, June 9, 2017); Uskudar Universitesi Girisimsel Olamayan Arastirmalar Etik Kurulum (Bahceci Group, Turkey, January 25, 2018). All participants provided written informed consent. Data were monitored by clinical research associates through on-site and/or remote visits, and the database was properly curated.

3.2.3. Statistical analysis

Analyses on an intention-to-treat and per-protocol basis were conducted for all data. One-way analysis of Variance (ANOVA) was used to compare non-categorical variables among groups by reproductive outcomes. Mean differences and standard deviation or median and interquartile ranges were used when the variables were not homogeneous, as well as the mean difference with 95% CI values. Categorical variables were described by counts (n) and percentages (%), and Chi-square test and two-sided Fisher's exact test were used to compare among groups by reproductive outcomes with respect to percentages. Multiple-comparison post-hoc correction (Bonferroni) was applied for all-pairwise comparisons. All analyses were conducted using SPSS 25 software (IBM, MD, USA) and R version 3.6.3 (The CRAN project).

3.3. Study 3: Functional evaluation of endometrial infections by an *in vitro* model

3.3.1. Study design

To investigate the mechanism by which pathogenic bacteria can affect embryo implantation, an *in vitro* model of bacterial colonisation in endometrial epithelial cells was performed. First, primary cultures of hEECs were established using individual EB from asymptomatic women, to model as closely as possible the normal endometrial epithelium. Primary hEEC cultures were then cultured to confluence and colonised with pathogenic or dysbiotic bacteria previously associated with NLD microbiota (*A. vaginae*, *G. vaginalis*, *P. acnes*, and *S. agalactiae*) alone or in combination with *L. rhamnosus BPL005* for 18 h in anaerobic conditions. Subsequently, the supernatant of the culture was used to measure pH and inflammatory molecules, while the pellet containing the bacterial cells was used to assess the inhibitory potential of *Lactobacillus* over selected populations by RT-PCR **(Figure 18)**.

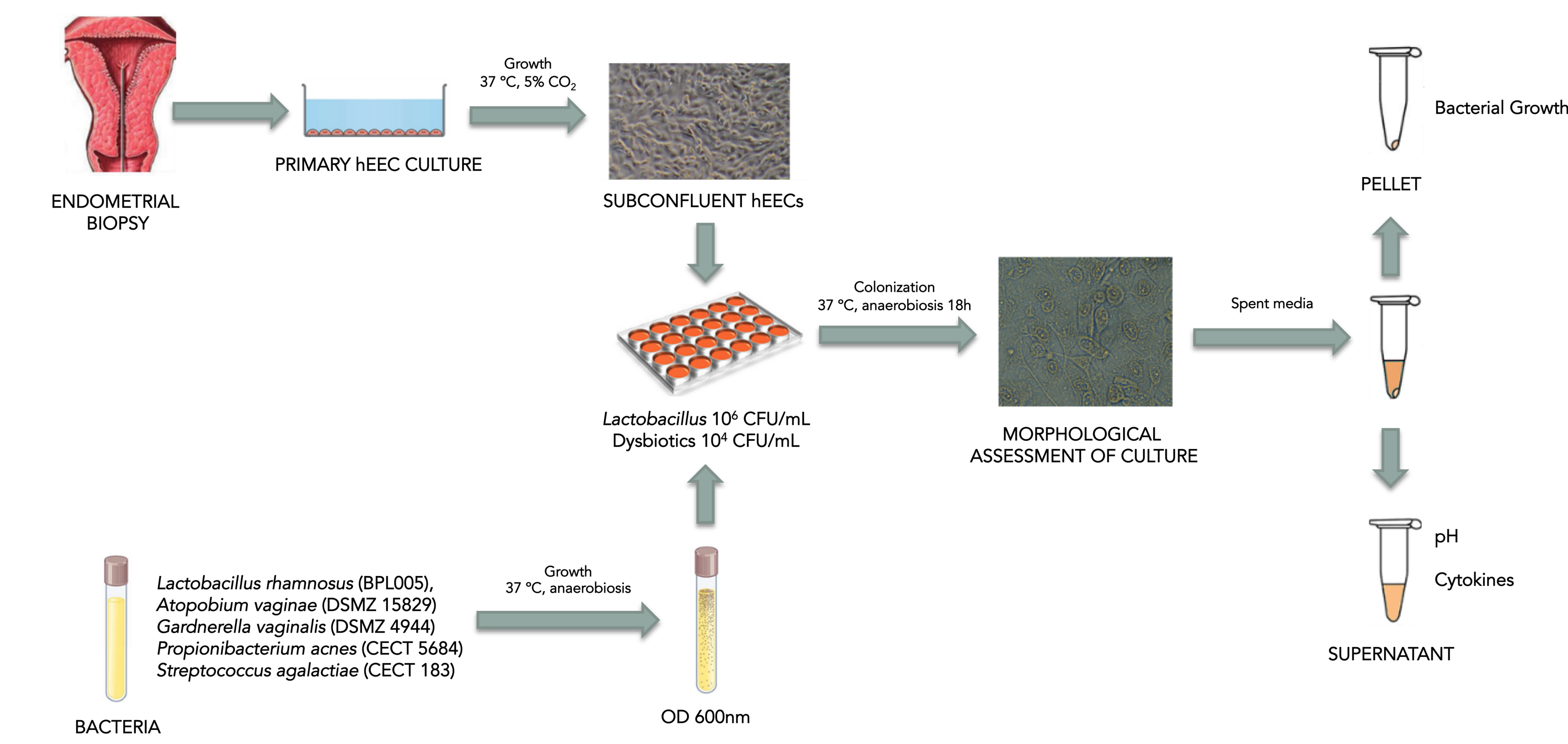


Figure 18 | *In vitro* model of bacterial colonisation of endometrial epithelium mimicking LD vs NLD conditions.

Primary cultures of hEECs were established and colonised with pathogenic or dysbiotic bacteria previously associated with NLD microbiota (*A. vaginae*, *G. vaginalis*, *P. acnes*, and *S. agalactiae*) alone or in combination with *L. rhamnosus* BPL005 for 18 h in anaerobic conditions. The supernatant of the culture was used to measure pH and inflammatory molecules, while the pellet was used to assess the bacterial growth. CFU, colony-forming unit; hEECs, human endometrial epithelial cells; NLD, Non-*Lactobacillus*-dominated; OD, optical density

3.3.2. Study participants and ethical committee

Endometrial tissue was obtained at day 15 of the menstrual cycle from healthy donors aged 18–35 years old. Participants diagnosed with endometriosis and/or endometritis were excluded. The study was carried out in accordance with the recommendations of the local Ethical Committee at IVI Valencia (Spain). All participants gave written informed consent in accordance with the Declaration of Helsinki. The protocol was approved by the local Ethical Committee at IVI Valencia, Spain (study code: 1404-FIVI-015-CS).

3.3.3. Statistics

Statistical analyses were performed with IBM SPSS Statistics version 22 software, with application of univariant ANOVA and Dunett's post-hoc test. Significance levels were established at p-value ≤ 0.05.

3.4. Studies 4 and 5: Functional evaluation of endometrial microbiota in infertile patients

3.4.1. Study design

To investigate the functional impact of the endometrial microbiota on fertility, two patients with infertility underwent microbiological follow-up analysing EF samples by 16S rRNA sequencing and WMS. In both cases, 16S rRNA sequencing was used to evaluate taxonomically the composition of endometrial microbiota, while WMS was included to further understand the functionality of the bacteria detected, the antibiotic resistance, and its impact on reproductive health.

The first clinical case describes the follow-up over 18 months of a patient with repeated reproductive failures (one ectopic pregnancy and two early clinical miscarriages), during which the endometrial microbiota was evaluated in a total of six

EF samples. The second clinical case describes the follow-up of an infertile patient during which the endometrial microbiota was evaluated in two EF samples: before an embryo transfer that resulted in a clinical miscarriage and, as an incidental case, during the fourth week of a gestation that yielded a live birth.

3.4.2. Study participants and ethical committee

We included two patients with infertility undergoing ART with repeated reproductive failures and indication of endometrial receptivity diagnosis by ERA test, who received embryo transfer with frozen blastocyst-stage embryos (day 5/day 6). The criteria for inclusion and exclusion of patients are shared with those described in section 3.2.2.

Patients were participating in a biomedical research study to evaluate the impact of endometrial microbiome on reproductive outcomes. This study was approved by the local ethical committee at Instituto Valenciano de Infertilidad (Federalwide Assurance number: FWA00027749) with protocol number 1606-IGX-044-CS. Patients provided written informed consent for the aspiration of the EF samples and the subsequent publication of the cases.

V. RESULTS

1. DIAGNOSIS OF ENDOMETRIAL INFECTIONS BY MOLECULAR METHODS

1.1. Process and evolution of the study

Endometrial samples from 113 patients with infertility subjected to classic CE diagnosis using histology, hysteroscopy, and/or microbial culture were blindly evaluated for the presence of pathogens most frequently causing CE by targeted RT-PCR.

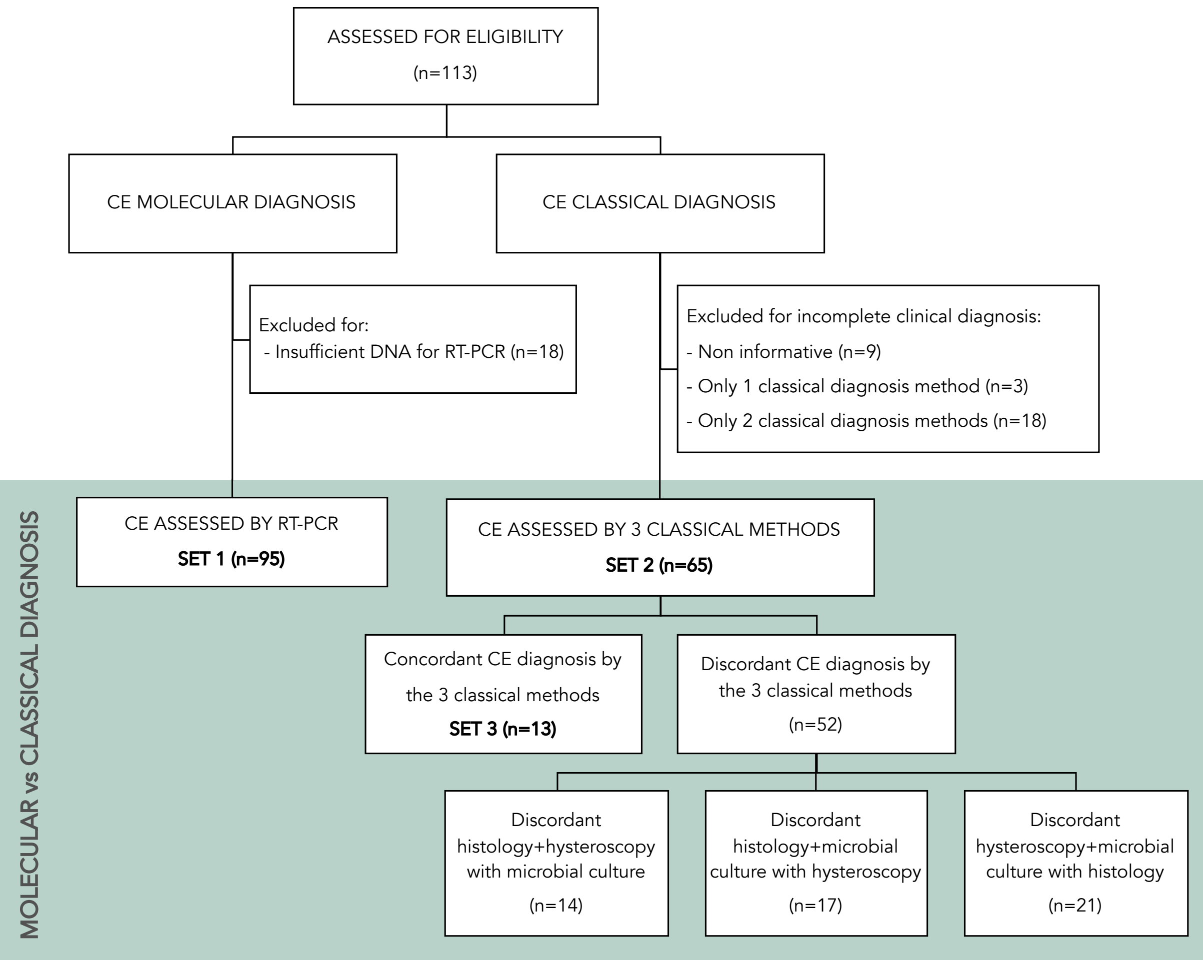


Figure 19 I Diagram of study and distribution of population investigated.
Endometrial samples from 113 patients subjected to classic diagnosis of CE by histology, hysteroscopy, and/or microbial culture were blindly evaluated by RT-PCR to detect the presence of CE pathogens. The molecular analysis and the classic diagnostic techniques were finally compared in 65 patients with CE results assessed by all three recognised classic methods. CE, chronic endometritis; RT-PCR, real-time polymerase chain reaction.

Eighteen patients were excluded from the molecular analysis because their samples did not yield enough standard-quality DNA for analysis, leaving a total of 95 samples assessed by RT-PCR (set 1). Then, the results of the molecular diagnosis were compared with the classic diagnostic methods. For this comparison, 30 patients were excluded for incomplete classic diagnosis (due to non-informative cases or lack of results in some of the techniques). Sensitivity and specificity of the molecular analysis were evaluated in 65 patients assessed by all three classical methods (set 2). Finally, the comparison was focused on the only 13 samples with concordant classic diagnosis (set 3) **(Figure 19)**.

1.2. Set-up and analytical performance of RT-PCR assays

The analytical specificity of the molecular microbiology assay was verified using specific primers for the most common bacteria causing CE (*C. trachomatis*, *Enterococcus* spp., *E. coli*, *G. vaginalis*, *K. pneumoniae*, *M. hominis*, *N. gonorrhoeae*, *Staphylococcus* spp., and *Streptococcus* spp.), with 30 ng of commercial bacterial DNA as a template.

All primer sets resulted in a specific amplification of their own bacterial DNA templates, with Cp values ranging from 12–20 compared to the negative control of ultrapure water. When each primer pair was tested against the other bacterial DNAs, insignificant cross-reactivity was detected between them, with a minimum difference of 10 amplification cycles between the specific and non-specific amplifications **(Figure 20)**.

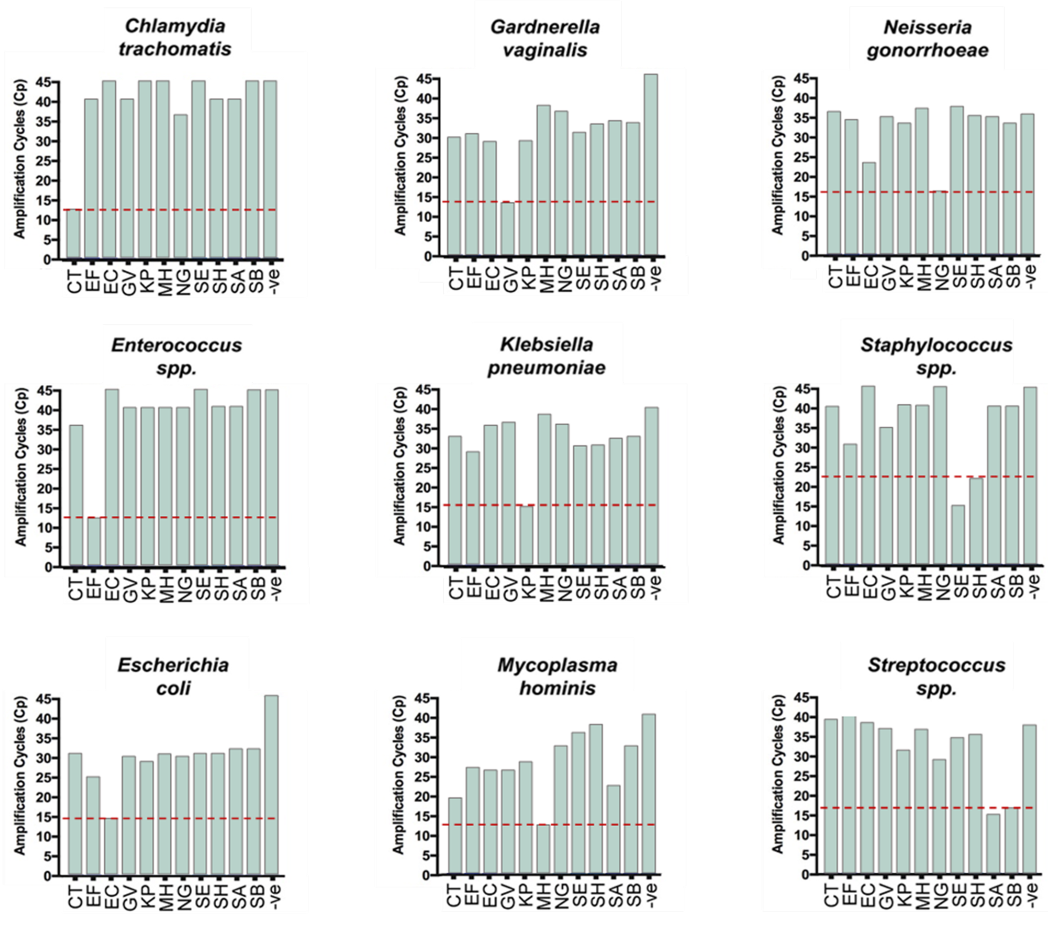


Figure 20 I Analytical specificity of molecular microbiology test.

Amplification cycles (Cp) of commercial bacterial DNA of most common bacteria causing CE using specific primers for these bacteria. CT, *C. trachomatis*; EC, *E. coli*; EF, *E. faecalis*; GV, *G. vaginalis*; KP, *K. pneumoniae*; MH, *M. hominis*; NG, *N. gonorrhoeae*; SA, *S. agalactiae*; SB, *S. bovis*; SE, S. *epidermidis*; SH, *S. hominis*; -ve, negative control.

The limit of detection of each microbe in the RT-PCR was assessed by amplifying increasing amounts of each DNA template (0–1,000,000 genomes) alone or in a complex mixture of DNA from all bacteria included in the molecular method. The detection limits of these RT-PCR reactions showed the high sensitivity of the molecular method for the different bacteria tested. The limits ranged from 10–1,000 genomes for all microorganisms, except for staphylococci, which showed a minimum detection limit of 10,000 genomes, equivalent to the microbial culture technique in which > 10,000 CFU is considered a positive test **(Figure 21)**.

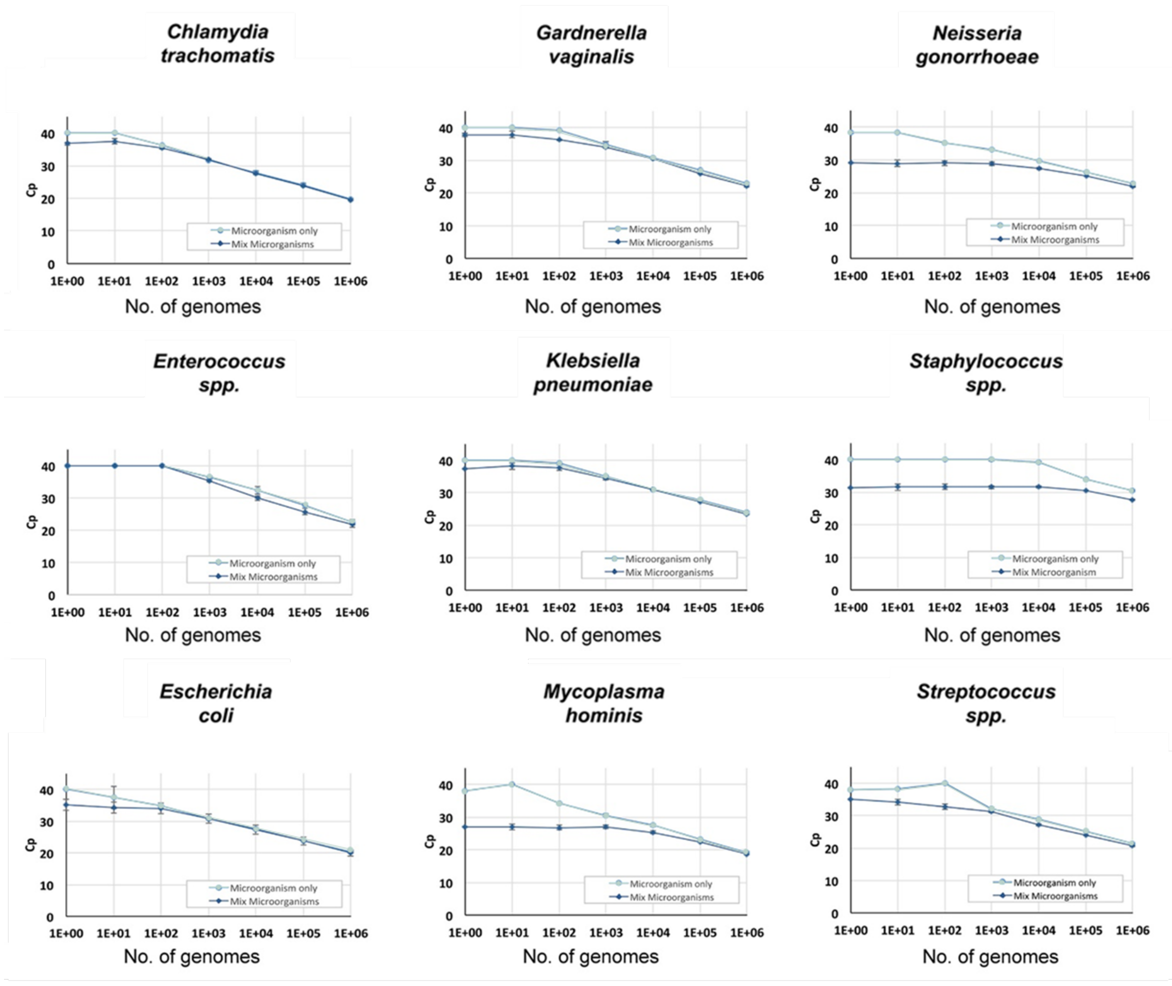


Figure 21 I Detection limit of microorganisms included in the molecular microbiology test. Detection limit of each bacteria tested in the RT-PCR using increasing amounts of bacterial DNA alone or in a mix of all bacteria included in the molecular method.

The positive control was a mix of all the bacterial DNA templates at a concentration of 10,000 genomes each, while the negative control was a mix of all DNA templates except for the bacterial DNA to be detected. For each independent assay, the melting peak profile and melting temperature (Tm) were determined. The amplified signal for each microorganism was considered positive when the threshold Cp obtained was equal or lower than the Cp value of the positive control and the Tm matched with that established in the positive control **(Figure 22)**.

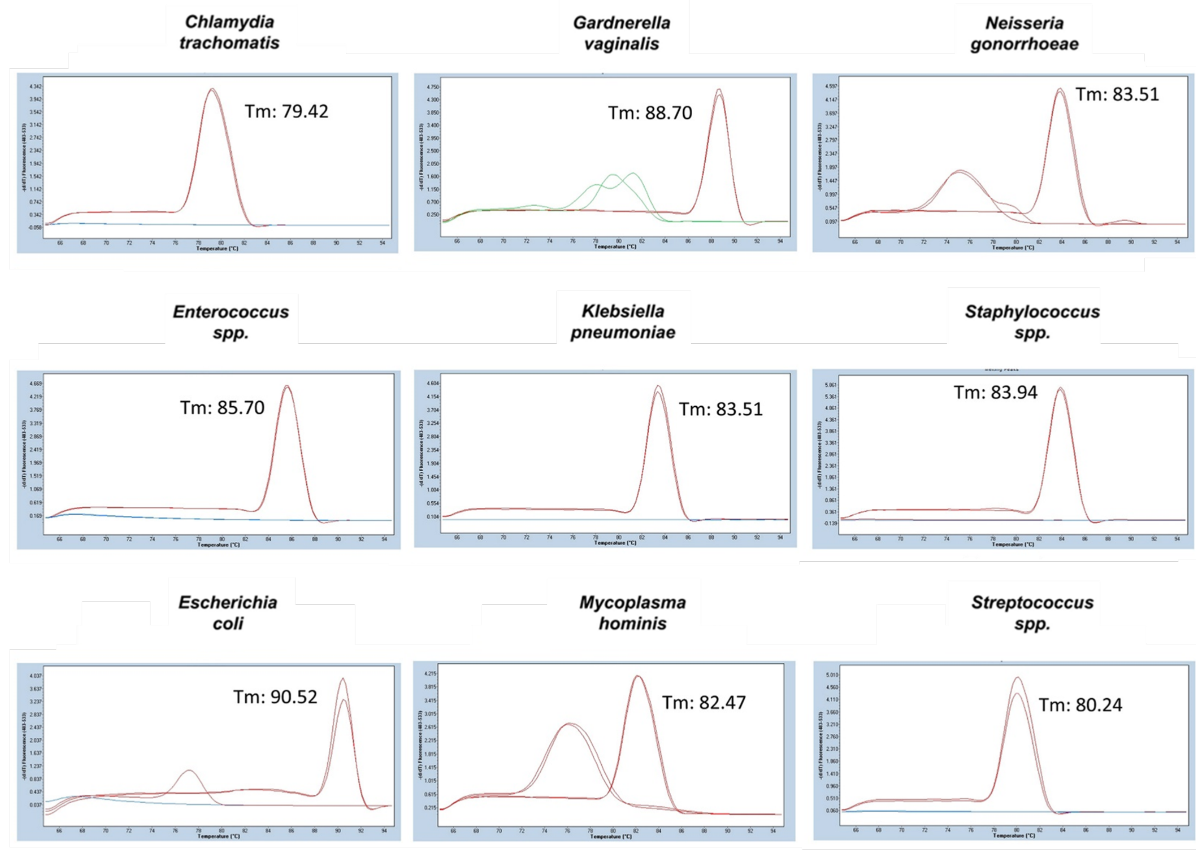


Figure 22 I Amplification signals of the controls of the molecular microbiology test.
Graphs obtained from RT-PCR showing melting peak profile and melting temperature for each amplicon, discriminating specific (positive) and unspecific (negative) amplification signals. Tm, melting temperature.

1.3. Molecular diagnosis of chronic endometritis using RT-PCR

CE was blindly analysed by RT-PCR in a total of 95 endometrial samples from women assessed for CE using any of the three classical methods (set 1). Using this molecular analysis, 42 of the 95 endometrial samples were negative for CE (44.21%), while 53 were positive (55.79%) for one or more pathogens tested (58.49% and 41.51%, respectively) **(Figure 23A)**. The pathogens most commonly represented in the samples analysed by RT-PCR were streptococci, while *C. trachomatis* and *N. gonorrhoeae* were undetectable in all tested samples **(Figure 23B)**. Of the 65 samples used for the comparison with the three classic techniques (set 2), 27 samples were negative for CE (41.54%) while 38 were positive (58.46%) for one or more pathogens

tested (55.26% and 44.74%, respectively) **(Figure 23A)**. The most represented bacteria in this set of patients was *Streptococcus*, which was found in 45.78% of the women with pathogens **(Figure 23C)**. The molecular analysis of the 13 samples with concordant results by three classic methods (set 3) showed that 4 endometrial samples were negative for CE (30.77%), while 9 were positive (69.23%) for one or more pathogens tested (66.67% and 33.33%, respectively) **(Figure 23A)**. The most detected pathogen in these samples was also *Streptococcus* spp. followed by *Enterococcus* spp. and *G. vaginalis* **(Figure 23D)**.

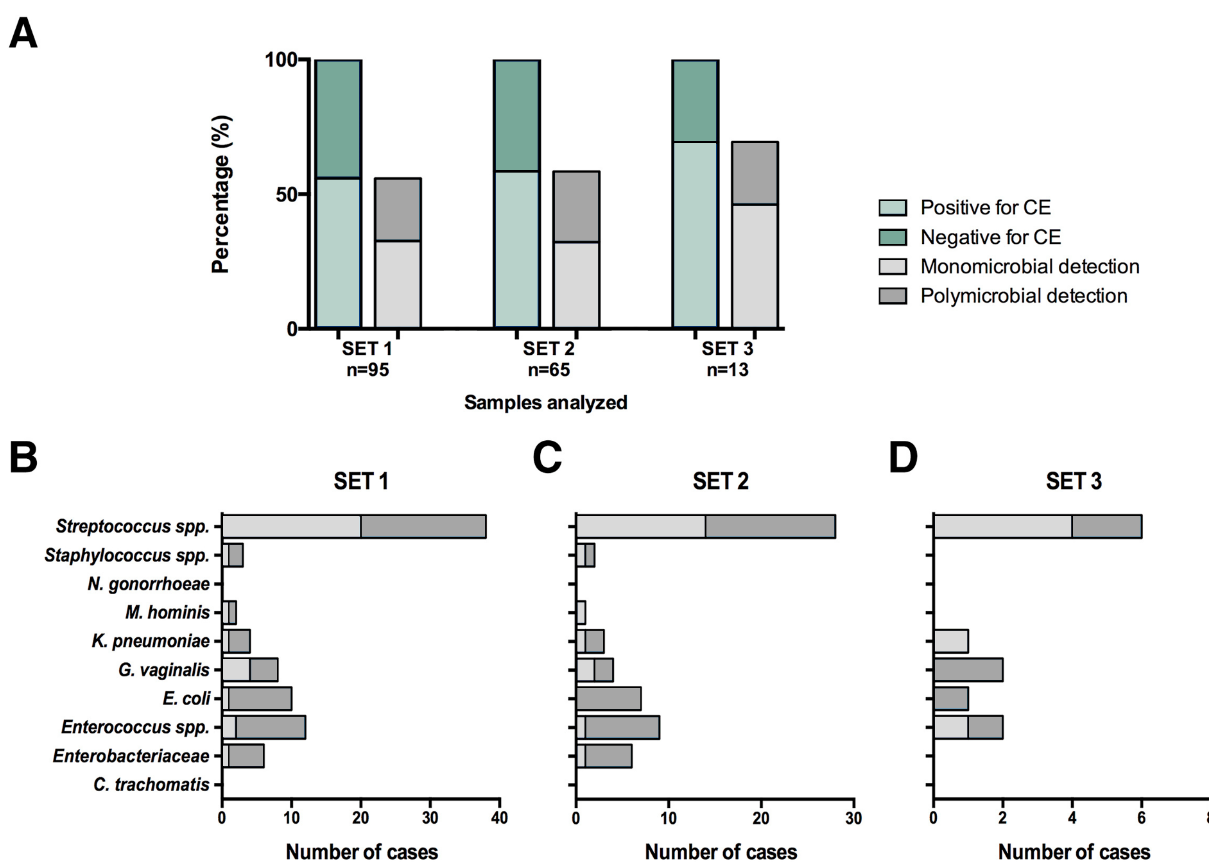


Figure 23 | Molecular diagnosis of chronic endometritis.

(A) Proportion of positive and negative cases for CE using targeted RT-PCR and percentage of positive cases with detection of one or more microorganisms in samples from set 1 (95 samples analysed by RT-PCR), set 2 (65 samples used for comparison with the three classical diagnosis), and set 3 (13 samples with concordant results between the three classical methods). Number of cases in which each targeted pathogen was detected alone or in combination with other bacteria in (B) set 1; (C) set 2; and (D) set 3. CE, chronic endometritis.

1.4. Comparison of chronic endometritis diagnosis based on molecular microbiology vs classic methods

Results of the molecular microbiology were compared with each of the classic diagnostic methods (histology, hysteroscopy, and microbial culture) based on negative/positive CE results of the 65 patients analysed (set 2) **(Table 4)**.

1.4.1. Molecular microbiology vs histology

The molecular microbiology was positive in 38 cases of 65 patients investigated (58.46%), while histological diagnosis alone was positive in 25 cases (38.46%). Concordant results were observed in 30 samples (14 double positives and 16 double negatives) giving a matching accuracy of 46.15%. Interestingly, in 24 (68.57%) of 35 samples with contradictory results, bacterial DNA was clearly identified by RT-PCR, while the histological analysis was negative **(Table 4)**.

1.4.2. Molecular microbiology vs hysteroscopy

Hysteroscopic-based diagnosis was positive in 63 of patients investigated (96.92%). Concordance with molecular microbiology was observed in 38 samples (37 double positives and 1 double negative) showing a matching accuracy of 58.46%. In 26 (96.29%) of 27 discordant patients, bacterial DNA was not identified in the paired endometrial sample obtained after positive hysteroscopic diagnosis of CE. Interestingly, only one sample with no signs of CE in the hysteroscopy was positive in the RT-PCR for *Streptococcus* spp. **(Table 4)**.

1.4.3. Molecular microbiology vs microbial culture

Microbiological culture was positive in 34 of patients tested for CE (52.31%). Concordance between molecular microbiology and microbial culture was present in 37 patients (22 double positive and 15 double negative) showing a matching rate of

56.92%. From the 22 double-positive cases, 11 RT-PCR cases were confirmed by isolation of the same pathogens.

When microbiological results were analysed in those cases with discordant diagnosis, we found several cases in which the culture was either contaminated by *S. epidermidis* (case 12 and 28) or presented microorganisms that were not tested in our targeted PCR panel such as *Ureaplasma* spp. (cases 12 and 38). Molecular microbiology also allowed for the identification of *G. vaginalis and M. hominis*, which are seldom cultured, and were not identified by classic microbial culture (cases 2, 5, and 36). Taking these cases into account, the comparison of these two methods demonstrated an accuracy of 66.15% **(Table 4)**.

1.4.4. Molecular microbiology vs histology + hysteroscopy

Because histology and hysteroscopy are subjective methods with the highest discordant results, the molecular diagnosis of CE using RT-PCR was compared with consistent histology + hysteroscopy. From the 65 patients analysed by all classic methods only 27 presented consistent histology + hysteroscopy results (41.54% concordance rate), with 25 double-positive and 2 double-negative results. From those 27 patients, RT-PCR showed concordant results in 15 cases (14 positives and only 1 negative) with an accuracy of 55.56%.

Interestingly, 38 of 65 patients (58.46%) had opposite results between histology and hysteroscopy, and from them all negative cases for histology were positive based on hysteroscopy. In these cases, the detection of bacterial DNA by RT-PCR (23 of 38 cases) was always coincident with positive hysteroscopy, while negative diagnosis of CE using the molecular method (15 of 38 samples) always matched with the negative histological diagnosis **(Table 4)**.

Table 4 | Results of chronic endometritis diagnosis by molecular and classic methods.

Patient	RT-PCR	Histology	Hysteroscopy	Microbial culture
1	*Enterococcus* species, *Staphylococcus* species	Negative	Positive	Negative
2	*Gardnerella vaginalis*	Negative	Positive	Negative
3	Negative	Positive	Positive	Negative
4	Negative	Negative	Positive	Negative
5	*Gardnerella vaginalis*	Negative	Positive	Negative
6	Negative	Negative	Positive	Negative
7	Negative	Negative	Positive	*Enterococcus faecalis*
8	*Streptococcus* species, *Gardnerella vaginalis*	Positive	Positive	*Streptococcus agalactiae*
9	*Streptococcus* species	Negative	Positive	*Enterococcus faecalis*
10	Negative	Negative	Negative	Negative
11	*Streptococcus* species	Negative	Positive	*Streptococcus agalactiae*
12	*Streptococcus* species	Negative	Positive	*Staphylococcus epidermidis*, *Ureaplasma*
13	*Staphylococcus* species	Positive	Positive	Negative
14	*Streptococcus* species, *Enterobacteriaceae*	Negative	Positive	*Streptococcus agalactiae*, *Ureaplasma*
15	*Gardnerella vaginalis*, *Escherichia coli*	Positive	Positive	*Escherichia coli*
16	Negative	Negative	Positive	*Enterococcus faecalis*, *Escherichia coli*
17	Negative	Positive	Positive	*Enterococcus faecalis*, *Ureaplasma*
18	*Streptococcus* species	Positive	Positive	*Streptococcus agalactiae*
19	*Streptococcus* species	Positive	Positive	*Escherichia coli*
20	*Streptococcus* species, *Enterococcus* species	Negative	Positive	*Ureaplasma*
21	*Streptococcus* species, *Enterococcus* species, *Escherichia coli*	Negative	Positive	*Enterococcus faecalis*
22	Negative	Positive	Positive	Negative
23	*Streptococcus* species, *Escherichia coli*, *Klebsiella pneumoniae*	Positive	Positive	Negative
24	*Klebsiella pneumoniae*	Positive	Positive	*Ureaplasma*
25	*Streptococcus* species	Negative	Positive	Negative
26	Negative	Positive	Positive	*Enterococcus faecium*
27	Negative	Negative	Positive	Negative

Table 4 | Continued

Patient	RT-PCR	Histology	Hysteroscopy	Microbial culture
28	Negative	Negative	Positive	*Staphylococcus epidermidis*
29	Negative	Positive	Positive	Negative
30	*Enterococcus* species	Positive	Positive	*Enterococcus faecalis*, *Streptococcus mitis*
31	*Streptococcus* species	Positive	Positive	*Klebsiella pneumoniae*
32	Negative	Positive	Positive	*Negative*
33	Negative	Negative	Positive	*Staphylococcus aureus*
34	Negative	Positive	Positive	Negative
35	Negative	Positive	Positive	Staphylococcus aureus
36	*Mycoplasma hominis*	Negative	Positive	Negative
37	*Streptococcus* species, *Enterobacteriaceae*	Negative	Positive	Negative
38	Negative	Negative	Positive	*Ureaplasma*
39	*Streptococcus* species	Positive	Positive	*Streptococcus* agalactiae
40	Negative	Negative	Positive	*Enterococcus gallinarum*
41	Streptococcus species, *Escherichia coli*	Positive	Positive	Negative
42	Streptococcus species, *Enterobacteriaceae*	Negative	Positive	Negative
43	Enterococcus species, *Enterobacteriaceae*, *Escherichia coli*	Negative	Positive	*Staphylococcus aureus*, *Ureaplasma*
44	Enterococcus species, *Streptococcus* species	Positive	Positive	*Escherichia coli*
45	*Streptococcus* species	Positive	Positive	Negative
46	*Enterococcus* species, *Streptococcus* species, *Escherichia coli*	Negative	Positive	*Enterococcus faecalis*
47	*Enterococcus* species, *Streptococcus* species, *Enterobacteriaceae*	Negative	Positive	Negative
48	*Streptococcus* species, *Klebsiella pneumoniae*	Negative	Positive	*Candida albicans*, *Ureaplasma*
49	*Negative*	Negative	Positive	Negative
50	*Streptococcus* species	Negative	Positive	*Escherichia coli*
51	Negative	Positive	Positive	Negative
52	*Enterococcus* species, Streptococcus species	Positive	Positive	Negative
53	*Streptococcus* species	Negative	Positive	Negative
54	*Streptococcus* species	Negative	Positive	Negative

Table 4 I Continued

Patient	RT-PCR	Histology	Hysteroscopy	Microbial culture
55	Negative	Negative	Positive	Negative
56	Negative	Positive	Positive	Negative
57	Negative	Negative	Positive	*Enterococcus faecalis*
58	Negative	Negative	Positive	Negative
59	Enterobacteria	Negative	Positive	Negative
60	*Streptococcus* species, *Escherichia coli*	Negative	Positive	*Streptococcus agalactiae*
61	Negative	Negative	Positive	*Staphylococcus aureus* , *Ureaplasma parvum*
62	Negative	Negative	Positive	*Streptococcus gallolyticus*
63	*Streptococcus* species	Negative	Negative	*Ureaplasma*
64	Negative	Positive	Positive	Negative
65	*Streptococcus* species	Negative	Positive	*Streptococcus agalactiae*

1.4.5. Molecular microbiology vs histology + hysteroscopy + microbial culture

From the 65 patients tested for CE by all three classical methods, only 13 (20%) had concordant results (set 3) **(Figure 19)**. The only patient diagnosed as negative based on all classic methods was also negative for the molecular evaluation of CE (Patient 10) **(Figure 24)**. From the remaining 12 patients who were positive for CE based on all classic methods, 9 were also positive based on RT-PCR, while 3 showed discordant results. In total, 10 of 13 cases presented similar results with an accuracy of 76.92%.

In 5 of the 9 cases with positive results for microbial culture and RT-PCR, the microorganisms detected were the same, and in 2 of them, *G. vaginalis* was also detected by RT-PCR together with other pathogens (cases 8 and 15) **(Table 4, Figure 24)**, adding information to that obtained from microbial culture.

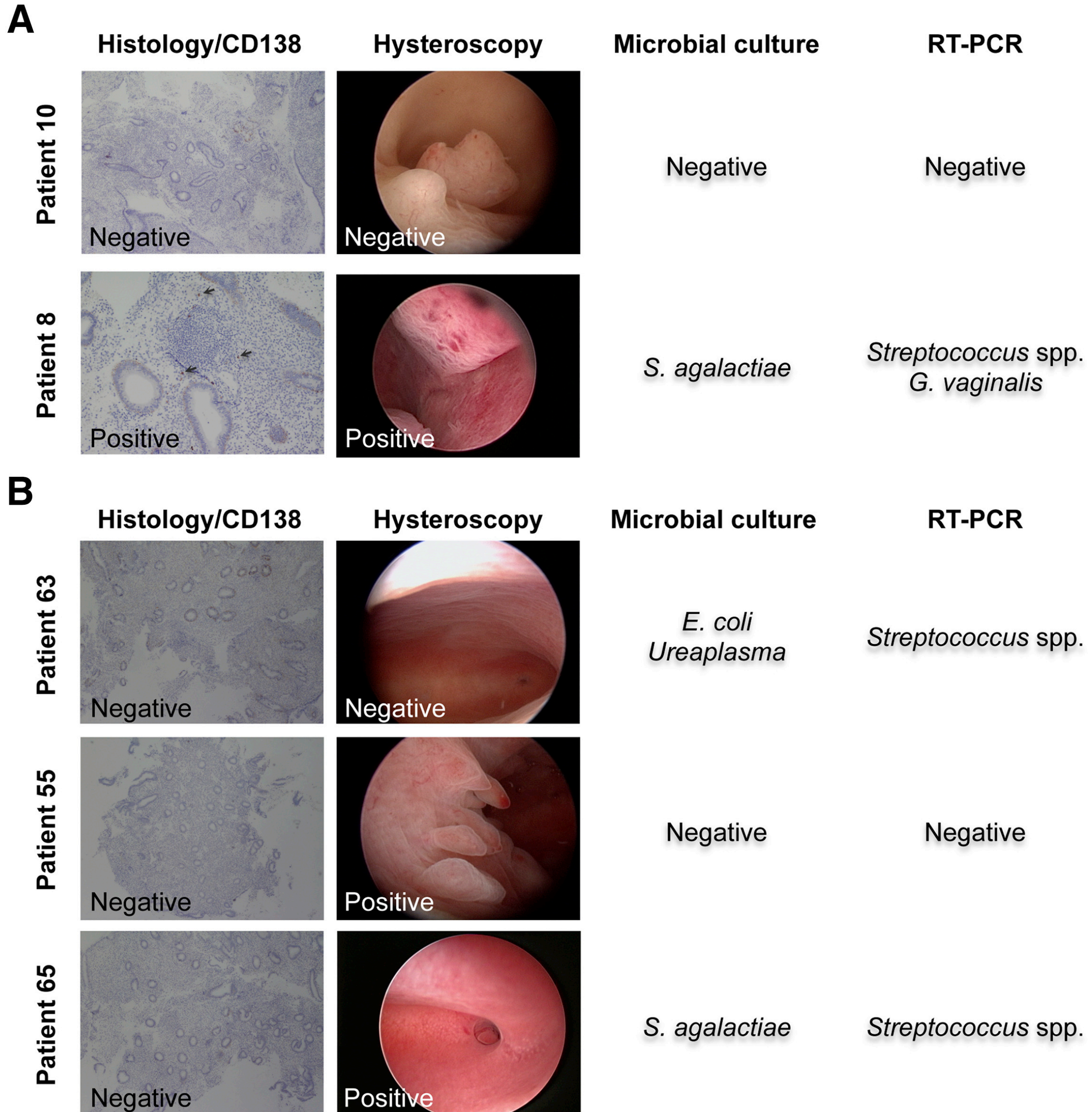


Figure 24 l Diagnosis of chronic endometritis depends on the method used.
(A) Examples of concordant and (B) discordant CE diagnosis in the patients analysed by the four methods compared in this study (three classical methods and RT-PCR evaluation).

Finally, sensitivity, specificity, positive and negative predictive values, and false-positive and -negative rates were assessed for each individual classic method and their combination in comparison to the RT-PCR diagnosis **(Table 5)**. The best results were obtained when RT-PCR was compared to the three concordant classic methods.

Table 5 | Index test of molecular diagnosis compared to classic diagnostic methods of chronic endometritis.

	Sensitivity (%)	Specificity (%)	Accuracy (%)	PPV (%)	NPV (%)	FPR (%)	FNR (%)
Histology (n = 65)	56.00	40.00	46.15	36.84	59.26	60.00	44.00
Hysteroscopy (n = 65)	58.73	50.00	58.46	97.37	3.70	50.00	41.27
Microbial culture (n = 65)	71.43	56.67	66.15	65.79	62.96	43.33	28.57
Histology + hysteroscopy (concordant results, n = 27)	56.00	50.00	55.56	93.33	8.33	50.00	44.00
Histology + hysteroscopy + microbial culture (concordant results, n = 13)	75.00	100.00	76.92	100.00	25.00	0.00	25.00

1.5. Molecular microbiology vs 16S rRNA sequencing

To confirm the results obtained by molecular microbiology, EB from these 13 patients with classic concordant diagnostic were subjected to bacterial 16S rRNA sequencing to assess their full endometrial microbiome. Data on the percentage of CE microorganisms detected, number of mapped reads, and Shannon index for each sample are detailed in **Table 6**.

The results of NGS for the bacterial 16S rRNA gene confirmed the detection of bacterial DNA in 12 of 13 endometrial samples since one sample did not yield sequencing results (case 24). The overall analysis of the endometrial microbiome in those 12 samples with concordant diagnosis in all the methods analysed showed that the most represented genus was *Lactobacillus*, followed by two bacterial pathogens associated with CE: *Streptococcus* and *Gardnerella*. Also, other bacteria previously reported to colonise the reproductive tract such as *Bifidobacterium*, *Megasphaera*, *Parvimonas*, *Prevotella*, *Propionibacterium*, and *Veillonella* were found **(Figure 25A)**. Interestingly, in two cases in which the RT-PCR was negative and the culture was positive (cases 17 and 26), the taxonomic assignment obtained in the sequencing confirmed the results of the RT-PCR, with no detection of bacterial DNA of the pathogens isolated in the microbial culture.

Table 6 | Microbiota profile of endometrial samples by 16S ribosomal RNA gene sequencing.

Patient	Microbial culture	RT-PCR	Relative abundance using 16S rRNA sequencing (%)												No. of mapped reads	Alpha diversity (Shannon index)
			Lactobacillus	*Enterococcus*	*Staphylococcus*	*Streptococcus*	*Mycoplasma*	Enterobacteriaceae	*Escherichia*	*Klebsiella*	*Gardnerella*	*Ureaplasma*	*Chlamydia*	*Neisseria*		
8	*Streptococcus agalactiae*	*Streptococcus* species, *Gardnerella vaginalis*	13.75	2.52	4.46	30.87	0.00	1.00	0.00	0.00	1.48	0.00	0.00	0.00	121624	7.32
10	Negative	Negative	99.94	0.00	0.00	0.00	0.00	0.01	0.00	0.00	0.01	0.00	0.00	0.00	218076	2.94
15	*Escherichia coli*	*Gardnerella vaginalis*, *Escherichia coli*	74.4	0.36	1.97	0.68	0.02	1.00	0.00	0.00	1.34	0.00	0.00	0.00	96697	6.12
17	*Enterococcus faecalis*, *Ureaplasma*	Negative	96.49	0.00	0.11	0.11	0.00	0.30	0.00	0.00	0.43	0.00	0.00	0.00	62033	3.79
18	*Streptococcus agalactiae*	*Streptococcus* species	0.32	0.00	0.25	81.53	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	102443	3.63
19	*Escherichia coli*	*Streptococcus* species	69.21	0.03	2.65	1.06	0.03	3.00	0.00	0.02	2.39	0.01	0.00	0.00	231538	7.78
24	*Ureaplasma*	*Klebsiella pneumoniae*	Not detected													
26	*Enterococcus faecium*	Negative	98.75	0.00	0.11	0.25	0.00	0.02	0.00	0.00	0.04	0.00	0.00	0.00	207848	3.41
30	*Enterococcus faecalis*, *Streptococcus mitis*	*Enterococcus* species	95.21	0.17	0.05	0.04	0.00	0.50	0.00	0.00	0.00	0.00	0.00	0.00	27178	4.67
31	*Klebsiella pneumoniae*	*Streptococcus* species	6.07	0.00	7.93	1.91	0.00	3.00	0.00	1.25	0.00	0.00	0.00	0.00	36673	8.03
35	*Staphylococcus aureus*	Negative	91.54	0.00	0.10	0.69	0.00	0.02	0.00	0.00	7.18	0.00	0.00	0.00	149621	4.14
39	*Streptococcus agalactiae*	*Streptococcus* species	1.33	0.00	0.04	97.54	0.00	0.01	0.00	0.00	0.00	0.00	0.00	0.00	91595	2.88
44	*Escherichia coli*	*Enterococcus* species, *Streptococcus* species	1.03	0.00	0.12	6.49	0.00	0.01	0.00	0.00	91.39	0.00	0.00	0.00	177273	4.06

On the other hand, the percentage of lactobacilli in these samples was also evaluated, based on previous reports showing that the presence of *Lactobacillus* spp. in the uterine cavity are associated with a healthy endometrial status (Moreno et al., 2016).

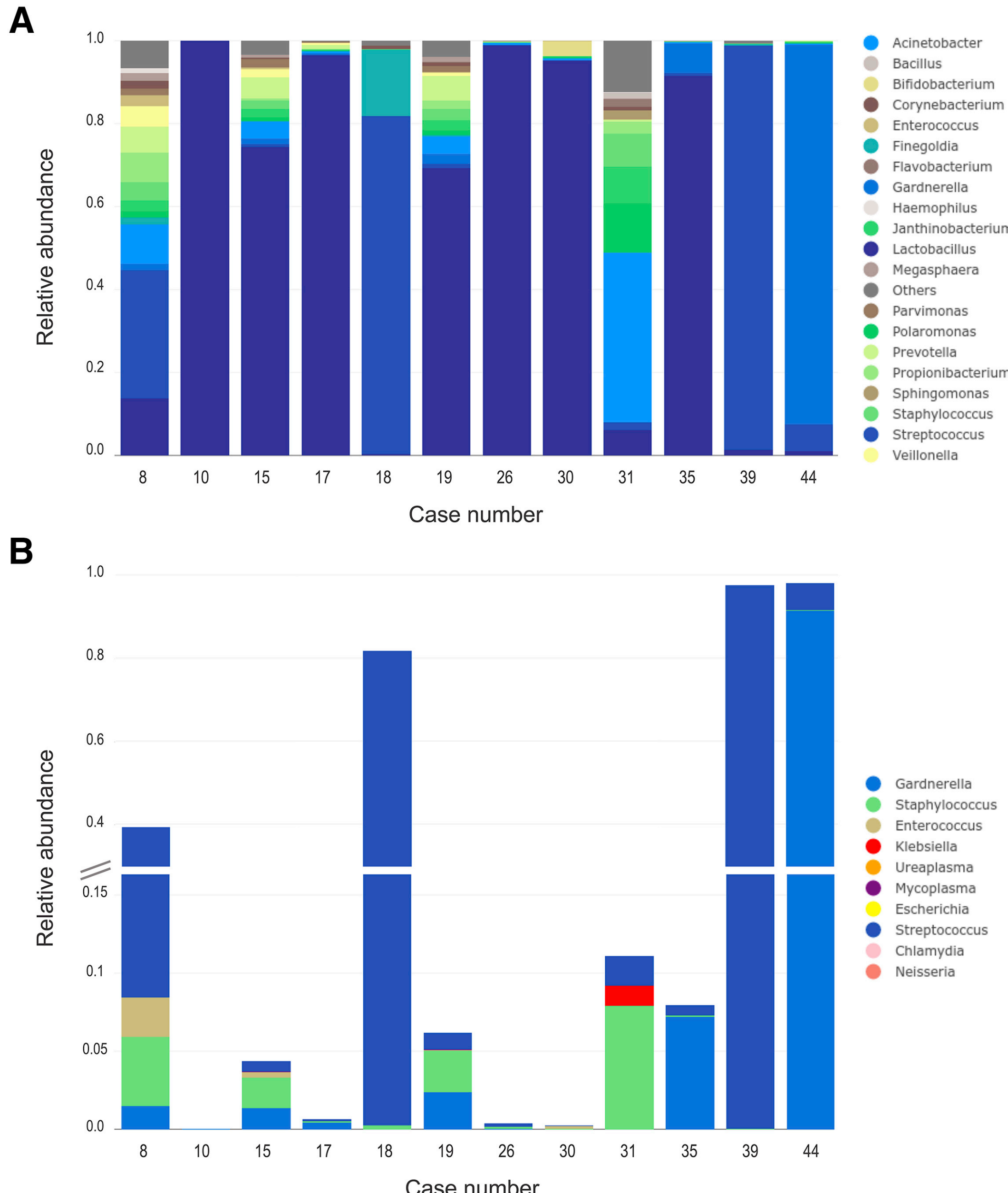


Figure 25 | Endometrial microbiome assessed by 16S ribosomal RNA gene sequencing.
(A) Microbiome composition profiles showing 20 most-abundant genera and their relative abundances in endometrial samples of patients with consistent CE diagnosis by three classic methods. (B) Relative abundance of most frequent bacteria causing CE in endometrial samples of patients with consistent CE diagnosis by three classic methods.

This analysis showed a low percentage of lactobacilli in all those samples with a positive diagnosis of CE by all the methods analysed, with the exception of one sample with 93.2% of *Lactobacillus* and a low percentage of the pathogens detected by either microbial culture or RT-PCR (case 30). By contrast, a high percentage of lactobacilli was detected in the samples that proved negative for the molecular diagnosis of CE (cases 10, 17, 26, and 35), supporting the results obtained from the RT-PCR analysis **(Figure 25B)**. In summary, when evaluating the presence of *Lactobacillus*, the microbiota profile using NGS was concordant with RT-PCR in 11 of 12 cases and coincided with the microbial culture in 9 of 12 cases, showing an accuracy of 91.67% and 75.00%, respectively.

1.6. Molecular microbiology in patients without chronic endometritis

From the 65 samples analysed for the three classic methods, only one presented concordant negative results for all the techniques. For this reason, to evaluate the efficacy of the RT-PCR method to detect negative cases of CE, EB samples from 10 control participants, that proved negative for histology and microbial culture, were subjected to the molecular method.

The molecular analysis of these samples showed negative results for the nine pathogenic bacteria tested **(Table 7)** consistent with their negative results for the classic methods analysed. However, RT-PCR was able to detect very small amounts of DNA from *G. vaginalis* (case C2) and *M. hominis* (case C5), but their levels were below the threshold of positive controls. These data support the efficacy of molecular microbiology to discriminate between positive and negative cases of CE, based on the detection of nine pathogens usually causing the disease.

Table 7 | Molecular diagnosis of endometrial pathogens in negative cases.

	Surgery indication/treatment	RT-PCR	Histology	Microbial culture
C1	Myomectomy; usual technique with Novak	Negative	Negative	Negative
C2	Oophorectomy (dermoid cyst); usual technique with Novak	Negative (low detection of *Gardnerella vaginalis*)	Negative	Negative
C3	Myomectomy; usual technique with Novak	Negative	Negative	Negative
C4	Myomectomy; usual technique with Novak	Negative	Negative	Negative
C5	Myomectomy; usual technique with Novak	Negative (low detection of *Mycoplasma hominis*)	Negative	Negative
C6	Myomectomy; usual technique with Novak	Negative	Negative	Negative
T1	Previous chronic endometritis, treated with antibiotics	Negative	Negative	Negative
T2	Previous chronic endometritis, treated with antibiotics	Negative	Negative	Negative
T3	Previous chronic endometritis, treated with antibiotics	Negative	Negative	Negative
T4	Previous chronic endometritis, treated with antibiotics	Negative	Negative	Negative

2. CHARACTERISATION OF THE ENDOMETRIAL MICROBIOME IN INFERTILE WOMEN

2.1. Patient cohort, characteristics, and outcomes

A total of 452 infertile patients undergoing IVF were assessed for eligibility in 13 reproductive clinics in Europe, America, and Asia. Forty-four patients were excluded from the analysis because they did not meet the inclusion criteria (n=42) or declined to participate (n=2). The remaining 408 women were recruited and subjected to an HRT cycle for assessment of both endometrial receptivity and microbiome, but 66 patients were lost in the follow-up. Of the 342 patients finally assessed, 198 (57.9%) became pregnant, while 144 (42.1%) did not become pregnant. For pregnant women, 141 (41.2%) had a live birth, 27 (7.9%) had a biochemical pregnancy and 28 (8.2%) had a clinical miscarriage. Moreover, 2 patients with an ectopic pregnancy were

identified, but this reproductive outcome was not considered as a group for further comparisons due to the small sample size **(Figure 26)**.

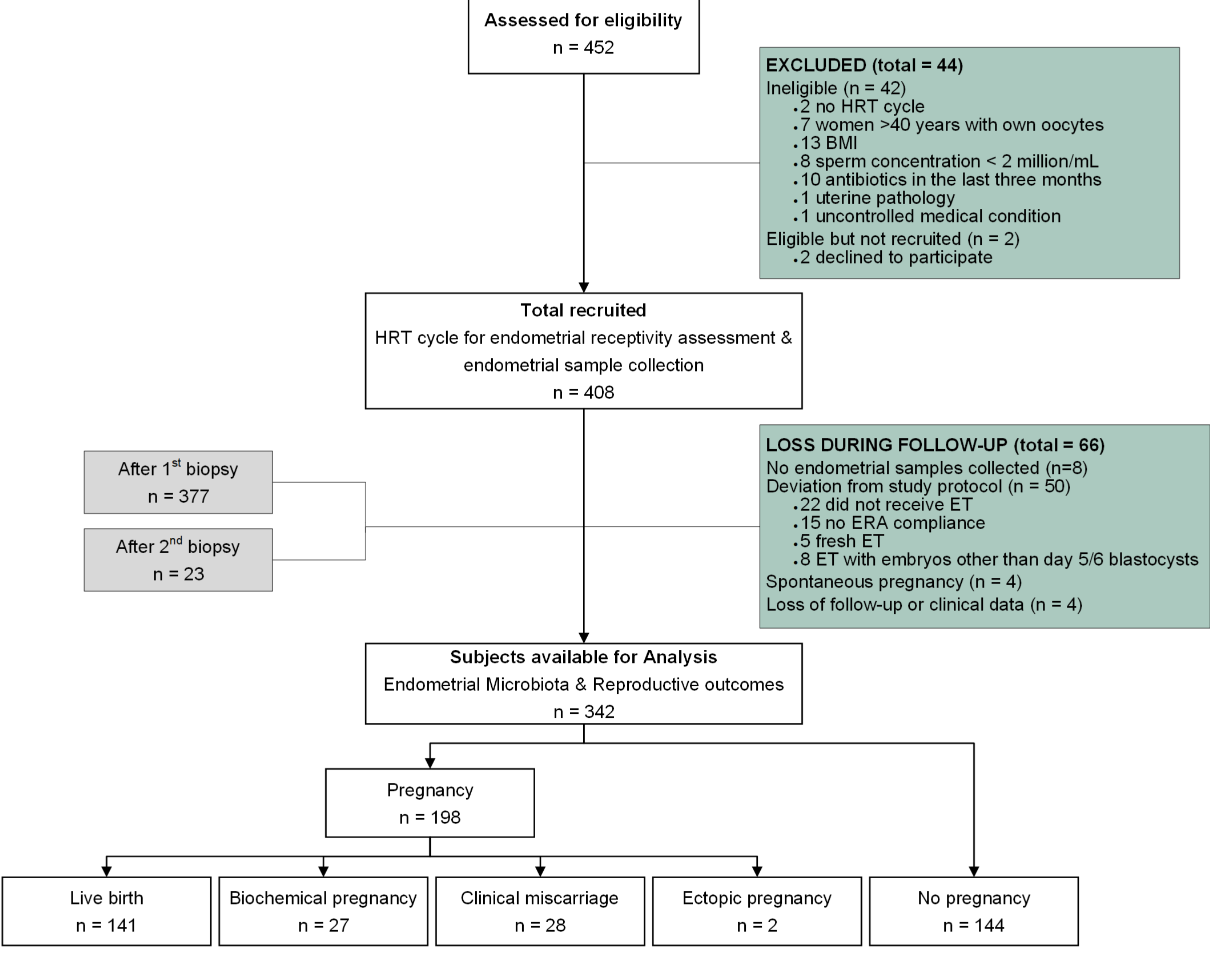


Figure 26 | Flowchart of the study population.
Of the total amount of patients assessed for eligibility, 342 patients were finally included to assess the impact of the endometrial microbiome on pregnancy outcomes. BMI, body mass index; ERA, endometrial receptivity analysis; ET, embryo transfer; HRT, hormonal replacement therapy.

Baseline demographics and clinical characteristics of these patients are detailed in **Table 8**. The recruited patients had a mean age of 36 years (range 21-49) and a mean BMI of 23.26 (range 18.5-30.0). The racial/ethnic distribution was Caucasian (57.3%), East Asian ancestry (14.0%), and Hispanic (11.4%) among others (17.3%). In general, the recruited patients had no previous miscarriages (74.9%), voluntary termination of pregnancies (94.7%), ectopic pregnancies (92.2%), stillbirth (99.7%), or curettage (89.8%), but many of them experienced one or more implantation failures

(20.2% and 57.5% respectively). The main indications for IVF were advanced maternal age, male factor, unexplained infertility, and ovarian pathology.

Table 8 | Demographic and clinical characteristics of the patients at baseline.

	Subjects available for analysis (n=342)
Age (y)	36.01 ± 4.98
Body mass Index (Kg/m^2)	23.26 ± 2.97
Ethnicity	
African	9 (2.63)
Ashkenazi	3 (0.88)
Caucasian	196 (57.31)
East Asian	48 (14.04)
Hispanic	39 (11.4)
Mulatto	2 (0.58)
South Asian	8 (2.34)
Other	37 (10.82)
No. previous pregnancies	
0	169 (49.42)
≥1	173 (50.58)
No. previous miscarriages	
0	256 (74.85)
≥1	86 (25.15)
No. previous voluntary termination of pregnancies	
0	324 (94.74)
≥1	18 (5.26)
No. previous ectopic pregnancies	
0	318 (92.18)
≥1	24 (7.82)
No. previous stillbirth	
0	341 (99.71)
1	1 (0.29)
No. previous live birth	
0	279 (81.58)
≥1	63 (18.42)
No. previous curettages	
0	307 (89.77)
≥1	36 (10.24)
No. implantation failure	
0	76 (22.29)
≥1	266 (77.71)
ART indication	
Advanced maternal age	38 (11.11)
Male factor	30 (8.77)
RPL	3 (0.88)
RIF	11 (3.22)
Tubal factor	9 (2.63)
Uterine factor	1 (0.29)
Ovarian pathology	20 (5.85)
Endometriosis	7 (2.05)
Genetic factor	8 (2.34)
Unexplained infertility	28 (8.19)
No indication	1 (0.29)
More than 1 indication	186 (54.38)
Treatment	
IVF	37 (10.82)
IVF/ICSI	305 (89.18)
Antibiotics last 3 months	
Yes	2 (0.58)
No	256 (74.85)
Probiotics last 3 months	
Yes	24 (6.36)
No	280 (81.87)

*Data are expressed as mean ± SD or n (%).

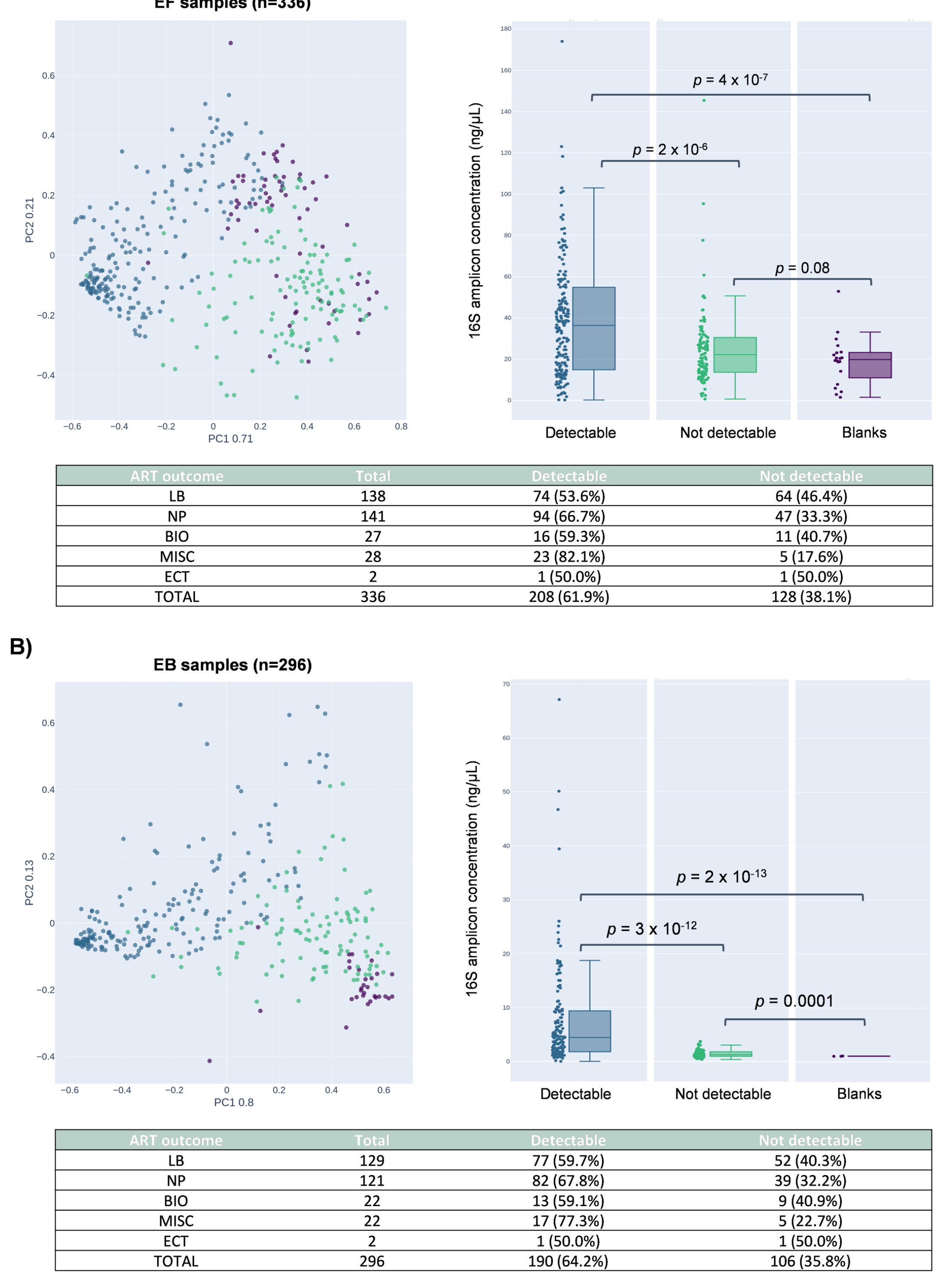
ART, assisted reproductive technology; RPL, recurrent pregnancy loss; RIF, recurrent implantation failure; IVF, *in vitro* fertilization; ICSI, intracytoplasmic sperm injection.

2.2. Endometrial microbiota composition in EF and EB samples

The endometrial microbiome was analysed in EF and EB samples from 336 (98.3%) and 296 (86.6%) patients, respectively, with paired samples in 290 (84.8%) of the participants. The mean of total sequencing reads per sample was 302,299 (range, 110,050–394,659) in runs of EF samples and 335,659 (range, 237,889–430,675) in runs of EB samples, with an average of filtered reads of 89,883 (range, 27,960–137,956) and 103,539 (range, 61,650–162,653), respectively.

Because endometrium presents a low-abundance microbiota, a stringent analysis was performed to ensure that contaminating reads did not interfere with downstream analysis. For that, samples were classified as detectable and non-detectable based on the results obtained from blank samples included in each run and certain quality parameters as a reference (see criteria in section 2.5.1. of Materials and Methods). Despite starting with equivalent amounts of extracted DNA, detectable samples showed a different clustering with higher 16S amplicon concentration, while in non-detectable/low-biomass samples clustering was roughly equivalent to blank controls. After applying these criteria, 208 EF samples and 190 EB samples were classified as detectable and included in the analysis of the association of the endometrial microbiota with reproductive results.

Then, OTU filtering criteria were applied and the low-abundance taxa and the genera that do not colonise humans or are associated with kitome contaminants were eliminated from each dataset. Finally, 15 and 19 OTUs were evaluated in EF and EB microbiota, respectively, with 13 OTUs shared between sample types **(Figure 28)**.



ART outcome	Total	Detectable	Not detectable
LB	138	74 (53.6%)	64 (46.4%)
NP	141	94 (66.7%)	47 (33.3%)
BIO	27	16 (59.3%)	11 (40.7%)
MISC	28	23 (82.1%)	5 (17.6%)
ECT	2	1 (50.0%)	1 (50.0%)
TOTAL	336	208 (61.9%)	128 (38.1%)

ART outcome	Total	Detectable	Not detectable
LB	129	77 (59.7%)	52 (40.3%)
NP	121	82 (67.8%)	39 (32.2%)
BIO	22	13 (59.1%)	9 (40.9%)
MISC	22	17 (77.3%)	5 (22.7%)
ECT	2	1 (50.0%)	1 (50.0%)
TOTAL	296	190 (64.2%)	106 (35.8%)

Figure 27 l Distribution of sequencing data.

Principle components analysis showing the clustering of the (A) EF samples (n=336) and (B) EB samples (n=296) and their corresponding blank controls, based on quality parameters such as percentage of empty reads, dispersion index for each sample, and ratio between the filtered and mapped reads. Samples are coloured as detectable (blue) and not detectable (green) using a filtered/mapped reads threshold of 0.65 for EF and 0.7 for EB. Blanks are coloured in purple. EB, endometrial biopsy; EF, endometrial fluid.

Lactobacillus was found as the major genus in both EF and EB samples. Bacterial genera such as *Anaerococcus*, *Atopobium*, *Bifidobacterium*, *Corynebacterium*, *Gardnerella*, *Haemophilus*, *Microbacterium*, *Prevotella*, *Propionibacterium*, *Staphylococcus*, and *Streptococcus* were also commonly identified in both sample types. Three taxa were detected in EF but not EB, whereas seven taxa were detected in EB but not EF. Unique taxonomies for EF microbiota were *Streptomyces*, *Clostridium*, and *Chryseobacterium*, and for EB microbiota were *Cupriavidus*, *Escherichia*, *Klebsiella*, *Bacillus*, *Finegoldia*, *Micrococcus*, and *Tepidimonas* (**Figure 28**).

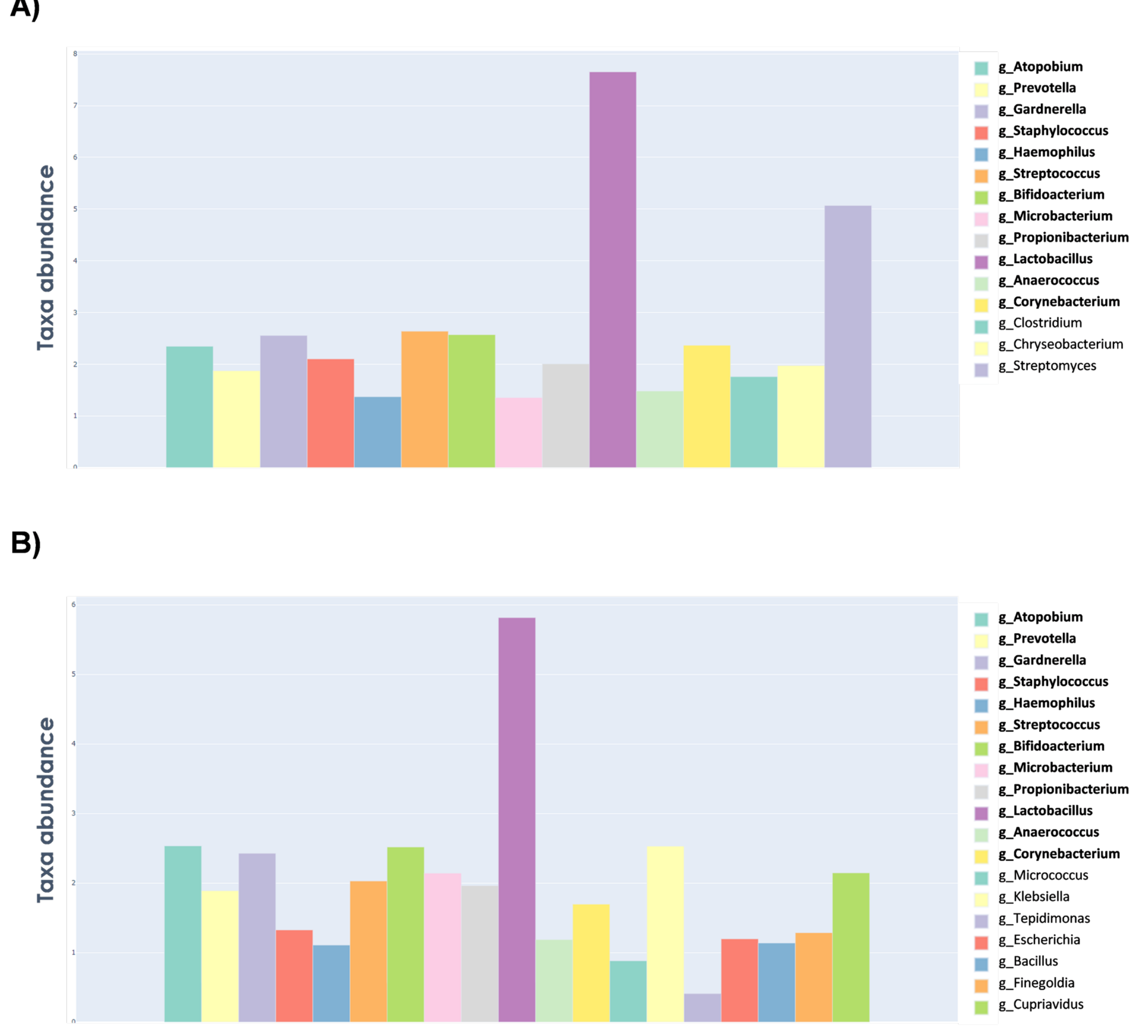


Figure 28 | Taxonomic composition of endometrial fluid and endometrial biopsy microbiota. Microbiota composition profiles showing the most-abundant genera and their relative abundance in (A) EF and (B) EB samples. Taxa common to both sample types are highlighted in bold.

To further analyse the EF and the EB microbiota composition, co-occurrence bacterial networks of these two microbiota data sets were built. The main differences between these two bacterial networks were: (i) the EF microbiota had two linked communities, while the EB microbiota had four linked communities and two isolated nodes; (ii) the EB microbiota network is more dispersed than the EF community; and (iii) *Lactobacillus* had positively and negatively related neighbours in the EF microbiota, while only negative relations in the EB microbiota. Interestingly, *Lactobacillus* was negatively associated with pathogenic bacteria (*Gardnerella*, *Bifidobacterium*, *Atopobium* in EB microbiota; and *Gardnerella*, *Bifidobacterium*, *Atopobium*, *Staphylococcus*, *Streptococcus*, *Chryseobacterium* in EF microbiota) while in EF, *Lactobacillus* was positively correlated to commensal bacteria (*Clostridium* and *Streptomyces*) **(Figure 29)**.

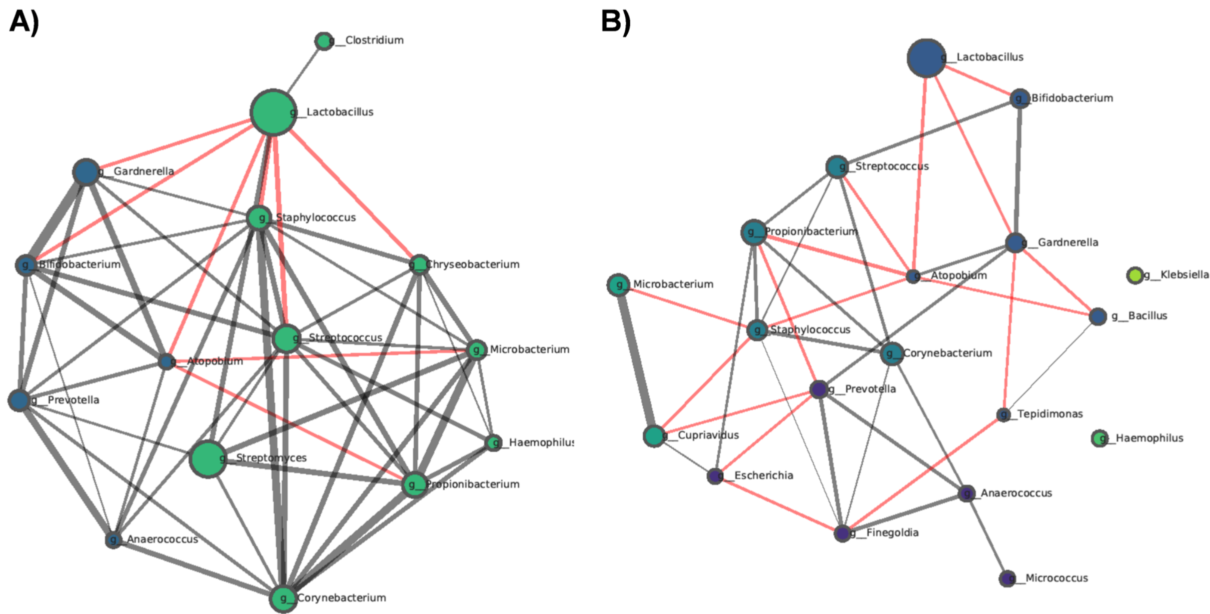


Figure 29 | Co-ocurrence bacterial networks for sample type.

Co-ocurrence bacterial networks for (A) EF and (B) EB samples. Each network was created by co-occurrence of the bacterial community evaluated having a significant Pearson correlation coefficient. Node properties: (i) circle size, normalised and standardised bacterial relative abundances; (ii) colour, communities as retrieved by Blondel algorithm. Edge properties: (i) thickness, proportional to p-value of Pearson correlation coefficient; (ii) colour, red for negative, and grey for positive correlations.

2.4. Endometrial microbiota composition and reproductive outcome

To analyse the association between the endometrial microbiota and reproductive outcome, taxa correlation analyses were also performed for each ART outcome **(Figure 30-31)**. The co-occurrence bacterial network of patients with live birth was denser and had a higher node degree distribution, in contrast to the number of isolated nodes in networks of unsuccessful outcomes. Additionally, potential interactions that only occur in patients with pregnancy success were found, reflecting that these relationships may be crucial for good pregnancy and that disruption of these interactions may lead to instability in the ecosystem.

In the EF microbiota of patients who had live birth, *Lactobacillus* was negatively related to pathogenic bacteria such as *Chryseobacterium*, *Staphylococcus,* and *Haemophilus*, and positively related to *Streptomyces*, which in turn was part of a dense community mainly comprising commensal bacteria (*Corynebacterium*, *Microbacterium*, *Propionibacterium*, and *Clostridium*). Similar behaviour was observed in nonpregnant patients, with *Lactobacillus* negatively correlated to *Gardnerella*, *Bifidobacterium*, *Atopobium*, *Staphylococcus*, *Streptococcus*, and *Chryseobacterium*, and positively related to *Streptomyces.* Interestingly, in the group of patients with biochemical pregnancy and clinical miscarriage, these interactions disappeared, and the resulting networks were disconnected and formed by sparse community **(Figure 30)**. Finally, EB microbiota networks were more dispersed as compared to the EF ones, with fewer interactions between *Lactobacillus* and other taxa. Thus, in this case the eventual beneficial/deleterious connections among taxa were less evident **(Figure 31)**.

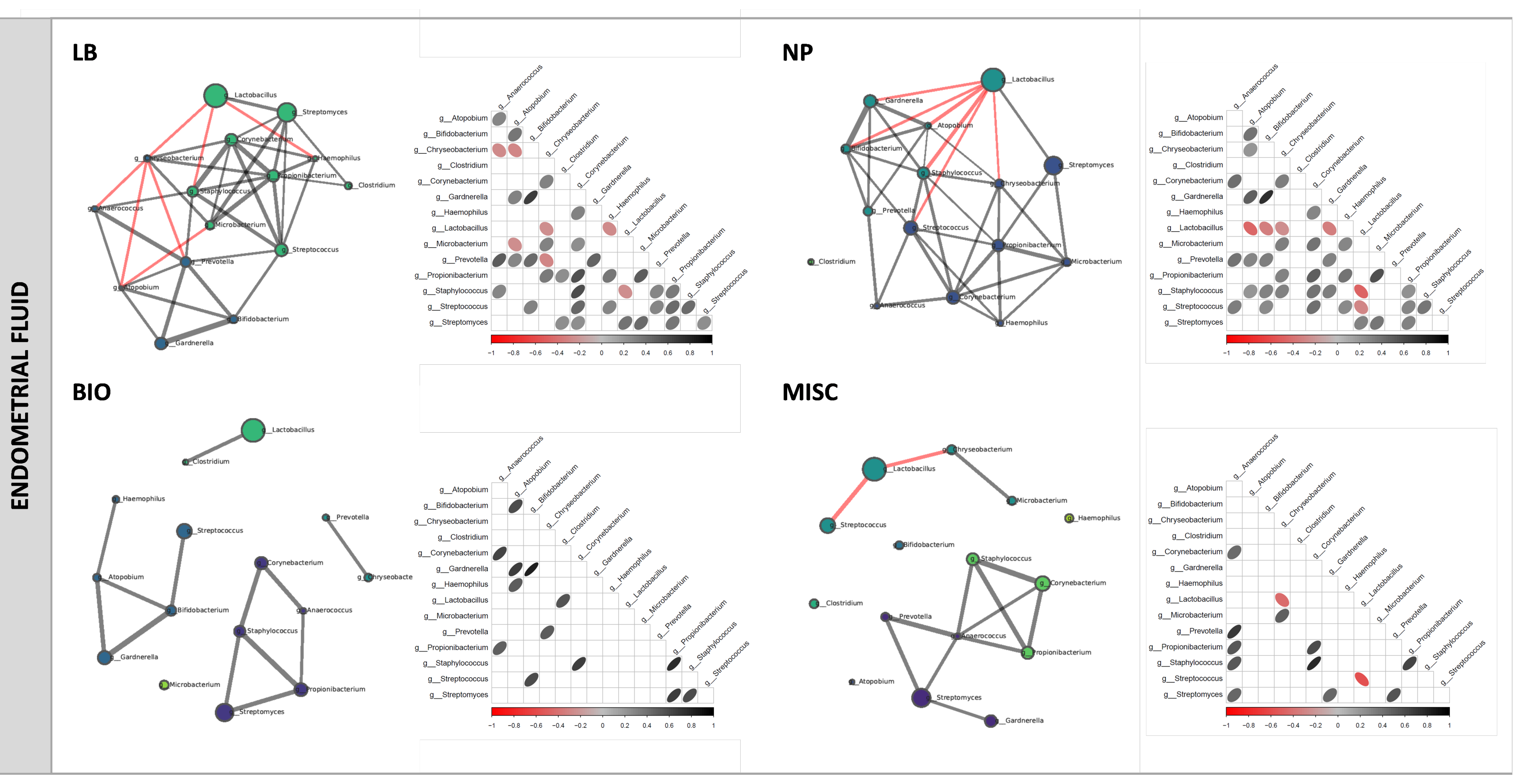


Figure 30 I Taxa correlation analysis by ART outcome in endometrial fluid samples.

Co-ocurrence bacterial networks together with an association analysis for each ART outcome in (A) EF and (B) EB samples. Each figure was created by co-occurrence of the bacterial community evaluated having a significant Pearson correlation coefficient. Node properties: (i) circle size, normalised and standardised bacterial relative abundances; (ii) colour, communities as retrieved by Blondel algorithm. Edge properties: (i) thickness, proportional to p-value of Pearson correlation coefficient; (ii) colour, red for negative, and grey for positive correlations. For association graphs, the same criteria were applied, with the thickness of the circle and colour intensity being proportional to Pearson correlation coefficients. Pairs of bacteria without circle have no significant Pearson correlation coefficient. BIO, biochemical pregnancy; LB, live birth; MISC, miscarriage; NP, no pregnancy.

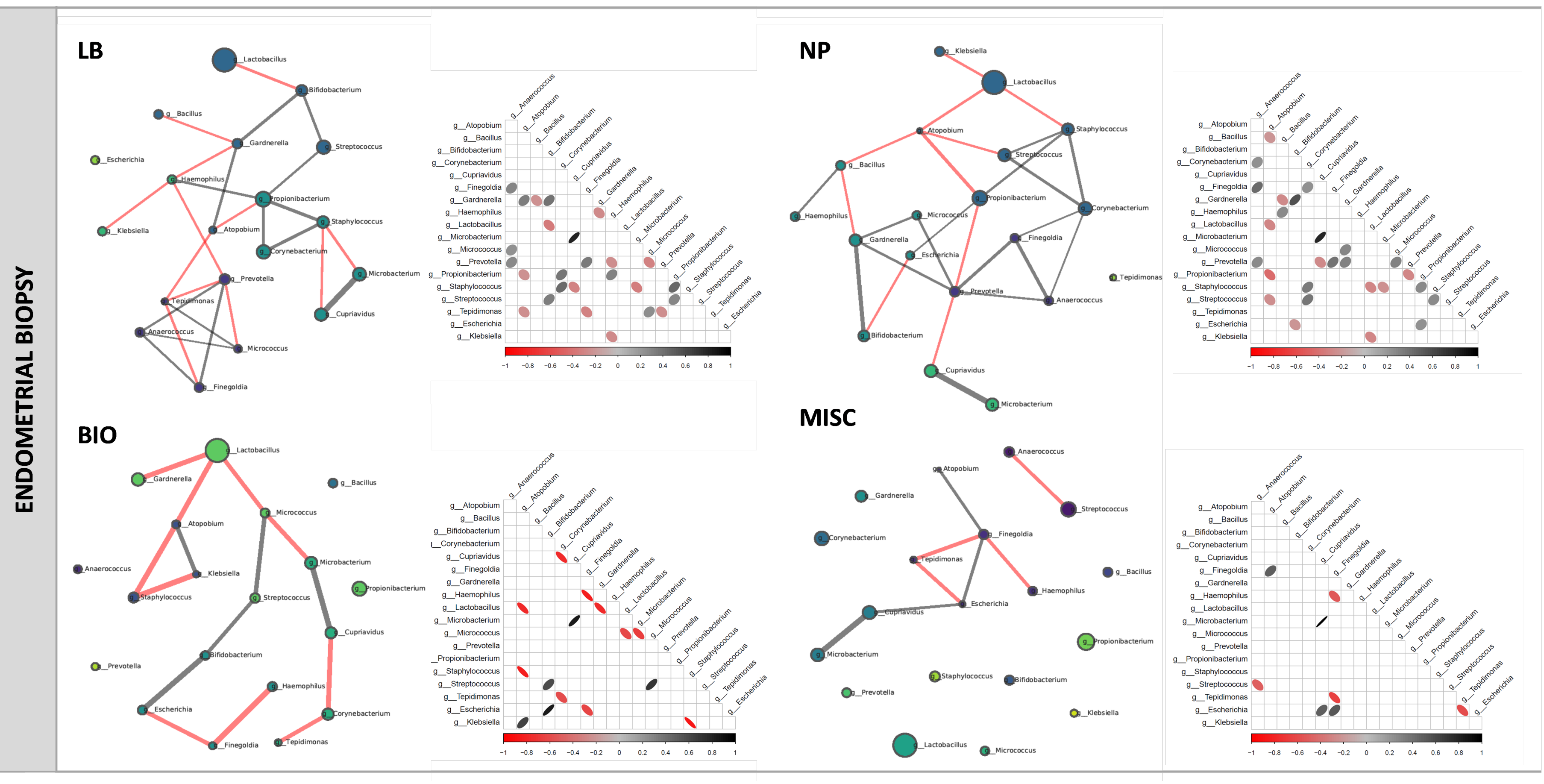


Figure 31 I Taxa correlation analysis by ART outcome in endometrial biopsy samples.

Co-ocurrence bacterial networks together with its associations analysis for each ART outcome in (A) EF and (B) EB samples. Each figure was created by co-occurrence of the bacterial community evaluated having a significant Pearson correlation coefficient. Node properties: (i) circle size, normalised and standardised bacterial relative abundances; (ii) colour, communities as retrieved by Blondel algorithm. Edge properties: (i) thickness, proportional to p-value of Pearson correlation coefficient; (ii) colour, red for negative, and grey for positive correlations. For association graphs, the same criteria were applied, with the thickness of the circle and colour intensity being proportional to Person correlation coefficients. Pairs of bacteria without circle have no significant Pearson correlation coefficient. BIO, biochemical pregnancy; LB, live birth; MISC, miscarriage; NP, no pregnancy.

To avoid potential bias when comparing samples analysed in different runs, bacterial profiles were transformed into clr data, and the bacterial communities were analysed according to the difference between *Lactobacillus* and other reproductive tract taxa using z-score normalised values.

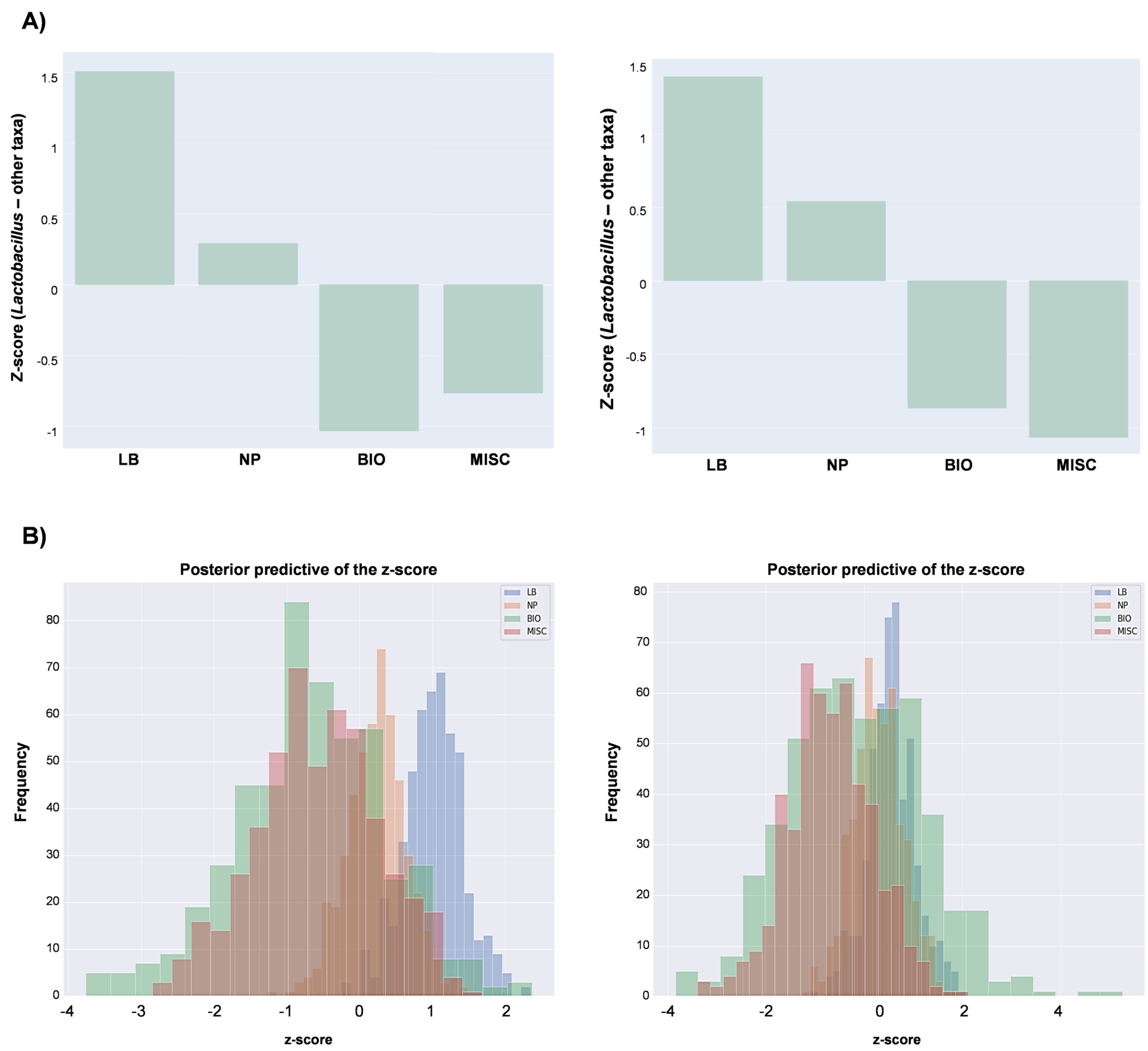


Figure 32 | Abundance of *Lactobacillus* is higher than other taxa in reproductive success.
(A) Difference between *Lactobacillus* and other reproductive tract taxa using z-score normalised values in EF (left panel) and EB (right panel) samples. (B) Posterior predictive distribution density plot of z-score differences between *Lactobacillus* and other reproductive tract taxa by reproductive outcome. BIO, biochemical pregnancy; LB, live birth; MISC, miscarriage; NP, no pregnancy

A higher abundance of *Lactobacillus* was observed in the group of patients with live birth compared to the groups with negative reproductive outcomes in both EF and EB **(Figure 32A)**. To calculate this difference, the taxa that presented a higher average abundance in bad outcomes compared to live birth were considered: *Streptococcus*, *Chryseobacterium*, *Corynebacterium*, *Haemophilus*, *Bifidobacterium*, *Staphylococcus*, *Atopobium*, *Gardnerella,* and *Propionibacterium* in the EF microbiota, and *Gardnerella*, *Klebsiella*, *Atopobium*, *Finegoldia*, *Escherichia*, *Propionibacterium*, *Haemophilus*, *Anaerococcus,* and *Bacillus* in the EB microbiota.

Predictive probability analysis using a Bayesian inference model showed a different highest posterior density (HPD) interval of the difference "*Lactobacillus*–other taxa" for each ART outcome: live birth (-0.12–1.51), no pregnancy (-1.05–0.65), biochemical pregnancy (-3.45–0.81), and clinical miscarriage (-2.81–0.55). These results reflect a greater probability of having a higher abundance of *Lactobacillus* in patients with a live birth. This probability is especially discriminative for EF samples where HPD for live birth samples shows less overlap with bad outcome intervals **(Figure 32B)**.

Finally, the bacterial profiles present in infertile patients that achieve a successful pregnancy with live birth were compared with that of patients with no pregnancy, biochemical pregnancy, and clinical miscarriage. We are based on the hypothesis that the microbiota composition in patients having a live birth is the physiological scenario and does not interfere with functional reproductive potential. Therefore, the distance between the bacterial abundance for each bacterial taxon and the upper and lower 95% CI established for patients with live birth was evaluated **(Figure 33)**.

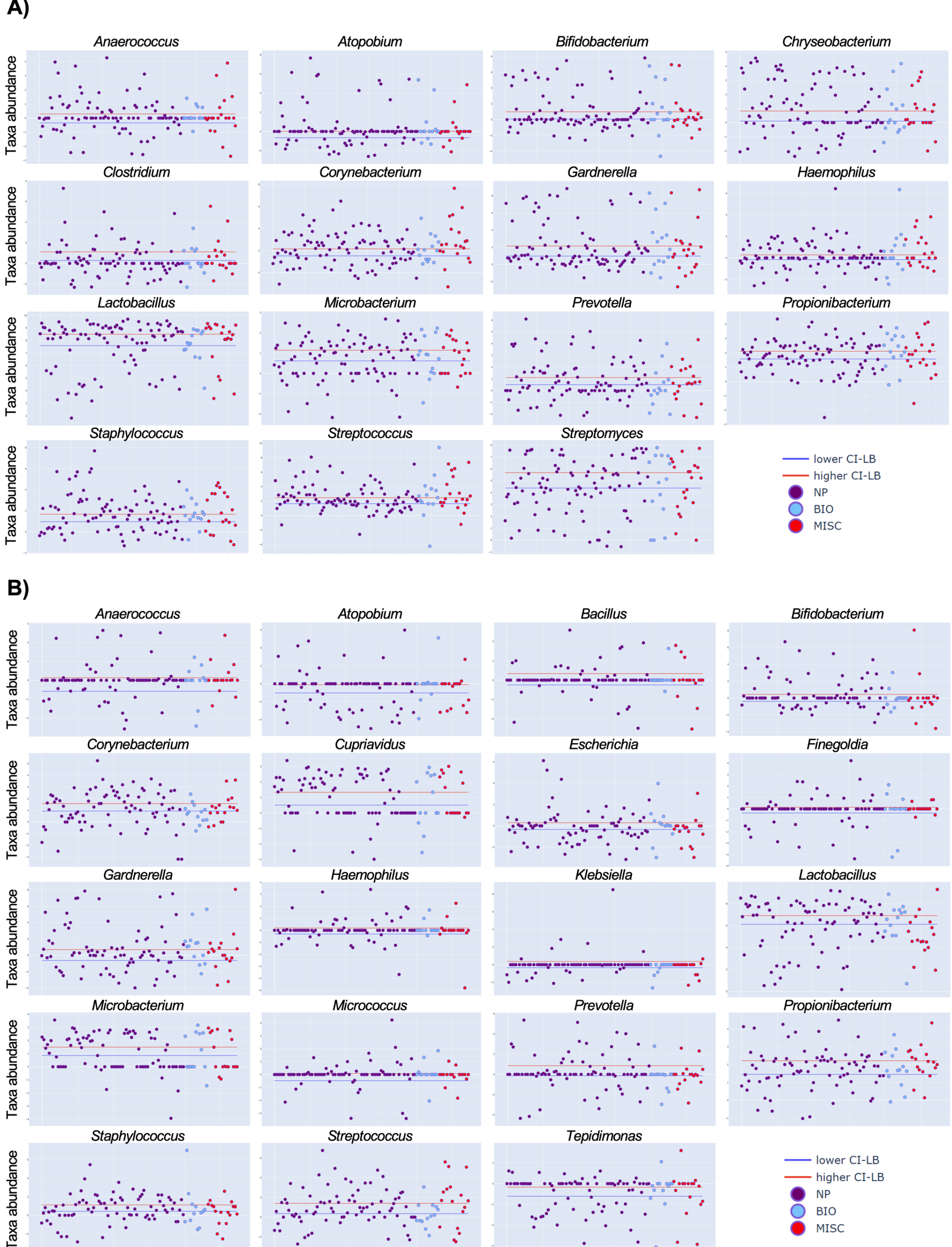


Figure 33 I Confidence intervals of bacterial taxa detected in patients with live birth.

The endometrial microbiome from patients with live birth was analysed in (A) EF and (B) EB samples to determine the reference ranges for each evaluated taxon. The CI displays the relative abundance for all the assessed patients, revealing the healthy ranges of abundance for the taxa in the tested panel. The healthy distribution was used to define the 95% CI (red line). Then taxa abundance in patients with poor reproductive outcomes were comparatively represented. BIO, biochemical pregnancy; LB, live birth; MISC, miscarriage; NP, no pregnancy.

In the EF microbiota, taxa with significant higher abundance exceeding the established upper CI were *Atopobium*, *Bifidobacterium*, *Chryseobacterium*, *Gardnerella,* and *Streptococcus* for non-pregnant women, and *Haemophilus* and *Staphylococcus* for women who suffered a miscarriage. By contrast, taxa with significant higher distance to the lower established CI were *Lactobacillus* and *Microbacterium* for non-pregnant women, and *Lactobacillus* for patients with miscarriage **(Figure 34A)**.

In the EB microbiota, taxa with significant abundance above the established CI for non-pregnant women were *Bifidobacterium*, *Gardnerella,* and *Klebsiella*, and taxa with significant abundance below the established CI were *Cupriavidus*, *Finegoldia*, *Lactobacillus,* and *Tepidimonas* **(Figure 34B)**. For the rest of the comparisons, the difference observed did not reach the statistical significance, possibly due to the small number of patients with biochemical pregnancy and clinical miscarriage.

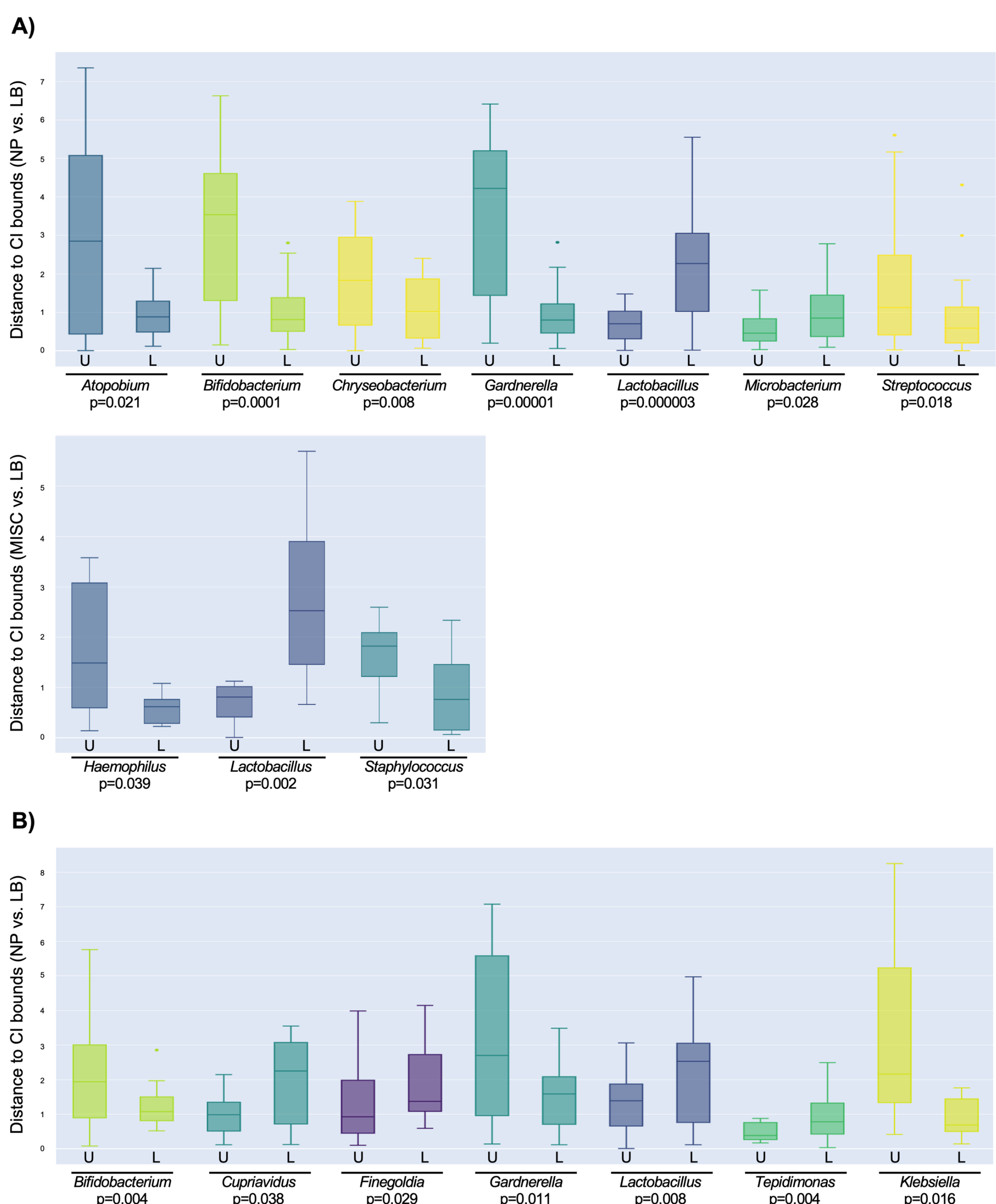


Figure 34 | Pathogenic bacterial profiles significantly associated with reproductive outcome.
Box plots showing taxa with significant differential abundance in no pregnancy, biochemical pregnancy, and miscarriage compared to live birth in (A) EF and (B) EB samples. Differential abundance was calculated using the distance of each value to the upper or lower bounds for the 95% CI in in LB (Figure 32). Only taxa with significant differential abundance, calculated with two-sided Mann-Whitney U-test are represented in the graphs. CI, confidence interval; L, lower confidence interval; LB, live birth; MISC, miscarriage; NP, no pregnancy; U, upper confidence interval.

2.5. Chronic endometritis bacteria with reproductive impact

The main pathogenic bacteria that have been reported to cause uterine infections were also evaluated comparing their abundance with the CI generated for patients with live birth **(Figure 35)**. Specifically, the bacterial genera assessed were: *Enterococcus*, *Escherichia*, *Klebsiella*, *Streptococcus*, *Staphylococcus*, *Gardnerella*, *Mycoplasma*, *Ureaplasma*, *Chlamydia*, and *Neisseria*, which have been reported as responsible for CE and are suspected to be a potential cause of infertility and obstetric and neonatal complications (Kitaya et al., 2016; Kitaya et al., 2018).

Compared to patients with a live birth, the CE pathogens significantly increased in EF microbiota were *Gardnerella*, *Klebsiella,* and *Streptococcus* for non-pregnant women, *Enterococcus* for biochemical pregnancy, and *Klebsiella* and *Staphylococcus* for clinical miscarriage. In the EB microbiota, *Gardnerella*, *Neisseria,* and *Klebsiella* were significantly enriched in non-pregnant women compared to live birth, while *Enterococcus* abundance was below the CI. In the other reproductive categories (biochemical pregnancy and clinical miscarriage) no significant taxa were detected in EB samples. Interestingly, *Gardnerella* and *Klebsiella* were the only common pathogens significantly enriched in both EF and EB from patients that did not become pregnant after pET.

2.6. Endometrial microbiota profiles associated with reproductive outcomes

In summary, the pathogenic profile associated with reproductive failure in our cohort of infertile patients consisted of *Atopobium*, *Bifidobacterium*, *Chryseobacterium*, *Gardnerella*, *Haemophilus*, *Klebsiella*, *Neisseria*, *Staphylococcus,* and *Streptococcus*. In contrast, *Lactobacillus* was consistently enriched in endometrial samples (both in EF and EB) from patients with a live birth. Also, commensal bacteria such as *Cupriavidus*, *Finegoldia*, *Microbacterium,* and *Tepidimonas* were positively associated with live birth.

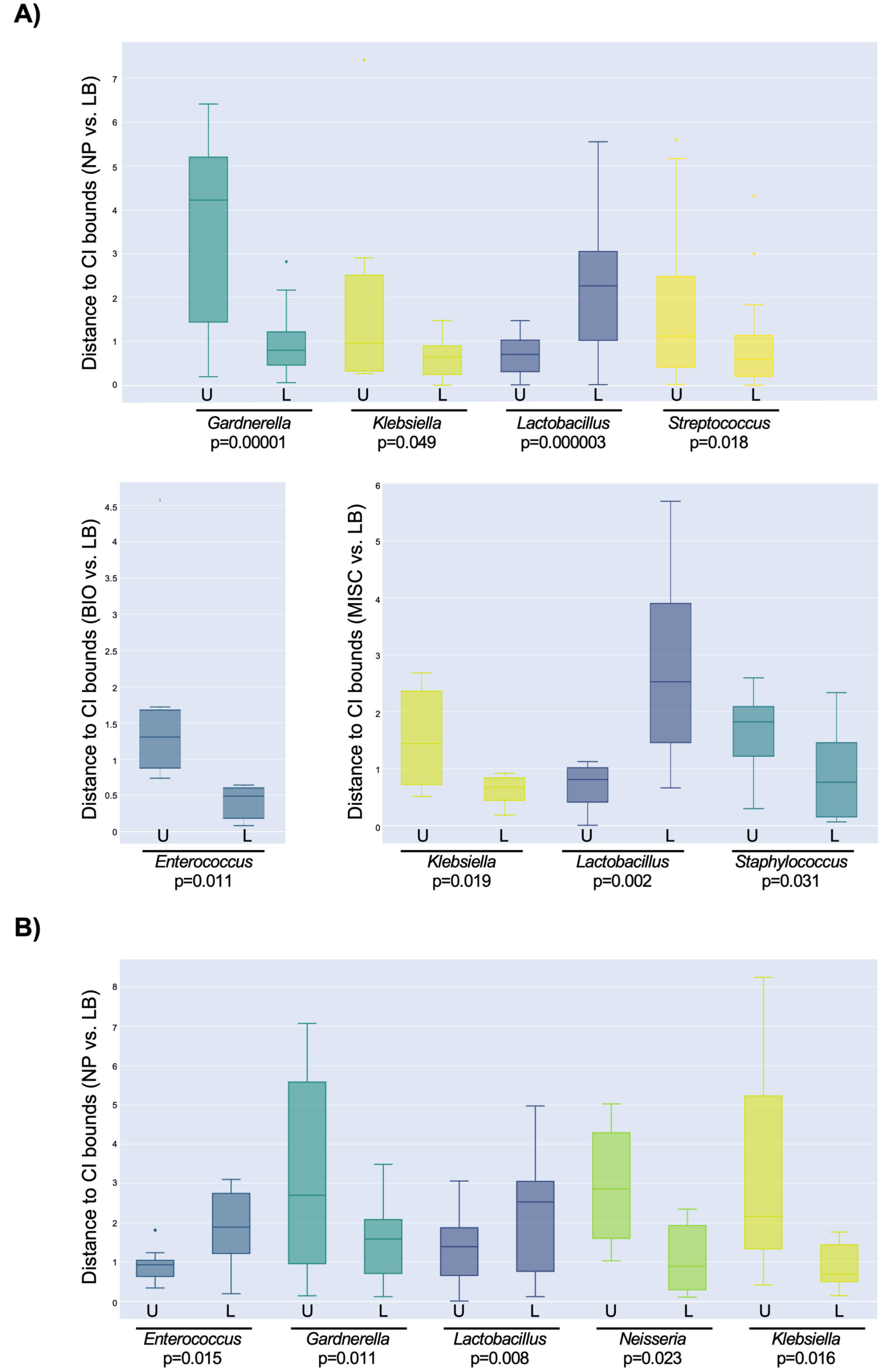


Figure 35 | Chronic endometritis bacteria significantly associated with reproductive outcome.
Box plots showing differential abundance in CE causing bacteria in no pregnancy, biochemical pregnancy, and miscarriage compared to live birth in (A) EF and (B) EB samples. Differential abundance was calculated using the distance of each value to the upper or lower bounds for the 95% CI in LB. Only taxa with significant differential abundance, calculated with two-sided Mann-Whitney U-test are represented in the graphs. BIO, biochemical pregnancy; CI, confidence interval; L, lower confidence interval; LB, live birth; MISC, miscarriage; NP, no pregnancy; U, upper confidence interval.

3. FUNCTIONAL EVALUATION OF ENDOMETRIAL INFECTIONS BY AN IN VITRO MODEL

3.1. Selection of *Lactobacillus* strains

Based on strains obtained from vaginal isolates (data not shown), six different *Lactobacillus* strains (*Lactobacillus casei*, BPL013; *L. jensenii* BPL016, BPL017, BPL035, BPL044; and *L. rhamnosus* BPL005) were tested to assess their potential probiotic capacity. In addition, given its reported relevance in the FGT, collection strains of *L. crispatus* CECT 4840 and *L. iners* DSM 13335 were also included in the study. To choose the candidate species, the capacity of strains to reduce pH level was studied in a preliminary *in vitro* assay. The pH levels decreased when all the different *Lactobacillus* strains were incorporated into the culture, reaching pH values around 4.5, similar to those found in vagina (Amabebe & Anumba, 2018). The highest pH reduction was observed in the case of *L. rhamnosus* BPL005, for a final pH 3.82. In the co-culture between *L. casei* BPL013 and *L. rhamnosus* BPL005, the results obtained did not showed differences compared to the results obtained with single cultures **(Table 9)**.

Table 9 l pH levels obtained in growth cultures of *Lactobacillus* strains.

Strain	pH (Mean ± SD)
Control (medium without culture)	6.10 ± 0.17
L. crispatus CECT4840	4.55 ±0.33
L. iners DSM13335	4.52 ± 0.51
L. casei BPL013	4.30 ± 0.30
L. jensenii BPL016	4.46 ± 0.41
L. jensenii BPL017	4.30 ± 0.15
L. jensenii BPL035	4.34 ± 0.37
L. jensenii BPL044	4.61 ± 0.34
L. rhamnosus BPL005	3.82 ± 0.24
L. casei BPL13 + *L. rhamnosus* BPL005	4.25 ± 0.34

Considering the sharp reduction in pH levels, *L. rhamnosus* BPL005 was selected to counteract the effect of dysbiotic or pathogenic bacteria on the endometrium and to investigate its beneficial role in endometrial infections. To this end we used an *in vitro* colonisation model of hEECs in which the pH, inflammatory molecules (supernatant), and the bacterial growth (pellet) were assessed after the co-infections.

3.2. Evaluation of pH-reducing capacity

After the colonisation of primary cultures of hEECS with pathogenic bacteria (*A. vaginae*, *G. vaginalis*, *P. acnes*, and *S. agalactiae*) alone or in combination with *L. rhamnosus* BPL005 for 18 h in anaerobic conditions, pH levels were measured **(Table 10)**. Except for streptococci infections, pH levels were not lowered by pathogen addition. However, when *L. rhamnosus* BPL005 was added to the plate, pH levels dropped to below pH 5. In the case of *S. agalactiae*, pathogenic strain reached levels close to those obtained with *Lactobacillus*.

Table 10 | pH levels obtained in infection endometrial assays.

Assays	pH (Mean ± SD)
Control (Broth + epithelium)	6.66 ± 0.16
A. vaginae	6.63 ± 0.00
G. vaginalis	6.64 ± 0.01
P. acnes	6.22 ± 0.73
S. agalactiae	4.42 ± 0.02[a*]
L. rhamnosus	4.31 ± 0.01[a*]
L. rhamnosus + *A. vaginae*	4.42 ± 0.03[b*]
L. rhamnosus + *G. vaginalis*	4.43 ± 0.02[b*]
L. rhamnosus + *P. acnes*	4.51 ± 0.06[b*]
L. rhamnosus + *S. agalactiae*	4.41 ± 0.02

[a] comparing control (broth + epithelium) vs. each pathogen, are statistically different
[b] comparing each pathogen vs. its grown together with strain BPL005, are statistically different
* $p < 0.001$

3.3. Quantification of secreted inflammatory molecules

Spent culture media obtained from co-cultures of hEECs with *L. rhamnosus* BPL005, the selected pathogens, or their combinations were also analysed for the secretion of cytokines, chemokines, and growth factors (GM-CSF, HB-EGF, IFN gamma, IL-1β, IL-1 RI, IL-1RA, IL-6, IL-8, MCP-1, and RANTES).

No detectable levels of GM-CSF, HB-EGF, IFN gamma, or IL-1 RI were recorded in this assay. The average concentrations of other molecules secreted to spent media by hEECs under control conditions (before colonisation with bacterial cells) were: IL-6, 868.25 pg/mL; IL-8, 29.81 μg/mL; MCP-1, 1.99 μg/mL; IL-1β, 9.39 pg/mL; RANTES, 10.67 pg/mL, and IL-1RA, 643 pg/mL. The amount of RANTES secreted by hEECs did not show changes upon colonisation with bacteria, either *L. rhamnosus* BPL005, dysbiotic/pathogenic bacteria, or combinations between them. The colonisation of *L. rhamnosus* BPL005 in the culture produced a decrease in IL-6, IL-8, and MCP-1, that was much more evident in the presence of pathogens, and presented significant values in the case of co-cultivation with *A. vaginae* (IL-6: 2-fold decrease, $p < 0.01$; IL-8: 3.5-fold decrease, $p < 0.001$; MCP-1: 16.5-fold decrease, $p < 0.001$), *G. vaginalis* (IL-8: 2.6-fold decrease, $p < 0.05$; MCP-1: 16.5-fold decrease, $p < 0.001$), *P. acnes* (MCP-1: 5-fold decrease, $p < 0.001$), and *S. agalactiae* (IL-6: 5-fold decrease, $p < 0.001$). On the other hand, the addition of *L. rhamnosus* BPL005 to the culture produced a generalised increase in IL-1RA and IL-1β, showing a statistically significant increase in the presence of *A. vaginae* and *G. vaginalis* (IL-1RA: 8.9 and 12.5-fold change increase respectively, $p < 0.05$) and *S. agalactiae* (IL-1β: 2.5-fold change, $p < 0.05$) **(Figure 36).**

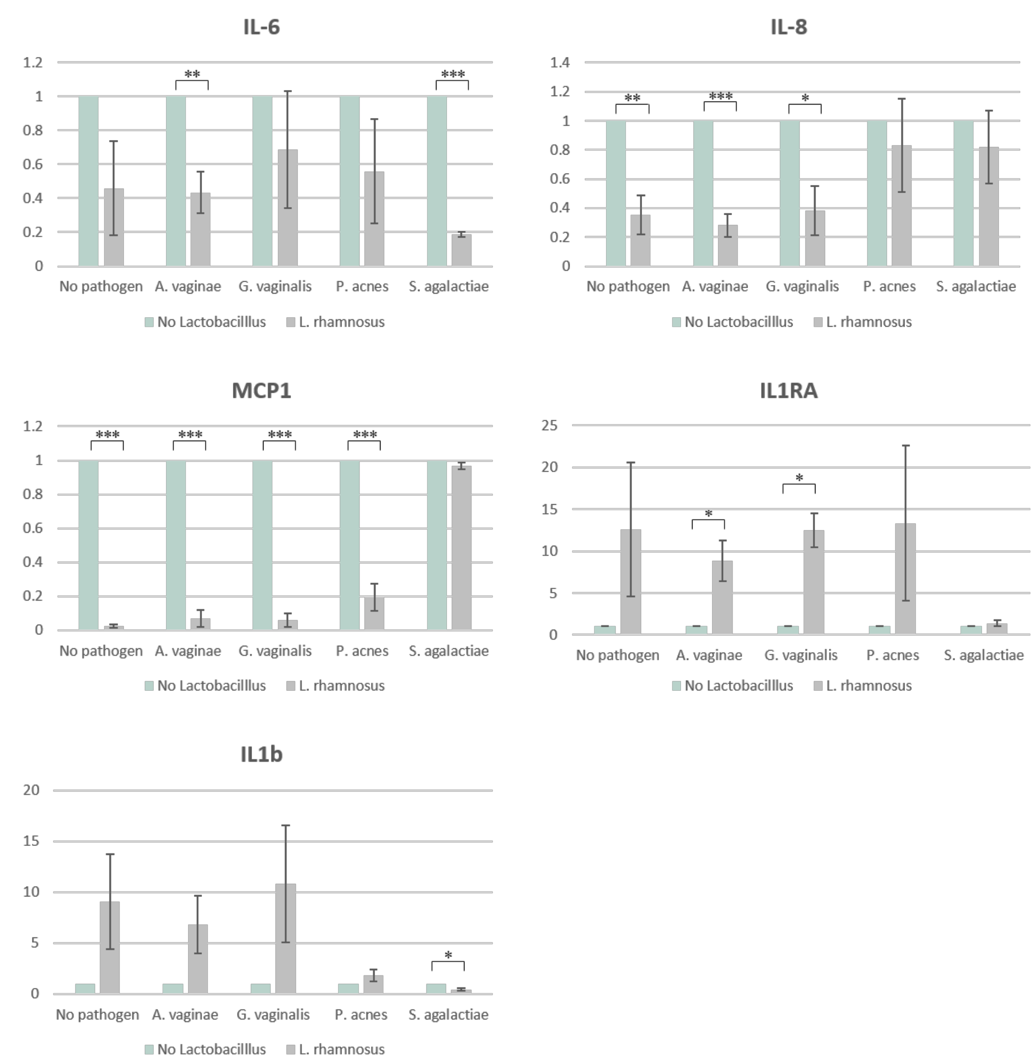


Figure 36 l Cytokine secretion after the infection endometrial assays.

Spent culture media obtained from co-cultures of hEECs with *L. rhamnosus* BPL005 and the selected pathogens were analysed for the secretion of cytokines, chemokines, and growth factors. Samples were analysed in duplicates in three biological replicates (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$).

3.4. Quantification of selected populations by RT-PCR

RT-PCR was used to quantify the evolution of pathogens in the infection assays. The co-cultivation of strain *L. rhamnosus* BPL005 with tested pathogens produced a significant reduction of *P. acnes* and *S. agalactiae* levels (a reduction of log_{10} 1.36 cells/mL and log_{10} 2.14 cells/mL, respectively) and a tendency to reduce *A. vaginae* (log_{10} 0.98 cells/mL) and *G. vaginalis* (log_{10} 0.64 cells/mL) although the differences observed in the last two cases were not statistically significant **(Figure 37)**.

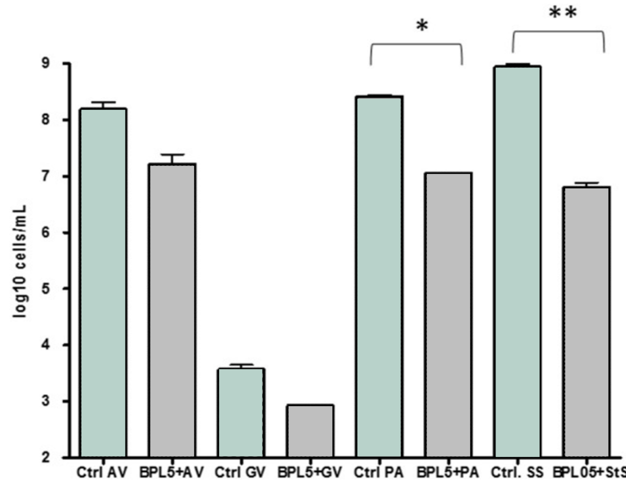


Figure 37 | Quantification of pathogen cells in infection assays with *L. rhamnosus*.

Pellet obtained after centrifugation of spent media from co-cultures of hEECs with *L. rhamnosus* BPL005 and the selected pathogens was analysed to evaluate the bacterial growth. Samples were analysed in duplicates in three biological replicates (*$p < 0.05$; **$p < 0.001$). AV, *A. vaginae*; BPL5, *L. rhamnosus*; GV, *G. vaginalis*; PA, *P. acnes*; StS, *S. agalactiae*.

4. FUNCTIONAL EVALUATION OF ENDOMETRIAL MICROBIOTA IN INFERTILE PATIENTS

4.1. Clinical case 1: Endometrial microbiota in a context of recurrent reproductive failure

4.1.1. Case Presentation

In March 2017, a 37-year-old woman with primary infertility of three years and a history of one IVF failure attended the clinic to undergo an ART **(Figure 38)**. Following intracytoplasmic sperm injection, two metaphase-II oocytes were recovered, but only one embryo reached the blastocyst stage. This embryo was identified by PGT-A as euploid and therefore the blastocyst was cryopreserved for transfer.

In April 2017, the patient was included in a biomedical research study, in which the endometrial microbiota was analysed from the EF collected at the time of ERA analysis to study the personalised window of implantation. As this was an observational study, the patient was prepared for a pET in the subsequent cycle, blinded to the microbiota results. In May 2017, the euploid blastocyst was transferred,

resulting in an ectopic pregnancy. The patient was treated with methotrexate (1 mg/kg, intramuscular) for the ectopic pregnancy and metronidazole (single vaginal dose of 500 mg). In October 2017, EB and EF samples were obtained to analyse the presence of pathogenic bacteria by microbial culture and sequencing, respectively. Analysis of the endometrial tissue confirmed the presence of endometritis with abundant Gram-negative bacilli and positive culture for *E. coli*. The patient was prescribed a course of amoxicillin/clavulanic acid (1,000/62.5 mg twice daily for seven days), followed by *Lactobacillus* probiotic tampons (three tampons daily for three days during menses). One month later, in November 2017, two new samples were taken to analyse the presence of pathogenic bacteria by microbial culture and sequencing. However, at this time the microbial culture was negative.

The patient decided to continue with a new ART cycle following the standard of care. The patient exhibited low ovarian reserve, and she elected to initiate an ovum donation cycle. Of 12 metaphase-II donor oocytes, 10 were fertilised and eight embryos reached the blastocyst stage. A pET with one blastocyst, performed in December 2017, resulted in a clinical pregnancy that ended in clinical miscarriage at the ninth week of gestation. In the following months, several antibiotics [amoxicillin/clavulanic acid (500/125 mg every 8 h for seven days) and metronidazole (500 mg twice daily for seven days)] together with *Lactobacillus* vaginal probiotic tampons (three daily for three days) were administered to the patient. After each treatment, an EF sample was taken to evaluate changes in the endometrial microbiota composition (April and June 2018). Finally, an EF was obtained in the same cycle as a third embryo transfer that was performed in October 2018. As in the previous attempts, pregnancy was achieved and the gestational sac was visualised at the fifth week, but a clinical miscarriage occurred at the sixth week of gestation.

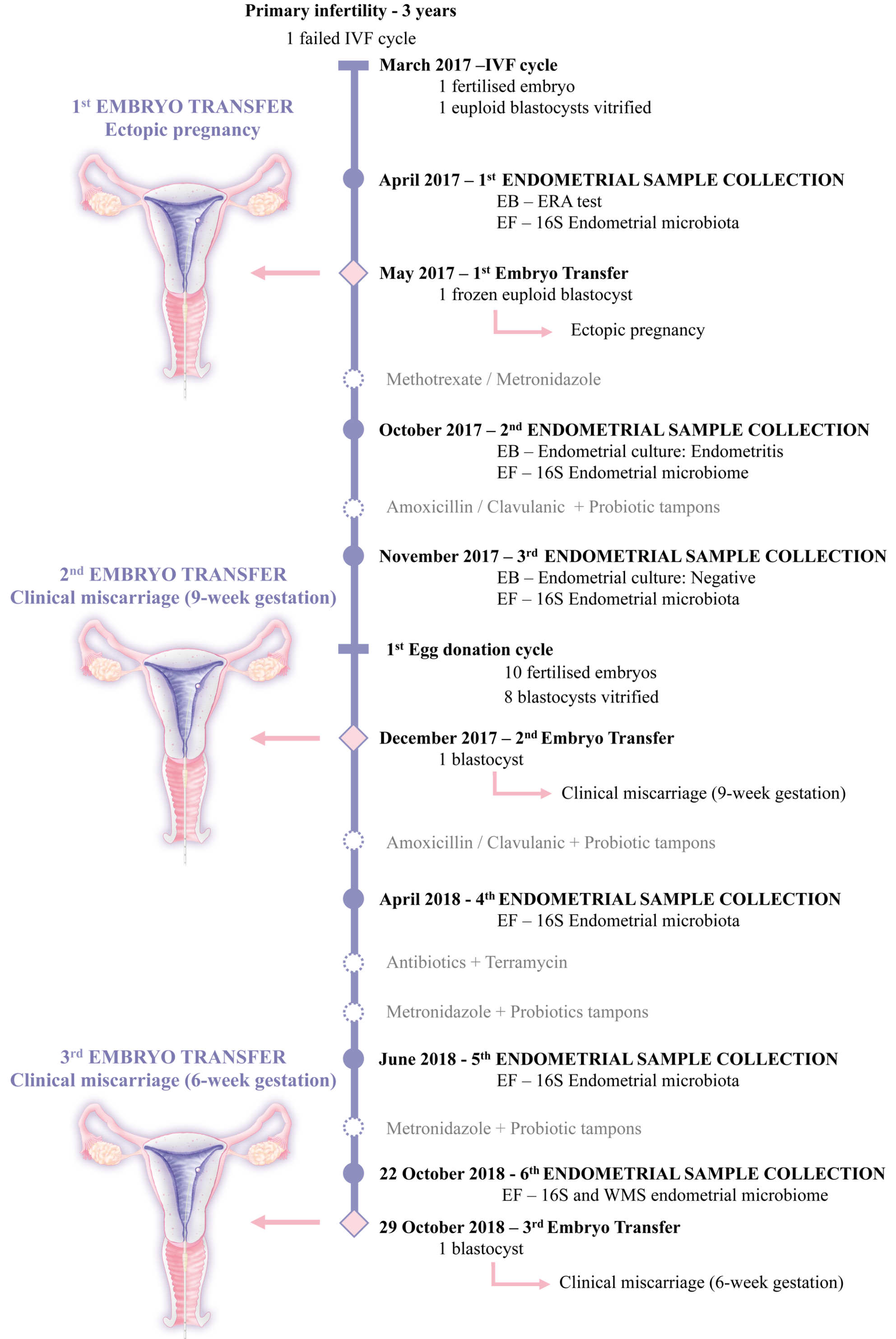


Figure 38 | Flow chart of the clinical evolution of an infertile patient with repeated reproductive failure.

The microbiological follow-up is presented over 18 months, in which the microbiota of an infertile patient was analysed in six EF samples. EB, endometrial biopsy; EF, endometrial fluid; ERA, endometrial receptivity analysis; ICSI, intracytoplasmic sperm injection; IVF, *in vitro* fertilisation; ET, embryo transfer; 16S, 16S rRNA sequencing; WMS, whole metagenome sequencing.

4.1.2. Taxonomic analysis by 16S rRNA sequencing

The microbiological follow-up of this clinical case with repeated reproductive failure is presented over 18 months, in which the microbiota was evaluated in six EF samples using 16S rRNA sequencing.

The first EF collected before pET showed a bacterial population with *Gardnerella* (32.8%), *Pseudoalteromonas* (14.2%), *Bifidobacterium* (8.8%), and *Rhodanobacter* (5.8%), with only 12.1% *Lactobacillus*. After the ectopic pregnancy, and its respective antibiotic treatment, EF and EB samples were taken from the patient to analyse the presence of pathogenic bacteria by microbial culture and sequencing, respectively. The sequencing results of the first EF, which had positive microbiological culture, revealed an altered microbial composition: *Lactobacillus* (18.8%), *Gardnerella* (16.5%), *Rhodanobacter* (15.2%), and *Pseudoalteromonas* (9.0%). The results of the second EF showed the microbiota composition with *Gardnerella* (28.8%), *Pseudoalteromonas* (16.8%), *Atopobium* (13.4%), *Rhodanobacter* (13.0%), and *Lactobacillus* (0.8%), although in this case the microbial culture was negative. After the clinical miscarriage at the ninth week of gestation, several antibiotic treatments were provided to the patient. After each treatment course, an EF sample was taken to evaluate changes in the endometrial microbiota composition (April and June 2018). In both samples, 16S rRNA sequencing detected a persistent colonisation of *Gardnerella* (21.8% and 46.1%, in respective time points), *Atopobium* (19.7% and 4.1%), and *Bifidobacterium* (0.3% and 15.5%) with suboptimal levels of *Lactobacillus* (50.2% and 33. 4%). Finally, in the sample collected before the last embryo transfer that resulted in a clinical miscarriage at the sixth week of gestation, sequencing still revealed the presence of *Lactobacillus* (48.1%), *Gardnerella* (32.8%), *Bifidobacterium* (6.2%), and *Atopobium* (5.4%) (**Figure 39**).

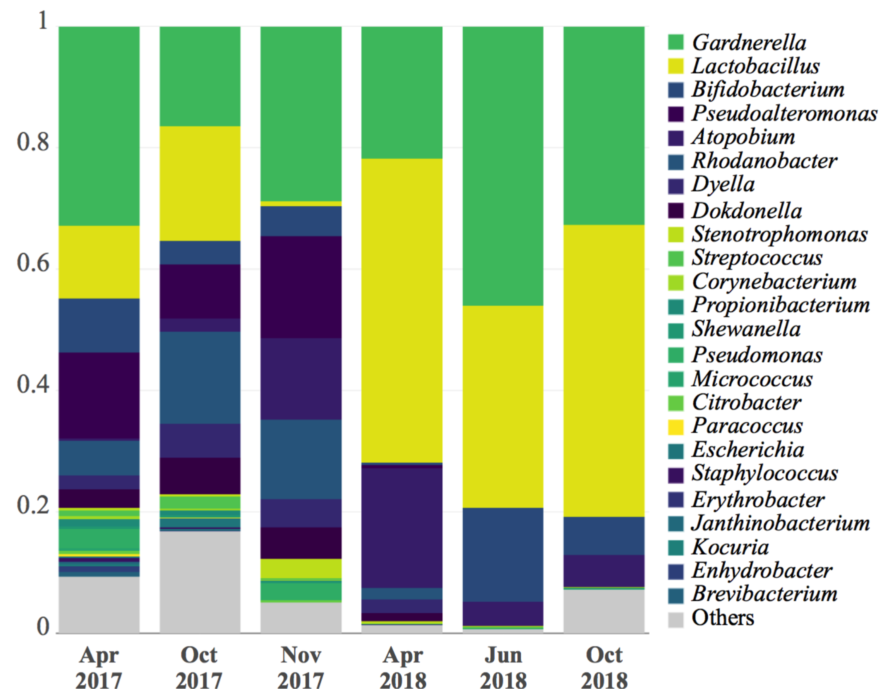


Figure 39 | Endometrial microbiota profile assessed by 16S rRNA gene sequencing.
The endometrial microbiota of an infertile patient with repeated reproductive failures was evaluated over 18 months in six EF samples by 16S rRNA gene sequencing, showing a persistent presence of *Gardnerella* and other bacterial taxa such as *Atopobium* and *Bifidobacterium*.

4.1.3. Taxonomic analysis by whole-metagenome sequencing

To further understand the proportion of *G. vaginalis*, its functionality, antibiotic resistance, and impact on reproductive health, a more complete taxonomic and functional analysis by WMS was performed. This analysis was conducted using the last EF sample collected before the embryo transfer that resulted in a clinical miscarriage at the sixth week of gestation. WMS analysis yielded a total of 99,127,515 reads. After quality control and filtering of human reads, only 0.4% of reads corresponded to bacterial DNA **(Table 11)**.

Table 11 | Sequencing reads obtained after sequencing, quality control, and elimination of human reads.

Sample	Raw reads	Cleaned reads (%)	Joined reads (%)	Nonhuman reads (%)
Oct 2018 WMS	99,127,515	*92,198,954 (93.0)*	39,791,044 (40.1)	*383,513 (0.4)*

Taxonomic analysis using WMS verified the dysbiotic profile dominated by *Gardnerella* (86.0%), *Lactobacillus* (8.2%), and *Atopobium* (5.1%) **(Figure 40)**. While these microorganisms were in agreement with those detected by 16S rRNA sequencing, the proportions differed. The taxonomic analysis by WMS also provided information on taxa at the species level, confirming that the most abundant bacterium in the sample was *G. vaginalis*.

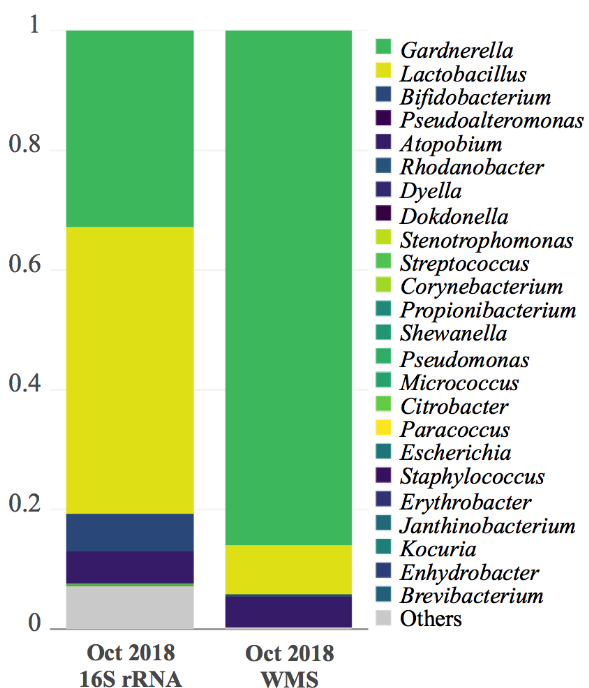


Figure 40 l Endometrial microbiota profile assessed by WMS.
Comparison between 16S rRNA sequencing and WMS in the EF sample taken seven days before the last embryo transfer (October 2018) that resulted in her third reproductive failure.

4.1.4. Assessment of *G. vaginalis* clades by whole-metagenome sequencing

Comparative genomic studies revealed that *G. vaginalis* has a population of four clades/ecotypes with distinct genomic properties that may confer different ecological functions (Ahmed et al., 2012). To assess possible antibiotic resistance, *Gardnerella* sequences obtained from the EF sample collected days before the last miscarriage were compared with the genomes of a panel of 17 *G. vaginalis* strains belonging to different clades (Ahmed et al., 2012).

A total of 613 significant sequence alignments were associated with genes from these strains, most of them matching with clade 1 (n = 452) and clade 4 (n = 309), with 232 and 140 unique genes of these two ecotypes, respectively. Conversely, clade 2 and clade 3 were annotated to a lesser extent, with only four genes specific to each of these two clades **(Figure 41)**.

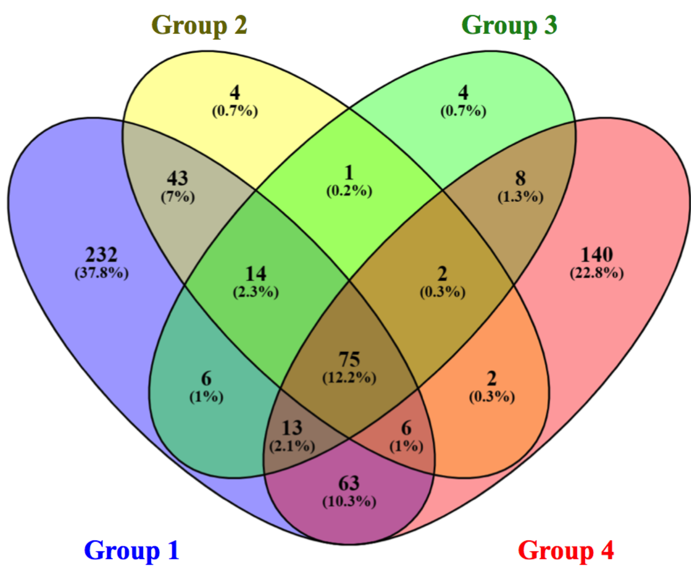


Figure 41 | Venn diagram showing the number of genes associated with the four *G. vaginalis* clades.

Sequences obtained from *Gardnerella* were compared with the genomes of a panel of 17 *G. vaginalis* strains belonging to different clades (Ahmed et al., 2012). A total of 613 significant sequence alignments were obtained, most of them match with clade 1 (n = 232) and clade 4 (n = 140).

To study the particular abundance of each ecotype, the genomic sequences obtained were searched for the presence of clade-specific genes, following the work by Balashov and collaborators (Balashov et al., 2014). Analysis of the genetic markers showed the concomitant presence of multiple *G. vaginalis* clades, clade 4 was the most represented, followed by clade 3 and clade 1 **(Figure 42A)**. Specifically, for clade 4 the chloride transporter ClC family and allantoate amidohydrolase were detected with 192 and 306 reads per kilobase million (RPKM), respectively. For clade 3, thioredoxin and α-β hydrolase fold proteins were interrogated, with 256 RPKM detected for the thioredoxin gene only, and for clade 1, α-L-fucosidase and

galactokinase genes were detected with 104 and 114 RPKM, respectively. No sequences belonging to the α-β hydrolase fold protein encoding gene (clade 3) or the clade 2 genes (hypothetical protein and cellulosome anchoring protein) were found in the sample. The predicted clades were also confirmed by multiplex RT-PCR for clade-specific genes (Balashov et al., 2014), showing positive detection of clades 1 and 4 **(Figure 42B)**.

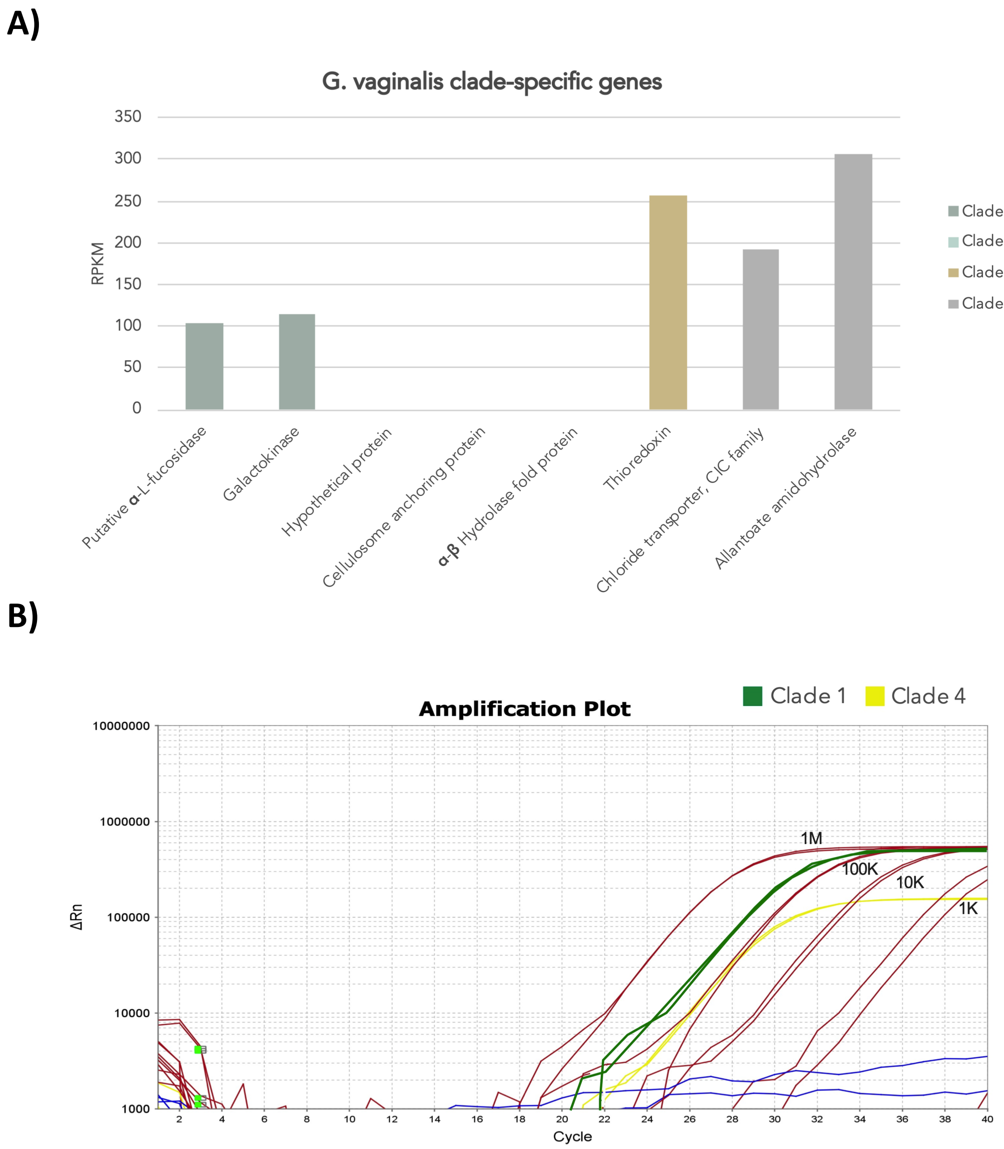


Figure 42 | Assessment of *G. vaginalis* clade-specific genes.

(A) Analysis of specific genetic markers for different *G. vaginalis* clades following the work by Balashov and collaborators (Balashov et al., 2014). (B) Confirmation of the predicted clades in the sample by multiplex RT-PCR for clade-specific genes. The presence of each clade was evaluated through a standard curve generated for *G. vaginalis* strain ATCC 14018 (Clade 1) at concentrations ranging from 0 to 10^6 genome copies.

4.1.5. Functional analysis by whole-metagenome sequencing

The functional metagenomics analysis by WMS was performed using the KEGG database. Most functional categories detected belonged to the genera *Gardnerella*, *Lactobacillus*, and *Atopobium* (representing 66%, 18%, and 14% of detected reads, respectively) **(Figure 43A)**.

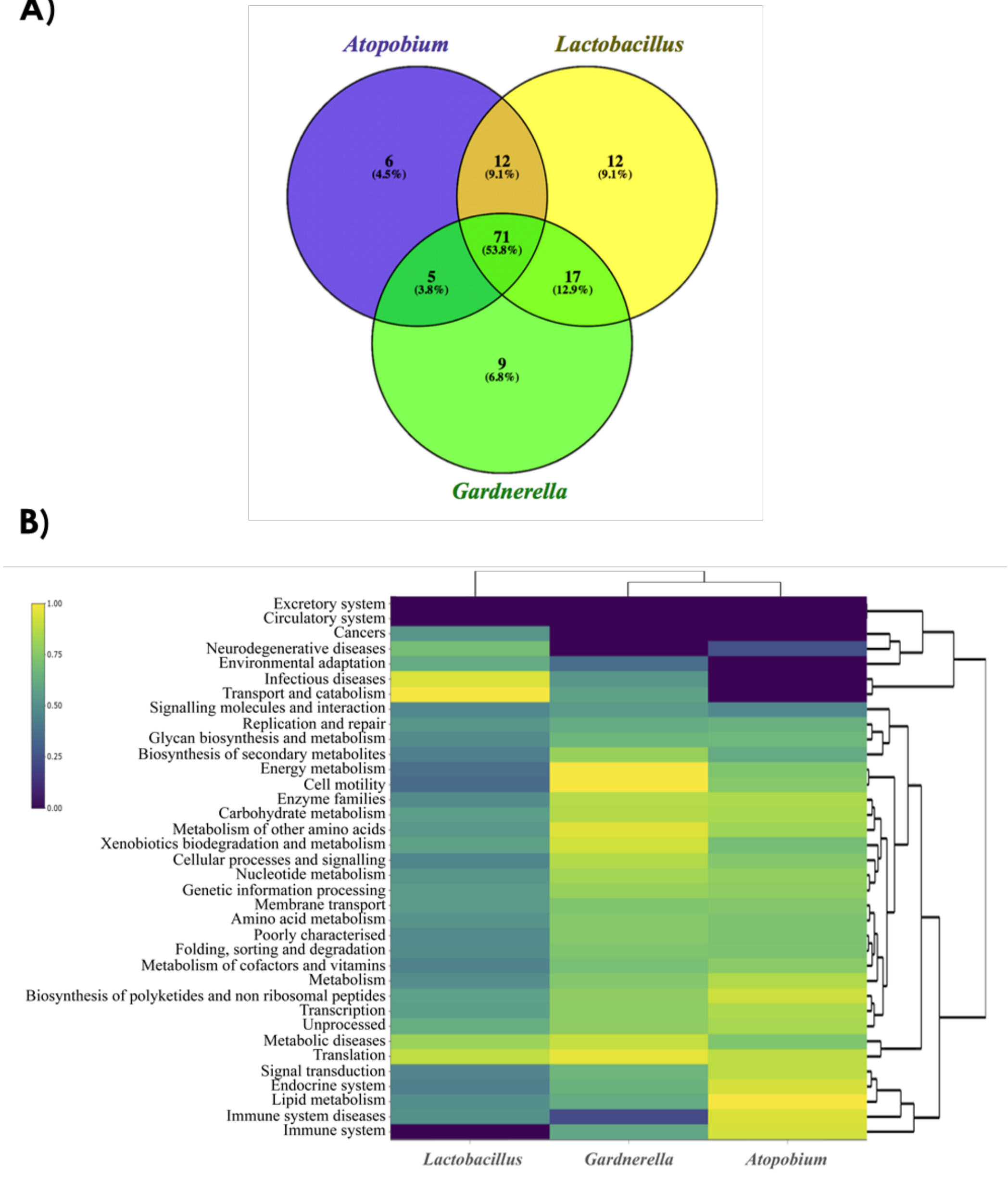


Figure 43 I Functional metagenomics analysis assessed by WMS.

(A) Venn diagram summarizing the number of KEGG functional categories found in *Gardnerella*, *Atopobium*, and *Lactobacillus*. (B) Heatmap representing the most abundant KEGG categories associated to each genus in the EF sample collected seven days before the embryo transfer that resulted in a clinical miscarriage.

Nine KEGG categories were exclusive of *Gardnerella*, comprising pathways related to metabolism (fatty acids biosynthesis, sulfur metabolism, and inositol phosphate metabolism), cell motility (including bacterial chemotaxis and motility proteins), signalling processes (such as sporulation and bacterial toxins) and immune system (NOD-like receptor signalling pathway). In contrast, 12 functional categories were exclusive of *Lactobacillus*, most of which were related to general functions such as metabolism, genetic information, and signalling processes **(Figure 43B, Table 12)**

Table 12: KEGG categories (Level 2-Level 3) exclusively expressed by the genera *Gardnerella*, *Atopobium*, and *Lactobacillus*.

9 categories exclusively expressed by *Gardnerella*	RPKM
Lipid metabolism-Fatty acid biosynthesis	729.54
Cell motility-Bacterial chemotaxis	559.63
Energy metabolism-Sulfur metabolism	303.44
Cellular processes and signalling-Sporulation	225.36
Signaling molecules and interaction-Bacterial toxins	207.02
Cell motility-Bacterial motility proteins	155.71
Environmental adaptation-Circadian rhythm - Plant	137.72
Carbohydrate metabolism-Inositol phosphate metabolism	132.10
Immune system-NOD-like receptor signalling pathway	115.83
6 categories exclusively expressed by *Atopobium*	**RPKM**
Metabolism-Nucleotide metabolism	92.61
Metabolism of cofactors and vitamins-Folate biosynthesis	68.42
Cellular processes and signalling-Cell motility and secretion	55.19
Immune system-RIG-I-like receptor signalling pathway	38.13
Metabolism of other amino acids-Beta-alanine metabolism	34.60
Neurodegenerative diseases-Alzheimer's disease	10.19
12 categories exclusively expressed by *Lactobacillus*	**RPKM**
Carbohydrate metabolism-C5-branched dibasic acid metabolism	34.35
Genetic information processing-Transcription related proteins	33.44
Metabolic diseases-Type I diabetes mellitus	25.47
Carbohydrate metabolism-Pentose and glucuronate interconversions	24.87
Glycan biosynthesis and metabolism-Lipopolysaccharide biosynthesis proteins	24.49
Environmental adaptation-Plant-pathogen interaction	22.52
Xenobiotics biodegradation and metabolism-Benzoate degradation via hydroxylation	16.80
Metabolism of cofactors and vitamins-Ubiquinone and other terpenoid-quinone biosynthesis	15.18
Carbohydrate metabolism-Citrate cycle (TCA cycle)	12.50
Metabolic diseases-Type II diabetes mellitus	10.66
Cellular processes and signalling-Pores ion channels	6.16
Metabolism-Amino acid metabolism	4.21

RPKM: reads per kilobase million associated with each function/taxon.

The sample was also interrogated for the presence of genes previously described to contribute to the pathogenic potential of *G. vaginalis* (Yeoman et al., 2010). Several detected genes were related to (i) toxin-antitoxin system and competitive exclusion, (ii) biofilm formation and epithelial adhesion, and (iii) virulence factors including cytotoxicity, antimicrobial resistance, iron acquisition, and mucin degradation, among others **(Table 13)**.

Table 13 | *G. vaginalis* pathogenic-associated genes detected in the sample collected in the same cycle of the second clinical miscarriage.

ADAPTATION TO ENVIRONMENT	
Mobile elements and horizontal gene transfer:	Recombinase (RecA); Transposase IS3509a; HK97 family phage major capsid protein; Site-specific recombinase phage integrase family; Helicase UvrD/REP; Phage related protein.
Competence:	Putative competence-damage inducible protein (CinA); ABC-type antimicrobial peptide transporter permease component; Glycoside hydrolase (GH) family; Lysozyme; Penicillin-binding protein; Fic-family protein; M13 family peptidase; ATP-binding subunit of Clp protease.
Toxin-antitoxin system:	RelB toxin/antitoxin family; Antitoxin/DNA-binding transcriptional repressor DinJ.
VIRULENCE	
Biofilm formation and exopolysaccharide formation:	Glycosyltransferase (GT) type II; Sortases; LPxTG domain; Actinobacterial surface anchored protein domain.
Epithelial adhesion:	Type I fimbrial major subunit precursor; Pilus assembly protein (PilY1); Tfp pilus.
Antimicrobial resistance:	Efflux transporter; ABC-type multidrug transport system; ABC-type bacteriocin/lantibiotic; Multidrug resistance transporter EmrB/QacA; Bleomycin hydrolase; SalY-type ABC-antimicrobial peptide transport system; Cadmium resistance transporter CadD family protein.
Mucin degradation:	Alpha-mannosidase; Beta-galactosidase.
Cytotoxicity and hemolysis:	Hemolysin-like protein.
Iron intake and utilisation:	FTR1-family iron permease; FtsK/SpoIIIE family protein.
Other virulence factors:	G-related albumin-binding (GA) modules; Virulence-associated E family; Oxygen-insensitive NAPDH nitroreductase (RdxA).

4.2. Case Report 2: Endometrial microbiota in early pregnancy

4.2.1. Case Presentation

In January 2017, a 28-year-old woman with primary infertility of two years and a history of one IVF failure attend to the clinic to undergo ART **(Figure 44)**. The patient did not have medical or surgical complications, had a BMI of 22 kg/m^2, and a negative serologic test result for human immunodeficiency virus, hepatitis B virus, hepatitis C virus, and syphilis. Her husband had normal semen analysis results, and did not present chromosomal abnormalities. As a result of her first intracytoplasmic sperm injection cycle, 14 metaphase-II oocytes were retrieved resulting in 13 zygotes. Of these, 10 embryos reached the blastocyst stage, and six were vitrified after their identification as euploid by PGT-A.

After the first embryo transfer of two euploid blastocysts in February 2017, the pregnancy test was negative. Two months later, an EF sample was collected and stored for microbiota analysis before the EB used for the endometrial receptivity analysis to guide pET. Subsequently, two euploid blastocysts were transferred in May 2017. Pregnancy was achieved, and the beta human chorionic gonadotropin concentration was 278.9 mIU/mL. One gestational sac, 8 mm in diameter, was visualised with the use of transvaginal ultrasound scanning during fifth week of pregnancy. However, a clinical miscarriage occurred at the eighth week of gestation, and dilation and curettage were performed. The patient received azithromycin, 500 mg per day for three days. Analysis of the products of conception confirmed that the embryo was chromosomally normal with a profile 46, XX of foetal origin.

Two months after the dilation and curettage, the patient was seen at the time of the expected menstruation to start a new embryo transfer cycle. In this visit, an EF sample was collected and stored to investigate changes in the microbiota. Subsequently, it became evident that the patient had conceived spontaneously and

was four weeks pregnant when the sample of EF was obtained. The pregnancy continued uneventfully, and the patient delivered a healthy male infant who weighed 3,700 g by caesarean section at 40 weeks of gestation.

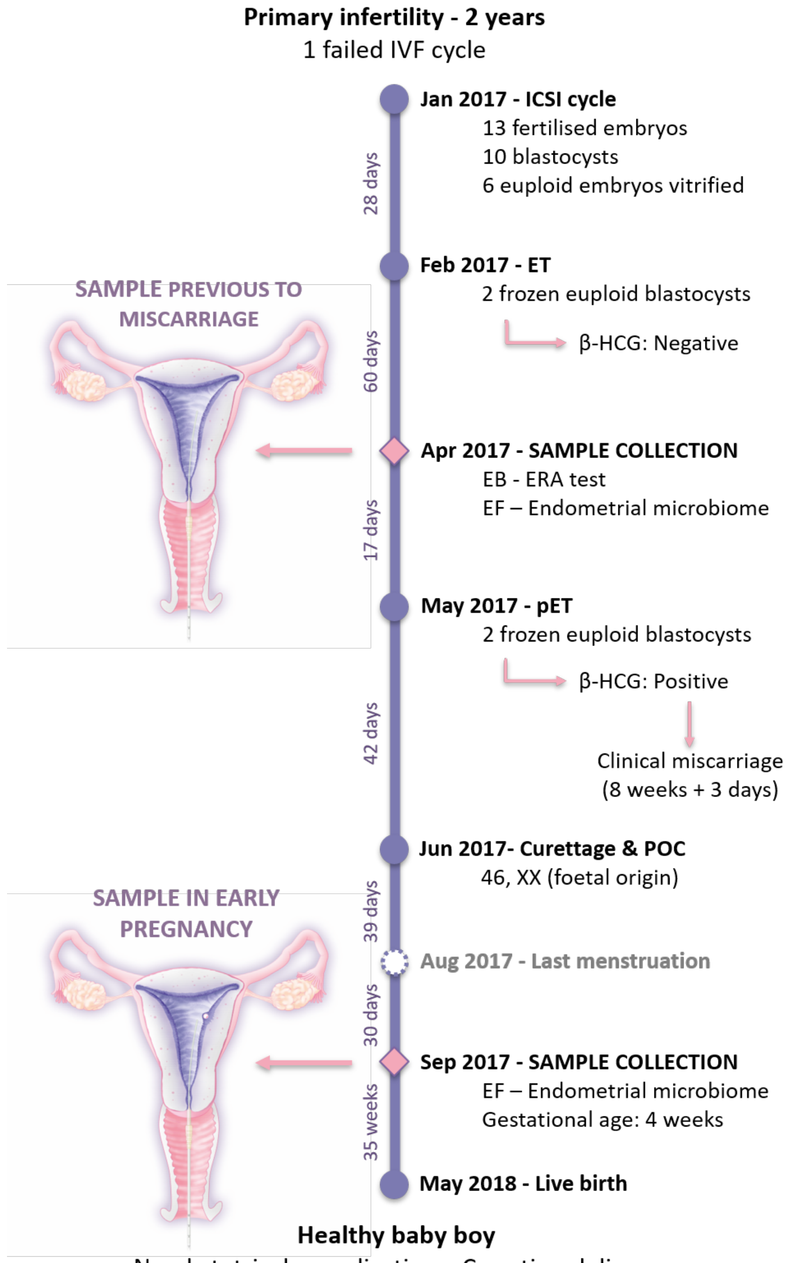


Figure 44 I Flow chart showing the clinical evolution of an IVF patient from clinical miscarriage to successful pregnancy.

EF samples were collected for microbiome analysis before an embryo transfer that resulted in clinical miscarriage and during a 4-week gestation in the same woman. C-section, caesarean delivery; EB, endometrial biopsy; EF, endometrial fluid; ERA, endometrial receptivity analysis; **β**-HCG, beta human chorionic gonadotropin; ET, embryo transfer; ICSI, intracytoplasmic sperm injection; IVF, *in vitro* fertilisation; pET, personalised embryo transfer; POC, product of conception.

4.2.2. Taxonomic analysis by 16S rRNA sequencing

The 16S rRNA sequencing of the EF obtained in the cycle before the clinical miscarriage showed an NLD profile with 5% Actinobacteria, 19% Firmicutes, and 76% Proteobacteria. From these phyla, 15% of lactobacilli was encountered together with several pathogenic bacterial genera previously reported to affect the reproductive tract such as Enterobacteriaceae (3%), *Streptococcus* (2%), *Pseudomonas* (2%), and *Staphylococcus* (0.8%). The microbiota in the sample collected at the fourth week of the successful pregnancy in the same patient revealed an LD profile with 91% of Firmicutes and only 9% of Proteobacteria. Interestingly enough, *Lactobacillus* was the only bacteria present under the Firmicutes phylum, accounting for 91% of the sample **(Figure 45)**.

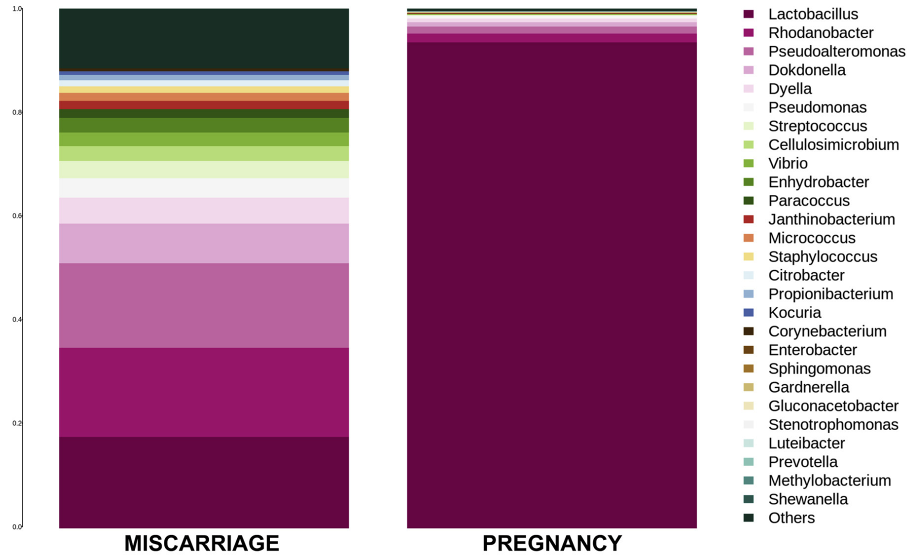


Figure 45 I Endometrial microbiota profile assessed by 16S rRNA gene sequencing. Microbiota composition profiles show the 20 most-abundant genera and their relative abundance in the sample preceding a spontaneous clinical miscarriage or a successful pregnancy in the same woman with the use of 16S rRNA sequencing.

4.2.3. Taxonomic analysis by whole-metagenome sequencing

The metagenomic analyses of both samples yielded a total of 238,778,133 reads. After quality control and filtering of reads, only 0.1%–1% of reads corresponded to bacterial DNA; the vast majority of the sequences mapped to human DNA **(Table 14)**.

Table 14 | Sequencing reads obtained after sequencing, quality control, and elimination of human reads.

Sample	Raw reads	Cleaned reads (%)	Joined reads (%)	Nonhuman reads (%)
Miscarriage	126,325,813	*115,991,731 (91.8)*	56,197,765 (44.5)	*1,291,879 (1)*
Pregnancy	112,452,320	102,731,745 (91.4)	41,138,063 (36.6)	76,160 (0.1)

As in the 16S rRNA sequencing results, the taxonomic analysis by WMS showed a dysbiotic NLD profile in the EF obtained before the clinical miscarriage and, alternatively, higher *Lactobacillus* abundance in the EF sample collected in the presence of an embryo with successful implantation. Nevertheless, when the complexity of the microbial communities with the WMS technology was analysed, certain bacterial genera not represented in the 16S rRNA sequencing were detected, such as *Cutibacterium*, *Acidovorax*, *Xanthomonas*, and *Aerococcus* **(Figure 46A)**.

Although the taxonomic assignment derived from WMS showed greater microbial diversity than 16S rRNA sequencing, when functional and taxonomic analyses were combined, the microbial diversity present in each sample was reduced. Because of this, the functional metagenomic analysis showed that the sample collected before the clinical miscarriage contained *L. crispatus* as the predominant *Lactobacillus* spp. (15%) and a variety of bacterial genera, such as *Propionibacterium (*21%), *Pseudomonas* (10%), and *Streptococcus* (3.5%). In contrast, in the sample collected during the successful pregnancy, *L. iners* was the only microbe found in the endometrium **(Figure 46B)**.

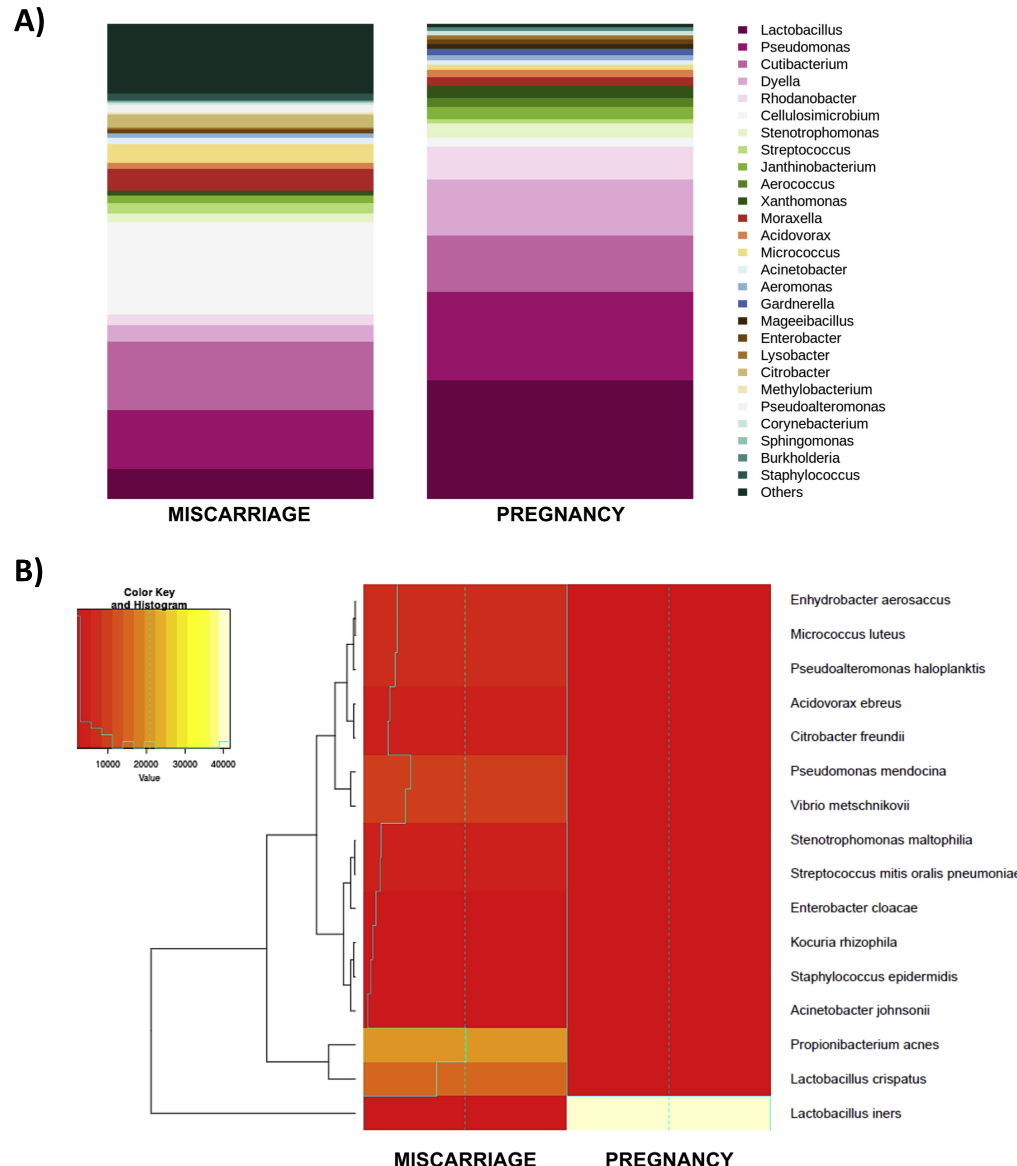


Figure 46 I Endometrial microbiota profile assessed by WMS.

(A) Microbiota composition profiles show the 20 most-abundant genera and their relative abundance in the sample preceding a spontaneous clinical miscarriage or a successful pregnancy in the same woman with the use of WMS. (B) Heatmap shows the bacterial composition with associated functional pattern analysed by WMS.

4.2.4. Functional analysis by whole-metagenome sequencing

The results of the metagenomic sequencing showed both taxonomic and functional differences in the two endometrial microbiomes from the same patient. The functional metagenomic analysis was performed with the use of information obtained from the UniRef database and clusters of orthologous (COG) groups, considering the proteins and functions associated with a specific taxonomy, respectively.

After analysis of the most represented proteins in each sample, a greater functional annotation associated with several bacteria was observed in the sample preceding the clinical miscarriage, whereas in the sample obtained during the successful pregnancy, only proteins associated with *L. iners* were detected **(Figure 47)**.

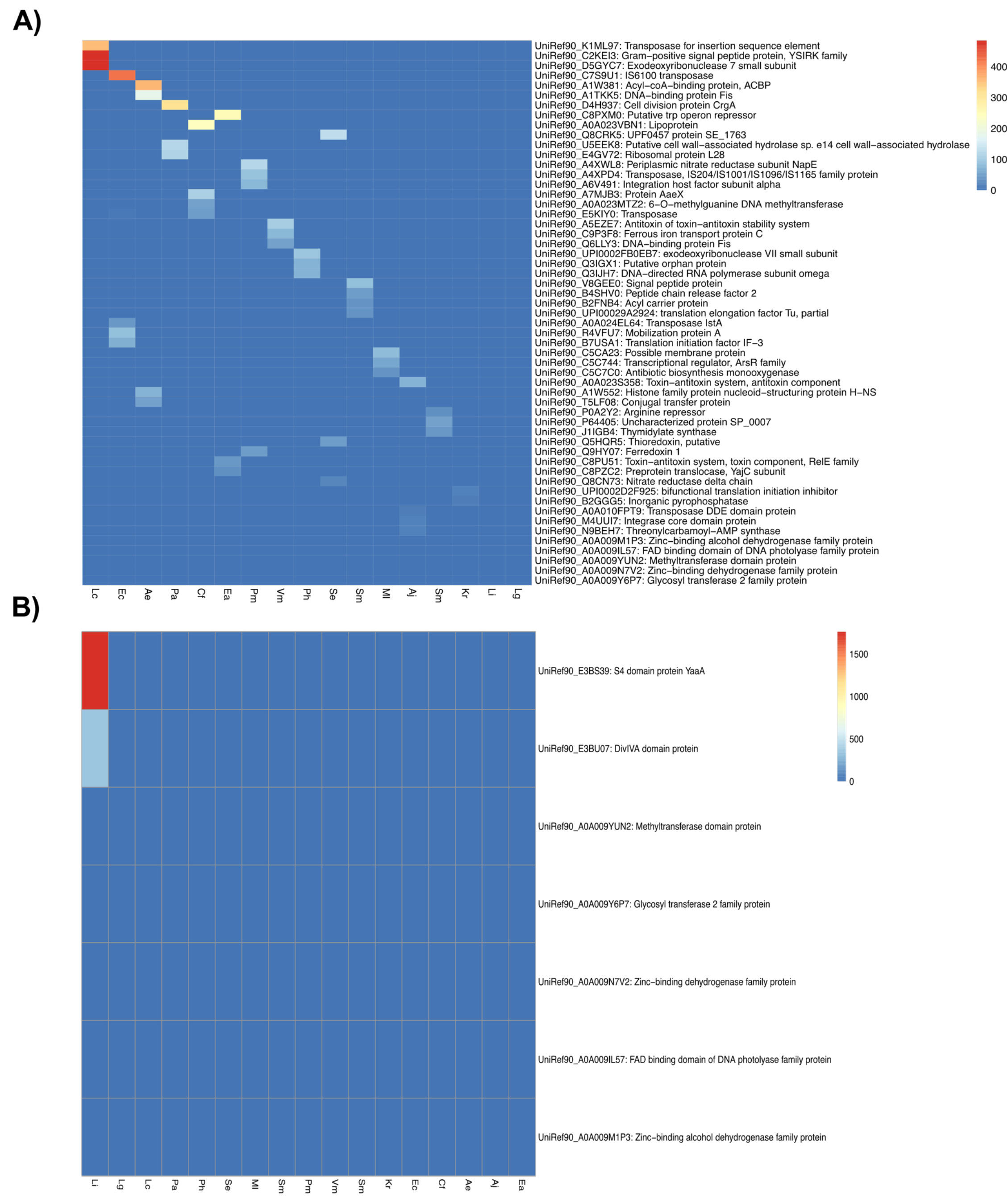


Figure 47 | Protein pattern associated with taxonomy assessed by WMS.
The functional metagenomic analysis was carried out in (A) the sample preceding a miscarriage and (B) a successful pregnancy with the use of the information obtained from UniRef database.

Different functional profiles were also observed when the main COG groups present in both samples were compared **(Figure 48A)**. "Information storage and processing" was the most represented functional category in both samples, with 2,285 and 798 counts per million in the samples associated with clinical miscarriage and successful pregnancy, respectively. Moreover, of the 25 COG subcategories established in the database, the endometrium before miscarriage showed an unstable functional pattern characterised by transposases and insertion elements belonging to the subcategory "[L] Replication, recombination and repair." For instance, we found transposases and mobile elements, like Tra8, the only member of the superfamily cl28582 (COG2826), and a member of the superfamily cl27435 (COG3547; **Figure 48B**). In contrast, the microbiome during early pregnancy subcategory "[J] translation, ribosomal structure and biogenesis" was the most represented. Notably, functions associated with defense mechanisms (subcategory [V]), carbohydrate metabolism and energy production (subcategories [C] [G]), and cell division (subcategory [D]) were represented only in the sample from the successful pregnancy, where the predominant bacterium was *Lactobacillus* **(Figure 48B)**.

Microbes produce GPCRs ligands to communicate with the human host and to regulate its physiologic condition (Cohen et al., 2017). For this reason, both EF samples were finally searched for sequences associated with the N-acyl synthase protein family PF13444, the consensus PFAM profile of the GPCRs. Specifically, the GNAT domain implicated in bacterial antibiotic resistance, chromatin remodelling, and anabolic and catabolic functions was searched. In the endometrial microbiome before the clinical miscarriage, 44 sequences that corresponded to molecules of the GNAT domain were identified; but in the microbiome of the early pregnancy, these sequences were not found. To study the clinical relevance of these molecules, ligands were searched in the ChEMBL database (The European Molecular Biology Laboratory, United Kingdom). Three putative ligands were found related to the GNAT domain:

Luspatercept, Ecallantide, and Rilonacept, which correspond to inhibitors of activin receptor type-2B, plasma kallikrein, and IL-1β, respectively.

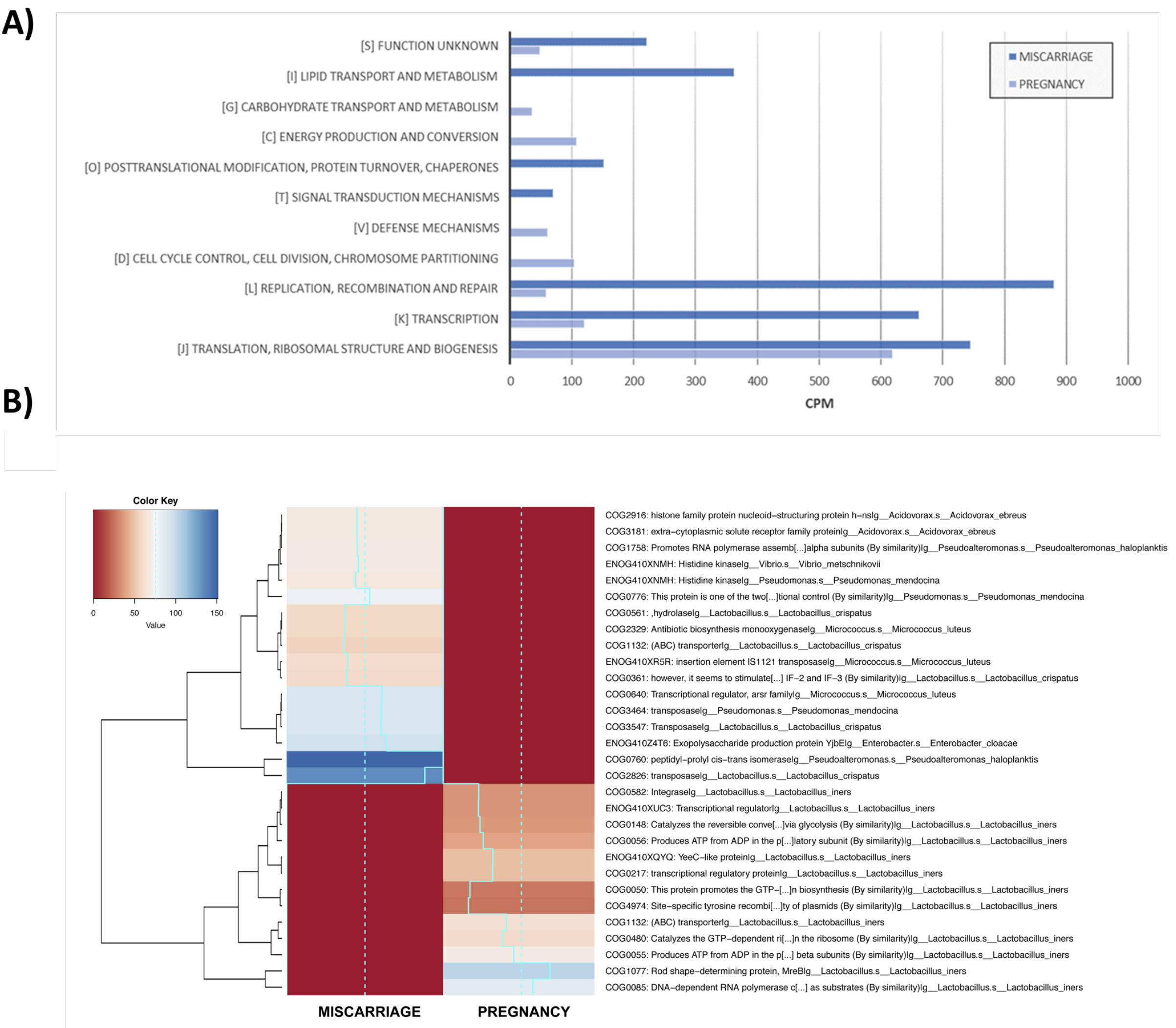
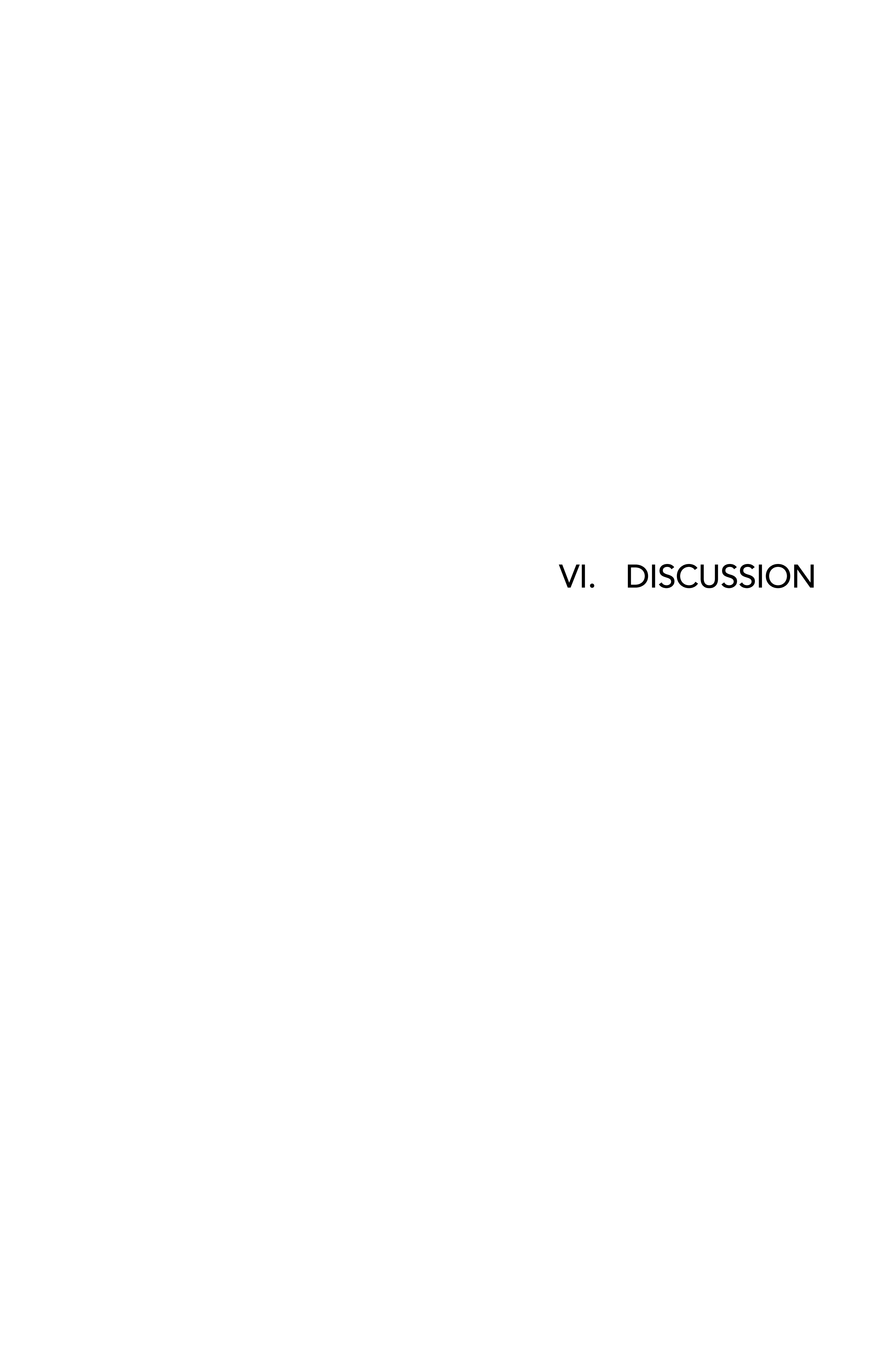


Figure 48 I Functional pattern associated with taxonomy assessed by WMS.

(A) Bar graph summarises the most detected COGs categories. (B) The functional metagenomic analysis was carried out in the sample preceding a miscarriage (*left panel*) and a successful pregnancy (*right panel*) with the use of the information obtained from COGs associated with a specific taxonomy. CPM, counts per million.



VI. DISCUSSION

Implantation is a complex process that requires the orchestration of several biological functions at cellular and molecular levels. Successful embryo implantation is dependent on the synchronised dialogue between a receptive endometrium and a developing embryo at blastocyst stage (Simón et al., 2000). The causes of implantation failure and pregnancy loss remain unclear in many cases; for this reason, the intrauterine environment is emerging as an attractive target for investigating mechanisms by which changes in the mother can impact the development of the offspring. Technical advances in NGS have allowed for the identification of microorganisms in the uterus, challenging the traditional view of human foetal development within a sterile environment (Tissier, 1900). Furthermore, emerging evidence indicates that the uterine microbiota may have important implications for reproductive health and disease (Baker et al., 2018; Koedooder, Mackens, et al., 2019). The identification of endometrial dysbiosis as a new cause of infertility opens a new microbiological field in the evaluation of endometrial factor, highlighting the relevance of assessing the uterine microbiota in infertile patients to improve and personalise their clinical care.

In most cases, women with intrauterine bacterial infections are asymptomatic or display mild symptoms such as pelvic pain, dysfunctional uterine bleeding, dyspareunia, and leukorrhea (Greenwood & Moran, 1981). Moreover, conventional infertility investigations such as ultrasound cannot detect intrauterine infections (Cicinelli et al., 2015). Thus, CE is often overlooked and not diagnosed in clinical practice (Kitaya & Yasuo, 2013). In addition, currently there are no accepted diagnostic standards, and their diagnosis seems to depend on the method used. The limitations of CE diagnosis using classic techniques and their misleading results were evident in the first study presented here, in which only 13 of 65 (20%) samples/patients analysed presented concordant results using all three diagnostic methods **(Figure 19 and Table 4)**.

The single use of classic diagnostic techniques (histology, hysteroscopy, or microbial culture) showed poor diagnostic accuracies (46.15%, 58.46%, and 66.15%, respectively) **(Table 5)**. Moreover, when the overall results were analysed in detail, histology and hysteroscopy yielded contradictory results in 58.46% of cases **(Table 4)**. In all the inconsistent cases, histology showed negative results while hysteroscopy showed positive results for the same patient, confirming that, compared to other methods, histopathological evaluation usually underdiagnoses CE, while hysteroscopy overdiagnoses the condition **(Table 4)**.

Histology and hysteroscopy are highly subjective, unspecific, and rely on the individual observations of the pathologist or endoscopic surgeon. Also, they cannot discriminate the causal agent, which in turn leads to broad-range therapy and likely results in recurrent CE. The main limitations of histology result from: dependence on the piece of endometrial sample analysed, variability of staining, observer experience, phase of the menstrual cycle in which the sample was collected, and unknown clinical relevance of a small amount of plasma cells (below the positive threshold) in the endometrium (Kitaya et al., 2018). Hysteroscopic diagnosis is based on the presence of hyperaemia, oedema, and/or micropolyps (Cicinelli et al., 2005), all of which are endometrial symptoms that could be due to other pathophysiological conditions or a non-infectious inflammation of the uterine cavity. This was observed in case 55 that was negative for all the methods used, except for hysteroscopy, in which the only sign of CE was the presence of micropolyps **(Figure 24B)**. In contrast, microbial culture allows to identify the microorganisms present in the endometrial tissue. But some important limitations of the microbial culture are the contamination with skin or environmental bacteria (i.e., *S. epidermidis*) and the inability to grow and isolate non-culturable bacteria. Some CE-causing bacteria such as *Ureaplasma* spp., *M. hominis*, and *G. vaginalis* are not culturable in standard laboratory conditions, which can lead to false-negative results.

Thus, a reliable diagnostic method based on molecular detection of DNA from pathogens present in the endometrium is needed to improve clinical management of infertile patients with CE. This study demonstrated the usefulness of a simple RT-PCR test for the CE diagnosis using a comprehensive panel of primers to detect the most common microorganisms involved. This panel was selected to cover > 80% of clinical cases, since the bacteria primarily responsible for the disease are streptococci, *E. faecalis*, *E. coli*, and staphylococci, accounting for 38.5%, 19.3%, 16.3%, and 5.9% of cases, respectively (Cicinelli et al., 2009). *Streptococcus* spp. were the most abundant bacteria detected (47%), followed by *Enterococcus* spp. (15%), *E. coli* (12%), *K. pneumoniae* (5%), *Staphylococcus* spp. (3%), and *M. hominis* (2%) **(Figure 23)**; these findings were consistent with previously reported microbial culture data (Cicinelli et al., 2009). Interestingly, *G. vaginalis*, a reproductive tract pathogen usually neglected in CE diagnosis, was detected in 7% of samples analysed using the molecular method. In contrast, *C. trachomatis* and *N. gonorrhoeae* were undetectable in all tested samples, which was expected since the patients recruited were not at risk of experiencing this type of infection. This is in agreement with other works describing a limited role for *C. trachomatis* and STIs pathogens in the origin of CE (Stern et al., 1996).

The results showed that RT-PCR is a robust and specific technique with an accuracy of 77%, a sensitivity of 75%, and a specificity of 100% compared to samples with concordant results for the three classical methods (histology + hysteroscopy + microbial culture) **(Table 5)**. Overall, these results indicated that molecular microbiology provides similar results to using the three classic methods together, overcoming the bias of using any of the classic methods alone. Other advantages of the molecular method are: (i) RT-PCR is highly sensitive and can identify and quantify small amounts of bacterial DNA, which may be useful to estimate the severity of the disease; (ii) it quantifies bacterial DNA instead of live bacteria, so it is able to detect CE pathogens in frozen or fixed samples, facilitating sample collection, shipment, and

storage (Zariffard et al., 2002); (iii) RT-PCR is a rapid assay that has comparable results to bacterial culture with turnaround times of hours rather than days (Cunningham et al., 2013), and (iv) RT-PCR may be useful for the detection of intrauterine germs when histology is negative, as well as for the decision of a target therapy when histology and/or hysteroscopy are positive.

On the other hand, several limitations must be addressed to improve the validity of this molecular test. The main limitation of molecular microbiology is: (i) the relatively low negative predictive value, estimated at 25%, compared to concordant histology + hysteroscopy + microbiology. This could be biased by the vast amount of positive CE cases determined by hysteroscopy, as the negative predictive value of the molecular method compared to histology or microbial culture alone was 59% and 63%, respectively. Also, (ii) molecular method does not guarantee that DNA comes from viable bacteria, and (iii) the minimum amount of bacterial DNA that causes the disease should be determined, as the presence of such DNA in some women could be innocuous depending on the host response to those pathogens. In fact, the copy numbers for the interrogated gene could be variable between different bacterial genera/species and could cause a slight deviation on the number of bacteria estimate (Kembel et al., 2012). Lastly, (iv) the topological localisation of bacterial growth should be considered; since bacteria such as *G. vaginalis* forms biofilms at the apical surface of the epithelial layer (Swidsinski et al., 2013), and therefore their detection may depend on the area of tissue analysed.

Taking all these features into account and given that the results of the RT-PCR were also confirmed by the microbiome assessed by NGS **(Figure 38; Table 6)**, 16S rRNA sequencing is proposed to address technical and clinical improvements on the molecular detection of endometrial bacterial communities. The analysis of the endometrial microbiota using 16S rRNA sequencing enables distinguishing between positive and negative species in a given sample, or even describing the microbial

signatures associated with a particular disease state. Using this approach, EF and EB samples from infertile patients undergoing ART were simultaneously subjected to microbiome analysis with the purpose of evaluating its impact on assisted reproduction by comparing the bacterial composition and the reproductive outcomes of those patients after the pET.

The results of this study showed a close relationship between the EF and EB microbiota, although some differences were found between both sample types. Specifically, 12 of the 15 most abundant taxa detected in the EF samples overlapped with the taxa identified in the EB samples, but three taxonomies were unique for EF microbiota (*Streptomyces*, *Clostridium* and *Chryseobacterium*) and seven taxa were detected only in EB microbiota (*Cupriavidus*, *Escherichia*, *Klebsiella*, *Bacillus*, *Finegoldia*, *Micrococcus,* and *Tepidimonas*) **(Figure 28)**. A possible explanation for the difference in bacterial taxa between samples is that bacteria present on the surface of the luminal epithelium may be somewhat different than those residing deeper close to the glandular epithelium and stroma. However, these differences may also be due to the sample processing and DNA extraction protocols, as they minimally differ for both types of samples.

The occurrence bacterial networks also revealed differences between the sample types **(Figure 29)**. The EB microbiota networks were more dispersed than the EF ones, suggesting that the microbiota found in the EF could be more stable (Naqvi et al., 2010). Previously, only one study compared the endometrial microbiota of EF and EB samples, suggesting also that the microbiota composition in EF does not fully reflect that in EB, and that sampling from both samples allows a more comprehensive view of microbial colonisation (Liu et al., 2018). Therefore, to fully understand the biological and clinical relevance of microbiota in the endometrium, both EF and EB samples should be carefully studied at the functional level.

While the bacterial composition of the vaginal microbiota is well established and is generally related to obstetric outcomes (Fettweis et al., 2019; Koedooder, Singer, et al., 2019), the uterine microbiota is now the focus of more extensive studies and debates. The few studies reported to date on the effect of the endometrial microbiota on reproductive outcomes have not reached consensus on the profile of bacterial pathogens and the mechanisms by which they could interfere with embryo implantation. Larger and well-designed studies are needed to better understand their potential role in reproductive competence (Benner et al., 2018).

In this study, the relationship between the endometrial microbiota composition and the reproductive success was investigated in a cohort of 342 infertile patients, ethnically diverse from three different continents. The results of this study showed that an unfavourable microbiota in endometrium at the time of conception associates with compromised implantation and pregnancy progress. The co-occurrence bacterial association plots built separately for each ART outcome showed that the live birth networks were denser and had a higher node degree distribution, in contrast to the networks of failed outcomes **(Figure 30-31)**. Moreover, the associations revealed that *Lactobacillus* was generally negatively correlated to pathogenic bacteria and positively correlated to commensal bacteria, which may be important for the stability in the ecosystem. *Lactobacillus* depletion and the presence of specific pathogenic bacteria such as *Atopobium*, *Bifidobacterium*, *Chryseobacterium*, *Gardnerella*, *Streptococcus,* and *Klebsiella* in EF microbiota, and *Bifidobacterium*, *Gardnerella*, *Klebsiella*, *Enterococcus,* and *Neisseria* in EB microbiota were associated with unsuccessful reproductive outcome **(Figure 33-35)**.

A. vaginae and *G. vaginalis* are major BV-associated bacteria; they stimulate an innate immune response from vaginal epithelial cells and contribute to the pathogenesis of BV (Libby et al., 2008). However, the deleterious impact of these pathogens is not restricted to the vagina, as a previous study showed that women

with BV present significant increased risk of developing endometrial polymicrobial biofilms with *G. vaginalis* and other bacteria (Swidsinski et al., 2013). *S. agalactiae*, *K. pneumoniae*, *Enterococcus*, *N. gonorrhoeae*, and in some studies also *G. vaginalis*, are regarded as the major pathogens of CE (Cicinelli et al., 2008; Kitaya et al., 2018). This condition impairs reproductive outcomes either in natural conceptions or after ART, further contributing to obstetric and neonatal complications (Kitaya et al., 2016). Moreover, bacteria like *S. agalactiae* are well-known as leading causes of neonatal infections by vertical transmission from colonised mothers (Patras & Nizet, 2018). Finally, it has been hypothesised that in the absence of *Lactobacillus*, *Bifidobacterium* might be able to guarantee a healthy vaginal balance by the production of lactic acid (Freitas & Hill, 2017), which would be consistent with the results published by Kyono and collaborators (Kyono et al., 2019). However, these results show a negative association of *Bifidobacterium* with live birth, in agreement with other reports demonstrating *Bifidobacterium* spp. as pathogens in various infectious conditions (Bhaskar et al., 2017; Chen et al., 2019; Pathak et al., 2014). Hence, the role of members of this genus should be further investigated.

This study is the first international and multi-center work that prospectively analyses endometrial microbiota in two types of samples taken simultaneously from the same patient and assesses the association of uterine microbial environment with pregnancy outcomes in infertile patients undergoing IVF. The data presented are robust because both types of samples were sequenced from the same bacterial 16S rRNA hypervariable regions and analysed using the same bioinformatics pipeline. Nevertheless, the high sensitivity of this technology might detect DNA contamination, which can confound the interpretation of microbiome data (Eisenhofer et al. 2019), specifically in low-biomass sites such as uterus. Therefore, we have ensured that contaminating reads, but not sample-related reads, are removed from downstream analyses. For that purpose, samples were first classified as detectable or non-detectable, excluding from the analysis those that clustered with

the blanks and had a roughly equivalent amplicon concentration **(Figure 27)**. Another strength of this work is that endometrial receptivity was analysed, and pET was performed to synchronise endometrial receptivity with embryo development, avoiding displacement of the implantation window (Díaz-Gimeno et al., 2011; Ruiz-Alonso et al., 2013; Simón et al., 2020). Additionally, all samples were collected in an HRT cycle, preventing the potential bias introduced by different hormonal status. There may be a concern that hormonal therapy may have influenced the endometrial microbiome, but the hormonal regimen and sampling day were consistent between the sample collection analysis and the embryo transfer cycles.

In addition to studying the microbial profiles associated with reproductive results, the functional interactions between the community of microorganisms and its host are also crucial to understand the effect of endometrial microbiota on infertility. The potential regulatory mechanisms of each microbial species on implantation are still not define, and currently we can only hypothesise that the presence of bacterial pathogens may trigger an inflammatory response in the endometrium that affects embryo implantation, as inflammatory mediators are tightly regulated during the adhesion of the blastocyst to the endometrial epithelium. To analyse the mechanism by which pathogenic bacteria can affect embryo implantation, an *in vitro* model of bacterial colonisation in endometrial epithelial cells was performed. This assay analysed the ability of a *Lactobacillus* probiotic strain to act against pathogen colonisation and modify the inflammation-related molecules.

Among all probiotic strains screened in a preliminary test, *L. rhamnosus* BPL005 was selected to counteract the colonisation of pathogenic or dysbiotic bacteria previously associated with NLD microbiota (*A. vaginae*, *G. vaginalis*, *P. acnes*, and *S. agalactiae*) based on its ability to sharply reduce pH **(Table 9)**. The beneficial role of lactobacilli on acidification has been reported, related with healthy Nugent scores and a balanced microbiome (Ravel et al., 2011). This strong acidification potential is

primarily associated with lactic acid production, which has positive effects including the control of pathogen populations, immune modulation, and colonisation (Borges et al., 2014). In this study, strain BPL005 also produced high levels of lactic acid (data not shown). This increase would explain its capacity to reduce pH quickly. Using this endometrial colonisation model, the capacity of strain BPL005 to lower pH was confirmed, reaching pH levels below 5.0 when BPL005 was co-cultured with pathogens except for *S. agalactiae*, in which no differences in pH were observed after co-culture with the probiotic strain, as this species is also a lactic acid producer **(Table 10)**.

To investigate whether these changes in the pH values result in differences in pathogen colonisation, the influence of *L. rhamnosus* BPL005 on pathogen colonisation was analysed by RT-PCR. Results showed an effect of L*. rhamnosus* BPL005 against *P. acnes* and *S. agalactiae* levels and lower but non-significant levels of *A. vaginae* and *G. vaginalis* **(Figure 37)**. As assays have been conducted with individual pathogenic strains, the potential interactions among them, or how the *L. rhamnosus* BPL005 strain could interact in a dysbiotic pathogenic niche, including both bacteria and yeast inhabitants, cannot be extrapolated with these results. In any case, the exposition of pathogens to *L. rhamnosus* BPL005 seems to block their colonisation in hEECs, conferring a potentially positive effect on biofilm inhibition.

Lactobacilli also use other pathogen inhibition pathways such as hydrogen peroxide and bacteriocins, but in this study it was not analysed whether these molecules exerted an effect against the pathogens tested. Although hydrogen peroxide has traditionally been considered a molecule responsible at least in part for the action against pathogens, its *in vivo* activity is questioned (Tachedjian et al., 2018). Regarding the potential effect of bacteriocins, the production of this type of substance has been observed in strains of the species *L. rhamnosus* with activity against vaginal pathogens (Ruíz et al., 2012); therefore, although not evaluated in this

work, its action would not be neglected. The effect of the probiotic strain on pathogens maybe also partly attributable to antimicrobial and anti-adhesive compounds production, as already observed for other *Lactobacillus* strains (Bertuccini et al., 2017; Coudeyras et al., 2008; Petrova et al., 2016).

On the other hand, the presence of *Lactobacillus* may also have an effect on inflammatory markers such as cytokines and chemokines that influence embryo implantation. Specifically, *Lactobacillus* can decrease the levels of pro-inflammatory molecules like IL-6, IL-8, MIP-3α, RANTES, and TNFα, while increasing the secretion of the anti-inflammatory cytokine IL-1RA (Hearps et al., 2017; Tachedjian et al., 2017). In this *in vitro* model, the colonisation of hEECs with different dysbiotic/pathogenic bacteria modulated the secretion of different cytokines and chemokines compared to colonisation with BPL005, showing that an NLD microbiota might trigger an inflammatory response in the endometrium characterised by increased proinflammatory cytokines IL-6, IL-8, and MCP-1 (**Figure 36**). Also, for some of these molecules (IL-6, IL-8, MCP-1, IL-1RA, and IL-1β), the addition of BPL005 to hEECs colonised with pathogens significantly restored equivalent levels to the *Lactobacillus*-only colonisation. This suggests a potential role of BPL005 probiotic to decrease the levels of pro-inflammatory cytokines produced by an NLD microbiota and maintain the inflammatory homeostasis of endometrial epithelial cells by increasing the levels of the anti-inflammatory molecule IL-1RA.

The changes detected are clinically relevant because alterations in the cytokine and/or chemokine levels have been related to infertility. IL-6 is one of the essential cytokines in embryo implantation, and women with unexplained infertility and/or endometriosis present increased levels of secreted IL-6 in the endometrium (Tseng et al., 1996). Also, elevated IL-8 and MCP-1 are found in peritoneal fluid of patients with endometriosis compared to controls, and their levels correlate with the severity of disease (Arici et al., 1996; Zeyneloglu et al., 1998). IL-8 could promote proliferation

and adhesion of endometrial cells outside the uterus, a hallmark of this inflammatory disease (Arici, 2002). Interestingly, elevated levels of pro-inflammatory cytokines have been described in gynaecological diseases of infectious origin, as is the case of high MCP-1 in abdominal-pelvic adhesions (Zeyneloglu et al., 1998), or increased IL-1β, IL-6, and TNFα in menstruation from infertile patients with CE (Tortorella et al., 2014). These findings support the implication of bacterial pathogens causing CE in the growth of these pro-inflammatory molecules in the endometrium. Overall, these results show the potential of *L. rhamnosus* BPL005 strain as a probiotic in gynaecological health and help us to molecularly characterise the effect of endometrial infections with pathogens/dysbiotic of the reproductive tract.

Finally, to directly determine what taxonomically and functionally constitutes an altered uterine microbiome and define its fluctuations over time, a couple of clinical cases are reported about the microbiota of infertile patients with repeated reproductive failures.

The first case report described the 18-month follow-up of a clinical case involving an ectopic pregnancy and two early clinical miscarriages, in whom the endometrial microbiota was evaluated in a total of six EF samples **(Figure 38)**. All samples analysed at different time points showed a persistent infection with *Gardnerella* and other bacterial taxa such as *Atopobium* and *Bifidobacterium* **(Figure 39)**. *G. vaginalis* is a sexually-acquired pathogen that produces infectious conditions such as BV, endometritis, and/or PID through the establishment of a polymicrobial biofilms along the FGT (Swidsinski et al., 2013). Biofilms are formed by a dense and tight network of bacterial cells enclosed within a fibrillar exopolysaccharide matrix, which confers a strong adherence to the host tissues and impedes access by antibiotics (Swidsinski et al., 2008). *G. vaginalis* biofilms can be present for prolonged periods of time without causing symptoms (Swidsinski et al., 2008), which could explain the recurrent nature of this condition and the subsequent treatment failures observed in this case.

Numerous studies report the inconsistent efficacy of routine therapies. Metronidazole is the first-line drug to treat this microbial condition because it is also effective against other anaerobes. However, treatment efficacy is limited to the short term, and symptoms usually return in 20% of patients with BV within one month (Koumans et al., 2002), and 58% within one year (Bradshaw, Morton, et al., 2006). Antimicrobial resistance could be due to the fact that strains present a variable susceptibility for metronidazole, and some even show intrinsic resistance (De Backer et al., 2006). Indeed, strains of *G. vaginalis* and *A. vaginae* resistant to metronidazole are detected in 100% and 75% of women with recurrent BV after antibiotic therapy respectively (Bradshaw, Tabrizi, et al., 2006).

Advances in NGS have enabled differentiation of four ecotypes of *G. vaginalis* according to sequence variations (Ahmed et al., 2012). After analysing clade-specific genetic markers by WMS in this patient **(Figure 41-42)**, the concomitant presence of multiple clades was detected, which has been identified as a risk factor in women with genital infections such as BV (Balashov et al., 2014). Specifically, a greater abundance of clade 4, followed by clade 3 and clade 1, was detected in the sample collected seven days before an embryo transfer that ended up in a six-week clinical miscarriage **(Figure 42)**. Interestingly, clades 3 and 4 have been reported to confer intrinsic resistance to metronidazole (Schuyler et al., 2016). The most abundant in this sample was a unique operon of clade 4 associated with allantoin utilisation as a nitrogen source under adverse conditions **(Figure 42)**. This unique scavenging/foraging ability is absent in other *Gardnerella* subtypes and may represent an advantage for its survival (Ahmed et al., 2012).

Potential alternative therapies to metronidazole include other compounds (amoxicillin/clavulanic acid or clindamycin), routes of administration, and antibiotic regimens (dose and duration) (Sfakianoudis et al., 2018). Nevertheless, antibiotic resistance is a growing problem, and there is a risk of disturbing normal bacterial flora

with cumulative usage of antibiotics. To avoid over-intervention with antibiotics, further studies are needed to properly diagnose a "true dysbiosis" of the endometrium that results in implantation failure. In addition, several studies also assessed the clinical and microbiological efficacy of probiotics to treat and/or prevent the recurrence of reproductive tract infections, denoting the value of including probiotics for the management of these diseases (MacPhee et al., 2010). Despite the need for additional clinical studies, probiotics were used here as an adjunct to antimicrobial treatment **(Figure 38)**, resulting in a gradual increase in the percentage of *Lactobacillus* in the three EF samples analysed after its administration **(Figure 39)**. However, the main problem of this clinical case was the resistance of *G. vaginalis* and other pathogens to the antibiotic and probiotic treatments administered to the patient. Therefore, this case shows the importance of identifying the genetic differences responsible for the production of particular pathogenicity traits to inform appropriate measures towards preventing adverse reproductive outcomes. A diagnostic tool that evaluates the presence of *G. vaginalis* and distinguishes its clades may be useful to treat infertile patients and identify those cases that may be at a higher risk for recurrent disease.

By contrast, the second case report described the endometrial microbiome in a patient who had an eighth-week clinical miscarriage with euploid embryos and, for the first time, during a pregnancy at four weeks of gestation in the same woman **(Figure 44)**. The microbial profile found on the endometrial sample before the clinical miscarriage had higher bacterial diversity and lower *Lactobacillus* abundance than that of healthy pregnancy **(Figure 45-46)**. Functional metagenomics revealed different *Lactobacillus* species between the two samples analysed, detecting *L. crispatus* in the endometrium before clinical miscarriage and *L. iners* during early pregnancy **(Figure 46B)**.

Lactobacillus spp. can have a protective role in genital infections; however, their level of protection varies by species or strain (Kroon et al., 2018), possibly due to a different capacity to produce antimicrobial factors (Tachedjian et al., 2018; Witkin et al., 2013). *L. crispatus* and *L. iners* are common inhabitants of the healthy reproductive tract. These two species are closely related and are thought to perform similar ecologic functions. Nevertheless, differences in their genomes can explain their specificity for a given niche. *L. crispatus* has the largest genome among those species studied, with unique DNA polymerase, bacteriocin, and toxin-antitoxin genes that encode mobile genetic elements, especially transposases (France et al., 2016; Petrova et al., 2015). This finding is consistent with the large number of functions related to mobile elements observed in the sample collected before clinical miscarriage **(Figure 48)**.

On the other hand, although the reproductive tract of healthy women can be colonised by *L. iners* (McMillan et al., 2013), this species has been often identified in transitional communities between BV and a normal microbiota (Gajer et al., 2012; Petrova et al., 2017). In this case, transition to a *L. iners*-dominated microbiota after a period of instability (clinical miscarriage, followed by dilation and curettage and antibiotic treatment) was observed during early pregnancy when the embryo was already implanted. The genome of *L. iners* contains an iron-sulfur cluster that limits iron availability. This system may be used as a defense mechanism by providing a competitive advantage against other bacterial pathogens, or it may play a role in providing nutrients and surviving in adverse conditions such as menstruation (Macklaim et al., 2011). Correspondingly, during menstruation, the abundance of *L. iners* in the vaginal community increases while the number of *L. crispatus* decreases (Gajer et al., 2012; Santiago et al., 2012). The potential of *L. iners* to sequester iron could confer this microorganism with an advantage in respect to other bacteria to colonise the uterine cavity after dilation and curettage, where the environmental conditions are characterised by the presence of blood, similar to menstruation.

Further studies are needed to determine the precise role of these interesting species in endometrial health and disease and whether these strains can serve as biomarkers of reproductive success or failure.

Finally, sequences associated with acetyltransferase GNAT domains were searched, which is implicated in bacterial antibiotic resistance, chromatin remodelling, and anabolic and catabolic functions. In the endometrial microbiome before the clinical miscarriage, 44 GNAT sequences were identified; but in the microbiome of the early pregnancy, these sequences were not found. Prior characterisation of the genomes of several *L. iners* strains revealed that they lack several proteins related to the GNAT family and various transcriptional regulators (Mendes-Soares et al., 2014), which is in agreement with our findings. Three putative ligands have been found in the ChEMBL database related to the GNAT domain: Luspatercept, Ecallantide, and Rilonacept, which correspond to inhibitors of activin receptor type-2B, plasma kallikrein, and IL-1β, respectively. Ecallantide (Kalbitor) and Rilonacept (Arcalyst) are U.S. Food and Drug Administration-approved drugs with important effects on human health. Specifically, Rilonacept blocks interleukin-1 signalling by trapping IL-1β before it can reach its membrane receptors and thereby preventing signal transduction leading to inflammation.

In summary, in addition to routine diagnostics, the use of molecular methods may help diagnose endometrial dysbiosis or infectious conditions as CE. Although the true core uterine microbiota has not yet been defined, the impact of the uterine microbiome in health and disease, and particularly its involvement in human reproduction, is becoming increasingly better characterised. Emerging data correlates the presence of certain bacterial taxa with worse fertility and pregnancy outcomes. Thus, the incorporation of the microbiological diagnosis in infertile patients is proposed as an option to improve the chances of a positive reproductive result.

The main strength of this thesis is the study of the impact of the endometrial microbiome on reproductive outcomes from different perspectives. Importantly, the prospective study presented here is the largest published to date, and it includes ethnic diversity and different IVF protocols, which had not been considered in studying the endometrial microbiome associated with pregnancy outcomes. Another important contribution of this investigation was to analyse *in vitro* the potential mechanism of bacterial colonisation in primary endometrial epithelial cells. This assay showed the ability of *Lactobacillus* to act against pathogen colonisation and modify inflammation-related molecules.

To complete the study with an *in vivo* approach, the endometrial community was characterised taxonomically and functionally in two clinical cases with the use of WMS and bioinformatics tools that provide resolution at the species level. Most of the high-throughput studies that characterise the endometrial microbiota have identified bacterial taxa to the genus, family, or order level but have not been able to distinguish between bacterial species. As has been shown in the two clinical cases presented, the genetic and functional differences between the strains could explain the lack of efficacy of the antibiotic treatments, the increased risk of recurrent infections, as well as the repeated reproductive failures. For this reason, analysing microbiota at the species-level resolution may be necessary for identifying the true pathogenic bacteria of the endometrium and avoiding over-intervention of patients. Moreover, in the second case report, the chromosomal status of the transferred embryo, assessed before embryo transfer and confirmed in the products of conception after clinical miscarriage, ruled out embryo aneuploidy as a possible cause of miscarriage and highlighted the relevance of the physiological endometrial microbiome in embryo implantation and pregnancy.

Finally, taking EF samples in a minimally invasive way and without affecting pregnancy rates (van de Gaast 2003) is another strength of this study, since it

facilitates the assessment of the endometrial microbiota in the same cycle of the pET and improves the clinical management of ART patients. The clinical cases presented here, in which the EF samples were taken in the same cycle of the pET (clinical case 1) or even in an early pregnancy in which the embryo had already implanted (clinical case 2), support the results presented throughout this thesis.

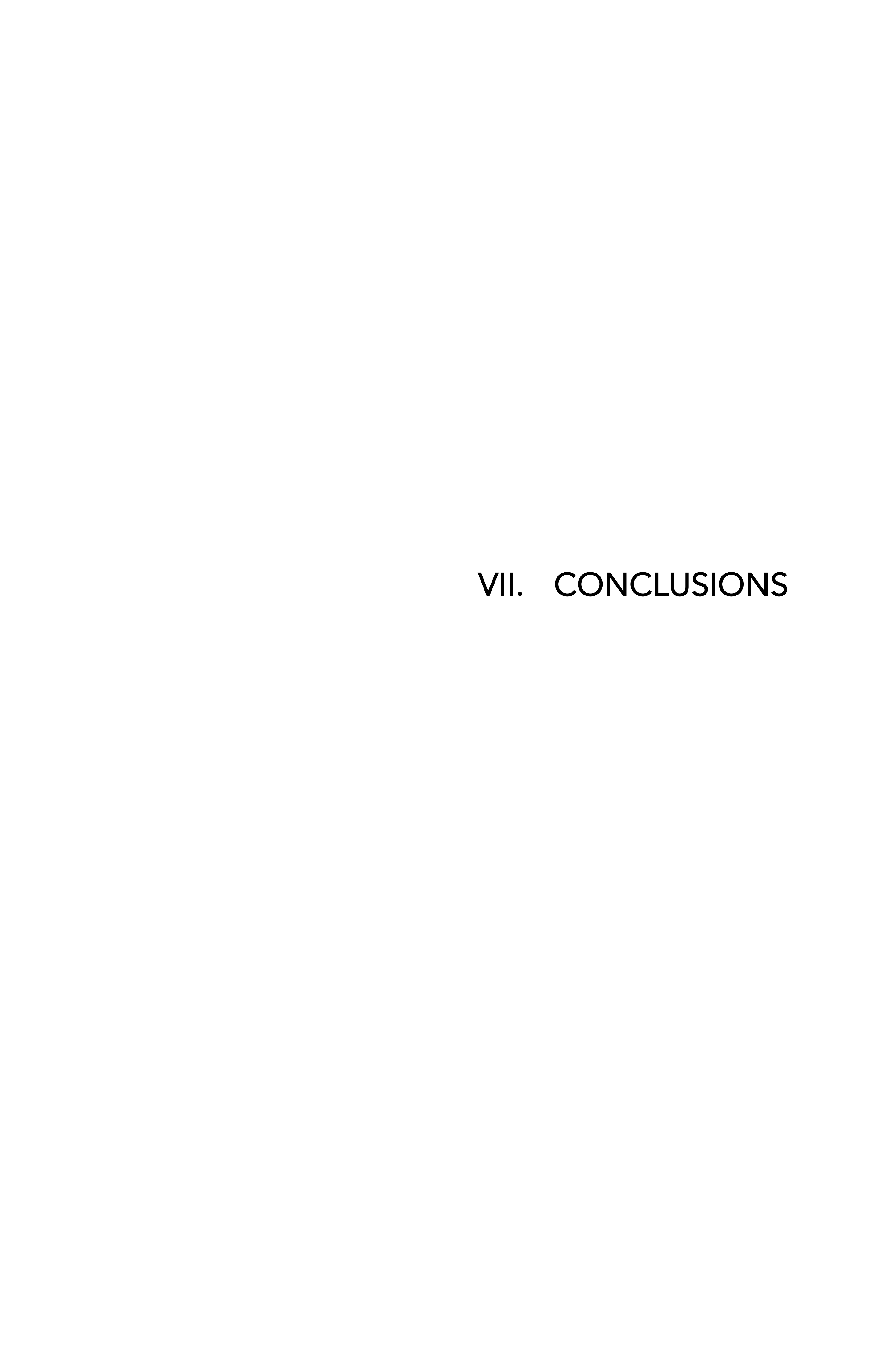
Nonetheless, some limitations must be acknowledged. The HMP revealed that samples collected from the vagina contain a large amount of human DNA (approximately 96%) (Consortium, 2012a). Considering that the endometrial microbiota is a low-biomass ecosystem, and its bacterial load is estimated to be between 100 and 10,000 times lower than the vaginal microbiota (Chen et al., 2017; Mitchell et al., 2015), the percentage of reads corresponding to bacteria found in WMS was not unexpected. Nevertheless, this scarcity did not prevent an in-depth analysis of bacterial DNA **(Table 11 and 14)**.

Differences were also observed between the microbial profiles obtained by 16S rRNA sequencing and WMS. A possible explanation for such differences could be the potential noise introduced in the sample by the DNA extraction kit (kitome). DNA from bacterial genera (such as *Methylobacterium*, *Stenotrophomonas*, *Janthinobacterium*) could be contained in laboratory reagents, affecting microbiota analysis in low-biomass samples at the taxonomic level (Salter et al., 2014). Moreover, the differences in experimental protocols, in gene amplification (16S rRNA sequencing only amplify one gene while WMS detects all genes expressed by the community), and in the bioinformatics pipelines applied to each sequencing method could also explain this divergence.

On the other hand, all the EF samples evaluated in this investigation were collected with a transcervical catheter. There may be a concern that transcervical collection of samples may have influenced the endometrial microbial results;

however, there are no alternative means to obtain endometrial samples from ART patients. Moreover, it is now accepted that the FGT presents a continuum microbiota (Chen et al., 2017) so even if endometrial samples would carry over some contamination from the cervix, the resulting microbial profiles will be consistent with the microbial environment of the uterine cavity. Finally, as for the timing of sample collection, the microbial analysis was performed in cycles before embryo transfer, but there are possibilities that the microbiomes change over time; therefore, this is also a factor to consider in future work.

Overall, this work may have implications for understanding the causes of implantation failure and clinical miscarriage. Likewise, these results facilitate the development of diagnostic tools that could be the basis for personalised therapeutic procedures that attempt to restore the physiological endometrial microbiota. Future research in this field will shed further translational mechanistic understanding onto the interplay of the uterine microbiota with women's health and reproduction.



VII. CONCLUSIONS

- Molecular microbiology allows the reliable characterisation of specific pathogens in clinical and subclinical chronic endometritis. Compared to hysteroscopy, histology, or microbial culture, RT-PCR effectively detects chronic endometritis-causing pathogens with an accuracy of 77%, a sensitivity of 75%, and a specificity of 100%.

- Microbiota composition in endometrial fluid shows a close relationship with that of the endometrial biopsy.

- *Lactobacillus* together with other commensal bacteria are consistently enriched in endometrial samples from patients with a live birth.

- The presence of pathogenic bacteria in the endometrium – *Atopobium*, *Bifidobacterium*, *Chryseobacterium*, *Gardnerella*, *Haemophilus*, *Klebsiella*, *Neisseria*, *Staphylococcus* and *Streptococcus* – is associated with negative reproductive outcomes and should be considered as emerging cause of implantation failure and pregnancy loss.

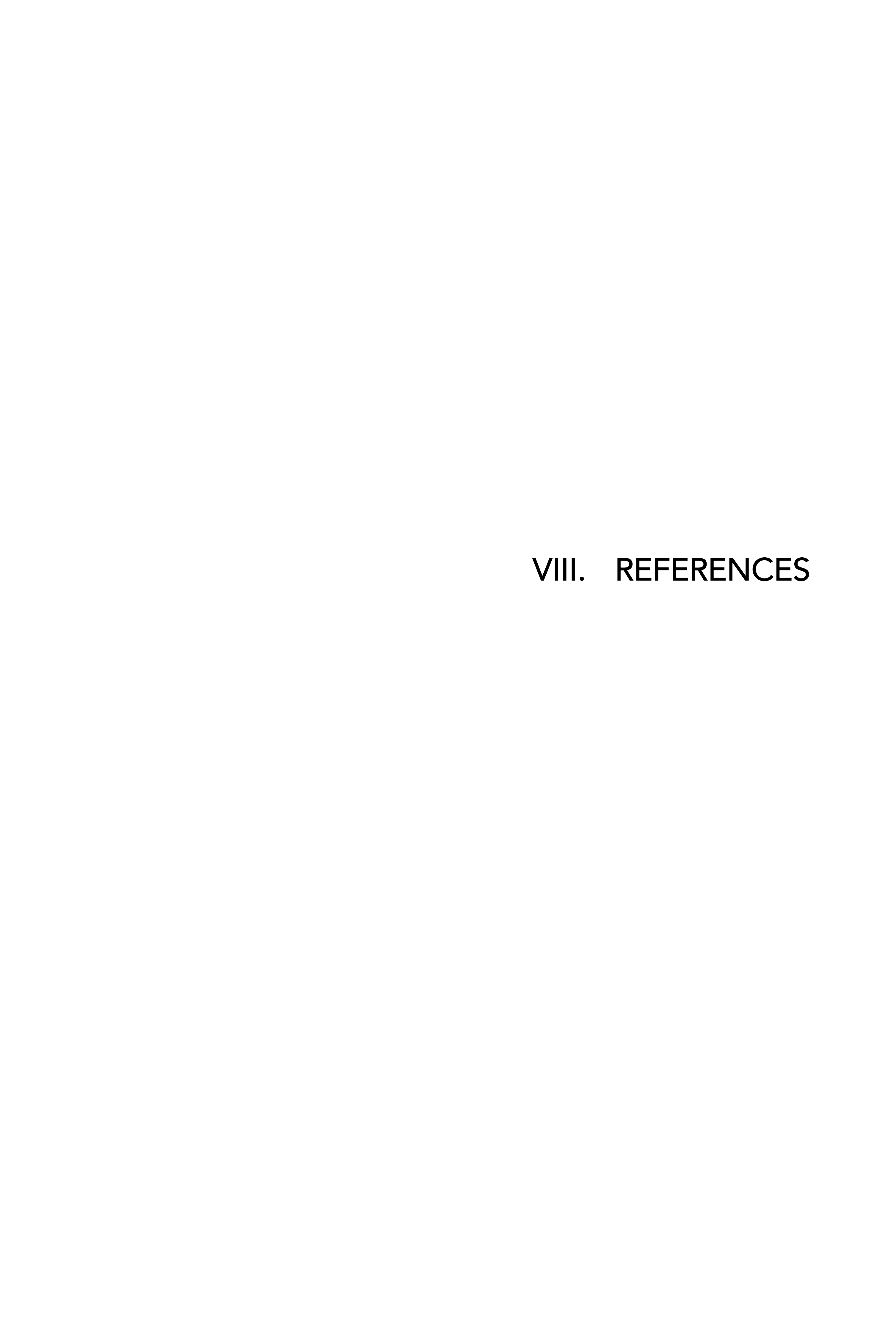
- *L. rhamnosus* BPL005 (CECT 8800) may have a protective role on endometrial infections with *A. vaginae*, *G. vaginalis*, *P. acnes*, and *S. agalactiae*. The co-cultivation of BPL005 with tested pathogens in an *in vitro* model of endometrial bacterial colonisation produces a reduction in the pH levels, the colonisation of pathogens, and modulation of the secretion of different cytokines and chemokines (decreased IL-6, IL-8, MCP-1, and increased IL-1RA and IL-1β).

- Functional microbiome study in an infertile patient with repeated reproductive failure shows a persistent colonisation with *G. vaginalis* during the 18 months of follow-up, expressing virulence factors and antimicrobial resistance genes.

- The endometrial microbiota in an early successful pregnancy and before clinical miscarriage with euploid embryos in the same patient reveals lower

community richness and diversity and higher *Lactobacillus* abundance during the pregnancy. Functional metagenomics shows distinct *Lactobacillus* species and functional profiles, in which basal metabolism and translation regulation associated with *L. iners* are the main functions in successful pregnancy.

- The implications of these results may contribute to medical awareness of the potential impact of microbial pathogens in the management of infertility. Likewise, it leads us to propose new diagnostic tools and personalised therapeutic procedures through molecular microbiological evaluation.



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