Female upper reproductive tract harbors endogenous microbial profiles

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Abstract

The vaginal milieu is known to have an active microbiome (>90% of *Lactobacillus*), but the microbial composition of the upper reproductive tract is not well established, especially in the Fallopian tubes. The first studies on the Fallopian tubes from women diagnosed with a benign disease or for prophylaxis suggest that this site supports an endogenous microbiome. However, today we lack the knowledge of the microbial composition in Fallopian tubes in the non-diseased conditions (as collecting samples from these sites may hamper the tissue and future fertility). Our study includes 24 fertile women with benign uterine pathology submitted to abdominal hysterectomy or tubal ligation at Hospital Universitario Virgen de Arrixaca Murcia, which endometrial and Fallopian tube samples were collected between January and July 2019. After DNA extraction, "Ion 16S Metagenomics Kit" (Ion S5™ System) was used to exploit the V5 to V9 regions of the 16S rRNA gene. Primary data analysis was performed with Torrent Suite™ Software v5.12.1 and advanced analysis using Ion Reporter™ software v5.18.0.2. In our study, distinct microbial community profiles in the Fallopian tubes confirm that this genital tract site harbors an endogenous microbiome and in big part is shared with the endometrial microbial profile (69% of the detected taxa). Nevertheless, 17 bacterial taxa were exclusively detected in the Fallopian tubes that included *Enhydrobacter, Granulicatella, Haemophilus, Rhizobium, Alistipes y Paracoccus,* among others, while 10 were found only in the endometrium, including *Klebsiella, Olsenella, Oscillibacter and Veillonella* (FDR <0.05). Regarding the endometrium samples, our study shows that method collection has an influence in results, where there is a *Lactobacillus-dominance* in fertile women with samples obtained transcervically while

44 Acinetobacter, Arthrobacter, Coprococcus, Methylobacterium, Prevotella, Roseburia, Staphylococcus,
45 Streptococcus were less abundant in patients which samples are collected by methods with lower
46 vaginal and cervical contamination. Although upper reproductive tract is a low microbial biomass site,
47 our results suggest that this upper reproductive site supports an endogenous microbiome that could be
48 characteristic of each individual.

49 **1 Introduction**

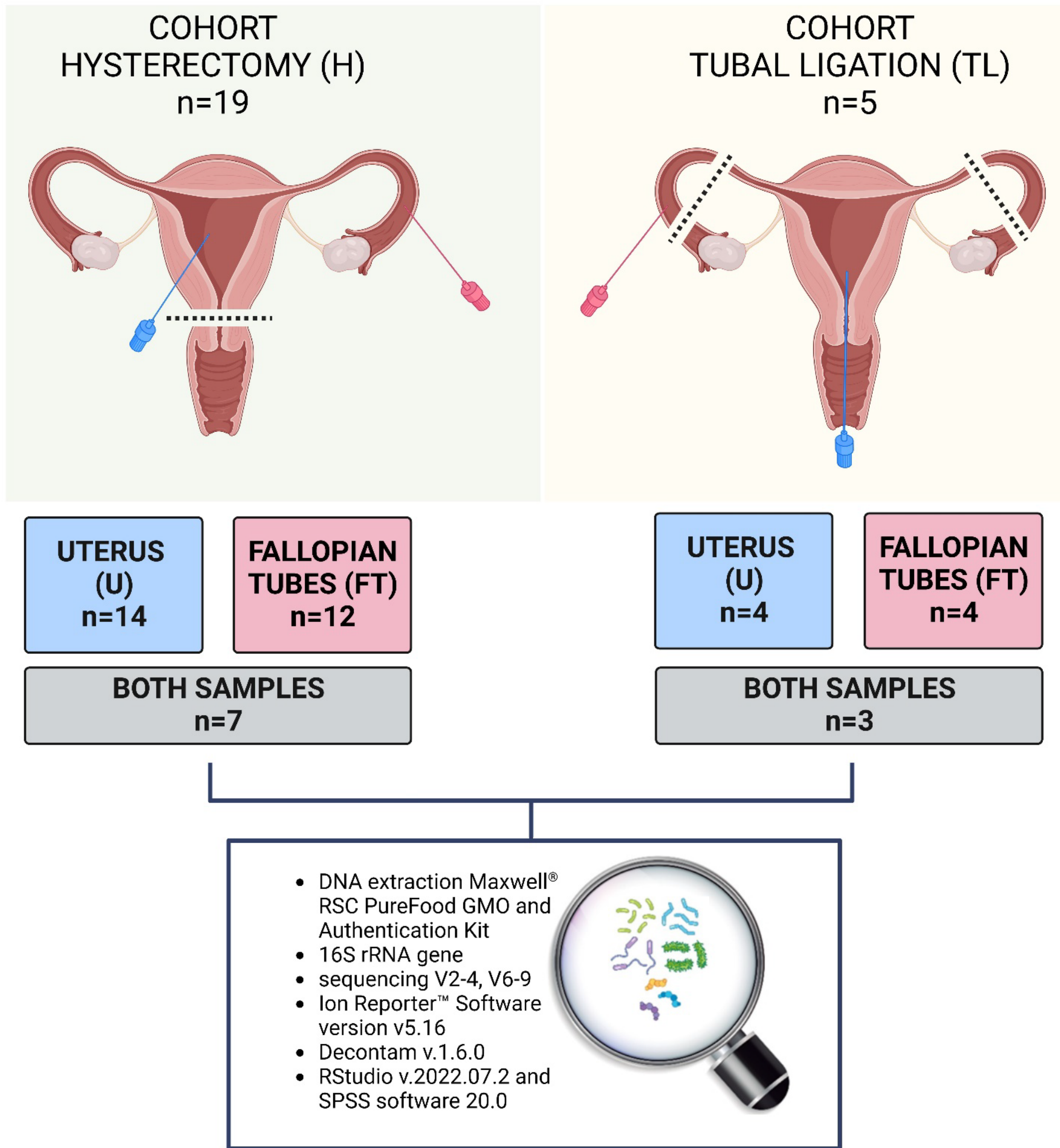
50 The more knowledge regarding the human microbiota (community of microbes/microorganisms)
51 is acquired, the clearer it becomes that it is ubiquitous and has a significant effect on human physiology
52 and pathophysiology (1–3). In the female reproductive tract, growing body of evidence is associating
53 the microbial composition with reproductive functions in health and disease (4–7).

54 While most of the microbiota studies corroborate the important role of the female lower
55 reproductive tract (vagina and cervix) microbes in the defense against pathogens, the upper
56 reproductive tract (endometrium, Fallopian tubes, ovaries) was traditionally considered a sterile cavity,
57 where the cervix acted as a barrier against the passage of bacteria (8). With the revolution of
58 microbiome (genomes of microbes) studies on human upper reproductive tract, mainly on
59 endometrium, it is clear now that it possesses its own microbial communities (7,9,10). Recent studies
60 are in agreement demonstrating that endometrium harbors a higher bacterial diversity and richness
61 when compared to the lower reproductive tract, mainly composed of bacteria of the phyla *Firmicutes*,
62 Bacteroidetes and Proteobacteria, where the dominance of *Lactobacillus* has been associated with a
63 higher probability of live births and *Gardnerella* or *Streptococcus* with early pregnancy loss or
64 implantation failure in IVF treatments (1,11). Nevertheless, there is a lack of consensus between the
65 studies due to differences in study design and lack of proper negative and positive controls (9,12), and
66 therefore the 'core' endometrial microbiome has not been identified and confirmed yet.

67 Fallopian tubes are less studied regarding the microbial composition due to the difficulty in
68 obtaining samples (sampling challenges future fertility). The characterization of its endogenous
69 microbiome has aroused special attention since it is known that this microenvironment provides a stable
70 temperature, optimal pH and dynamic fluid secretions to support oocyte fertilisation and the first
71 embryo developmental steps (13–15). The few studies analysing samples from women with a benign
72 disease or for prophylaxis indicate that Fallopian tubes present an endogenous microbiome, where the
73 taxa *Firmicutes* (especially *Staphylococcus sp*., *Enterococcus sp*., and *Lactobacillus sp*.),
74 *Pseudomonads (Pseudomonas sp. and Burkholderia sp*.) and *Propionibacterium sp.* and *Prevotella*
75 *sp.*are predominant (15–18). However, it is still debatable whether Fallopian tubes harbor an
76 endogenous microbiome and to what extent it impacts the oocyte fertilisation and the first steps of
77 embryo development.

78 It is reasonable to hypothesize that the microbiome of the Fallopian tubes could be similar to the
79 uterus since there is a smooth communication between these anatomical regions as the intramural
80 portion of the uterine tube does not allow a real physical separation between the two sites (19–21).
81 Therefore, comparative studies of uterine and Fallopian tubes samples collected simultaneously from
82 the same donor are warranted to evaluate if the organs that constitute the female upper reproductive
83 tract hold specific endogenous microbial profiles in disease free conditions. We set out to study the
84 endometrial and Fallopian tubes' 16S rRNA gene V2-4, V6-9 regions in fertile women in order to
85 identify the female upper reproductive tract microbiome in disease-free sties.

86 **2 Materials and methods**



87

88 Figure 1. Study design. In total 24 women participated in the study and 34 samples from the upper
89 reproductive tract were retrieved. In the hysterectomy cohort seven women provided both endometrial
90 and Fallopian tube samples, and in the tubal ligation cohort three women provided both samples. The
91 rest of the participants provided only one of the samples due to the tissue damage in laparoscopic
92 procedure, non-sterile condition or blood contamination.

93 ## 2.1 Study population

94 In this prospective study, the sampling was carried out at the Service of Obstetrics and
95 Gynaecology of the University Clinical Hospital Virgen de la Arrixaca in Murcia, Spain. Patients who

96 underwent a planned abdominal hysterectomy together with bilateral salpingectomy or tubal ligation,
97 from January 2016 until June 2018, were invited to participate in the study. Inclusion criteria were the
98 following: women with no hormonal treatment during the three months before surgery, normal
99 menstrual cycles, and absence of fertility problems, endometriosis or any other adnexal pathology
100 detected by transvaginal ultrasound analysis and confirmed after the histological study. Nineteen
101 participants underwent a total laparoscopic hysterectomy with bilateral salpingo-oophorectomy to
102 remove the uterus, cervix, ovaries, and Fallopian tubes due existence of uterine fibroids and consequent
103 abnormal bleeding (see Figure 1 for the study design). While five participants underwent tubal ligation
104 to remove just the Fallopian tubes for permanent contraception, or sterilization purposes, through
105 laparoscopy techniques. This study was approved by the Ethics Research Committee (CEIC) of
106 Clinical University Hospital "Virgen de la Arrixaca" (HCUVA), Murcia, Spain (Approval No. EST:
107 04/16) and all the participants provided their written informed consent. Patient data and samples
108 included in this study were registered, stored, and processed by the Biobanco en Red de la Región de
109 Murcia, BIOBANC-MUR, registered on the Registro Nacional de Biobancos – ISCIII, with registration
110 number B.0000859, following standard operating procedures with appropriate approval of the Ethical
111 and Scientific Committees.

112 **2.2 Collection of Fallopian tubes (FT) and endometrial samples (E)**

113 The collection procedure of the upper reproductive tract samples (FT and E) differed according
114 to the type of surgery indicated for each patient (laparoscopic hysterectomy with bilateral salpingo-
115 oophorectomy or laparoscopic tubal ligation). In both types of the surgery, the patients were placed in
116 dorsal lithotomy position and if the patient did not void immediately prior to the procedure, the bladder
117 was drained with a urinary catheter.

118 The collection method for the FT samples was the same for both types of surgery. Upon
119 laparoscopic intervention, FTs were removed, transferred to ice-cold Petri dishes, and dissected. Once
120 dissected, FTs were clamped in both extremities to avoid sample waste. Next, with an ascendant
121 manual mechanical pressure between the extremities, the FT content that accumulated at the upper
122 portion of the ampulla was aspirated through the sterile Mucat device (CDD Laboratorie, France). This
123 class I medical device, complying with Directive 93/42/EEC, indicated for direct exocervical or
124 endocervical aspiration and Hühner test, was adapted to be easily introduced into the tubes. Once
125 introduced, aspiration of content was performed with the integrated plunger, which slides up and down
126 when pushed by a flexible acetal resin shaft, without a syringe. The content was immediately aliquoted
127 in 1,5ml Eppendorf Safe-Lock® Tubes, frozen in liquid nitrogen until further analysis.

128 The collection method of the endometrial samples differed depending on the surgery. During the
129 hysterectomy, all the upper reproductive tract was removed, and it was possible to access directly into
130 the uterus with sterile Mucat device (CDD Laboratorie, France) avoiding the uterine fibroids tissue
131 (clearly identified with eye) and possible contamination of vaginal/cervix microbiota when sampling
132 endometrial tissue. On the other hand, in patients undergoing only Tubal ligation (uterus was not
133 removed), a speculum was inserted and gently spread apart their vagina so that the cervix could be
134 viewed. The cervix was cleaned with saline solution and then the sterile Mucat device (CDD
135 Laboratorie, France) was inserted into the cervix to reach the interior of the uterus. The aspiration of
136 the uterus content was performed with the integrated plunger as previously described (22). The content
137 was aliquoted in 1,5ml Eppendorf Safe-Lock® Tubes, and frozen in liquid nitrogen until further
138 analysis.

139 **2.3 DNA extraction, amplification, library preparation and sequencing**

140 DNA extraction from the stored samples was performed with Maxwell® RSC PureFood GMO
141 and Authentication Kit and Maxwell® RSC Equipment (Promega, USA). NanoDrop
142 spectrophotometer was used to determine the DNA yield (A260) and purity (A260/A280 ratio)
143 (Supplementary Table 1).

144 The bacterial identification was performed by Genomics Platform of the Instituto Murciano de
145 Investigación Biosanitaria Virgen de la Arrixaca (IMIB-Arrixaca). The multiplex PCR using Ion
146 Torrent 16S Metagenomics kit (Thermo Fisher Scientific Inc. USA) was used to amplify the 16S rRNA
147 gene, using two sets of primers, which targeted the regions V2, V4, and V8, and V3, V6-7, and V9,
148 correspondingly (Supplementary Table 2). Amplification was performed in a SimpliAmp thermal
149 cycler (Applied Biosystems, USA) by running the following program: denaturation at 95°C for 10 min,
150 followed by a cyclic 3-step stage consisting of 25 cycles of denaturation at 95°C for 30 s, annealing at
151 58°C for 30 s, and extension at 72°C for 20s; at the end of this stage, the program concluded with an
152 additional extension period at 72°C for 7 min and the reaction was stopped by cooling at 4°C. The
153 resulting amplicons were tested by electrophoresis using 2% agarose gels in tris-acetate-EDTA (TAE)
154 buffer, purified with AMPure® XP Beads (Beckman Coulter Inc., USA), and quantified using
155 QubitTM dsDNA HS Assay Kit in a Qubit 3 fluorometer (Invitrogen, Thermo Fisher). Afterwards, the
156 Ion Plus Fragment Library Kit (Thermo Fisher Scientific Inc. USA) was used to generate a library from
157 each sample. Each library was indexed by ligating Ion Xpress ™ Barcode Adapters ((Thermo Fisher
158 Scientific Inc. USA to the amplicons. Libraries were purified with AMPure® XP Beads and quantified
159 using the Ion Universal Library Quantitation Kit (Thermo Fisher Scientific Inc. USA) in a QuantStudio
160 5 Real-Time PCR Instrument (Applied Biosystems, USA).

161 Next, the libraries were pooled and clonally amplified onto Ion Sphere Particles (ISPs) by
162 emulsion PCR in an Ion OneTouch™ 2 System (Thermo Fisher Scientific Inc. USA) according to the
163 manufacturer´s instructions. Sequencing of the amplicon libraries was carried on an Ion 530™ Kit
164 (Thermo Fisher Scientific Inc. USA) on an Ion S5™ System (Thermo Fisher Scientific Inc. USA).

165 2.4 Data processing

166 After sequencing, the individual sequence reads were filtered by the Torrent Suite ™ Software
167 v5.12.1 to remove the low quality and polyclonal sequences. The quality filtered data were analyzed
168 using Ion Reporter™ Software version v5.16. Clustering into operational taxonomic units (OTUs) and
169 taxonomic assignment were performed based on the Basic Local Alignment Search Tool (BLAST)
170 using two reference libraries, MicroSEQ® 16S Reference Library v2013.1 and the Greengenes v13.5
171 database (Life Technologies Corporation, USA). For an OTU to be accepted as valid, at least ten reads
172 with an alignment coverage ≥ 90% between the hit and query were required. Identifications were
173 accepted at the genus and species level with sequence identity > 97% and > 99%, respectively.

174 Since the characterization of the low microbial biomass like is upper reproductive tract requires
175 meticulous contamination control, *in-silico* decontamination approach using Decontam v.1.6.0(23,24)
176 was applied to discern between the true bacterial sequences and potential contaminants. To use this
177 method, first a table of the relative abundances of OTUs (columns) in each sample (rows) was created
178 from the raw data. Next, we included into the model DNA concentration of each sample (from
179 Supplementary Table 1). The Decontam score threshold was set to 0.1 as a default setting to define
180 contaminating phylotypes (23). The relative abundance of the considered contaminant phylotypes were
181 set to zero as described previously (24). Furthermore, for diversity and abundance analyses we
182 additionally filtered out those taxa that were detected in less than 30% of the remaining samples, as
183 previously described (25).

184 ## 2.5 Statistical analyses

185 Statistical analyses were performed using the R statistical software v.4.2.1 under RStudio
186 v.2022.07.2 and SPSS software 20.0 (SPSS, USA). Microbiome data were aggregated to genus level
187 for diversity and abundance comparisons. All relative abundances are expressed as median and first
188 and third quartiles (q1, q3). Normal distribution of the variables was tested by using the Shapiro-Wilk
189 test. Relative abundances of identified genera did not meet normality and were analyzed using the
190 nonparametric Mann-Whitney *U* test. Differences were considered statistically significant between
191 groups when $p < 0.05$. Benjamini-Hochberg method (false discovery rate [FDR]) was used to obtain
192 adjusted p-values in multiple comparisons. Alpha-diversity indexes (Shannon diversity index and
193 OTUs number [i.e., richness]) were calculated using the diversity function of the *vegan* R package,
194 both in FT and endometrial samples. Differences among the groups of samples' diversity indexes were
195 tested using Mann-Whitney *U* test. Further, alpha-diversity was compared between women with both
196 types of samples using a Wilcoxon signed-rank test for paired data. Bray-Curtis dissimilarity was
197 calculated using *vegdist* R function and Permutational Analysis of Variance (PERMANOVA) was
198 performed to analyze beta-diversity using *adonis* R function.

199 # 3 Results

200 ## 3.1 Samples

201 In total thirty-four samples were collected from 24 recruited patients. The patient characteristics
202 are presented in Table 1 and Supplementary Table 3. As indicated in Figure 1, from the laparoscopic
203 tubal ligation group, it was possible to collect four FT samples and transcervically four endometrial
204 samples. From the hysterectomy group all the upper reproductive tract was extracted, and 12 FT
205 samples and 14 endometrial samples were collected from the uterus, avoiding the uterine fibroids
206 tissue. It was not always possible to collect both types of samples from each patient because some
207 anatomical pieces after being removed by laparoscopic techniques were damaged and due to the
208 impossibility of collecting some samples with the required sterile conditions and without blood
209 contamination. However, both samples were collected in seven out of 19 patients of hysterectomy
210 cohort and in three of the five patients of tubal ligation cohort (Figure 1).

211

212 **Table 1**. Demographic characteristics of study population and collected samples from two group of
213 patients: patients who underwent a total laparoscopic hysterectomy with bilateral salpingo-
214 oophorectomy and patients submitted to a laparoscopic tubal ligation.

215

Study Population Groups	Hysterectomy n=19	Tubal ligation n=5
Age (years)	45 ± 3	37 ± 4
BMI	28,5 ± 4,7	28,3 ± 4
Parity	1,8 ± 0,9	2,2 ± 0,5
Fallopian tube samples	12	4
Endometrial samples	14	4
Both tissue samples	7	3

216

217 ## 3.2 Data processing

218 In total, 245 and 252 genera were identified in the endometrial and FT samples, respectively. The
219 average number of reads per sample in the fallopian tubes was 25241,44±10845,46 (mean±SD). The
220 average number of reads per sample in the endometrium was 30845±18702,56 (mean±SD). Applying
221 the decontamination method using Decontam, two genera, *Aerococcus* in FT and *Acidovorax* in the
222 endometrial samples were identified as contaminant phylotypes. Further, to ensure the identification
223 of the "core" microbiome of both sites an additional filtering step was applied by eliminating those
224 bacterial taxa present in less than 30% of the participants as previously described (25). Finally, a total
225 of 70 bacterial genera were identified in the endometrial samples, and 77 bacteria in the FT samples
226 (Supplementary Table4).

227

228 **3.3 Microbial profiles of FT samples**

229 The microbial composition in FT samples varied between samples, while the sampling method
230 (hysterectomy or tubal ligation) did not seem to influence the microbiome (Figure 2). The most
231 abundant taxa among all samples were *Lactobacillus* (relative abundance =14.3 [3.48;24.4]),
232 *Prevotella* (9.29 [0.31;12.7]), *Acinetobacter* (relative abundance =3.20 [1.36;11.7]),
233 *Propionibacterium* (relative abundance =3.09 [2.45;5.86]) and *Faecalibacterium* (relative abundance
234 =3.09 [0.68;4.97]) (Supplementary Table4).

235

Fallopian tubes samples

100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%

1 2 3 5 9 10 11 12 13 15 16 17 20 21 23 24

Hysterectomy
Tubal ligation

Lactobacillus, Massilia, Prevotella, Pseudomonas, Acinetobacter,
Propionibacterium, Streptococcus, Oxalicibacterium, Faecalibacterium, Methylobacterium,
Clostridium, Catenibacterium, Staphylococcus, Corynebacterium, Micrococcus,
Eubacterium2, Dorea, Ruminococcus2, Ruminococcus, Sphingomonas,
Bacteroides, Kocuria, Arthrobacter, Haemophilus, Blautia,
Gemmiger, Eubacterium, Enterococcus, Cloacibacterium, Parabacteroides,
Dolosigranulum, Pelomonas, Collinsella, Microbacterium, Lactococcus,
Granulicatella, Paracoccus, Roseburia, Finegoldia, Gemella,
Bacillus, Enhydrobacter, Brevundimonas, Coprococcus, Bilophila,
Megasphaera, Helicobacter, Neisseria, Ruminiclostridium, Rhizobium,
Ralstonia, Anaerococcus, Campylobacter, Fusobacterium, Dialister,
Burkholderia, Aeromonas, Butyricimonas, Subdoligranulum, Rothia,
Desulfovibrio, Moraxella, Alistipes, Pseudoflavonifractor, Bifidobacterium,
Brachyspira, Lachnoclostridium, Phascolarctobacterium, Sutterella, Shewanella,
Parasutterella, Flavonifractor, Actinomyces, Porphyromonas, Herbaspirillum,
Comamonas, Mitsuokella

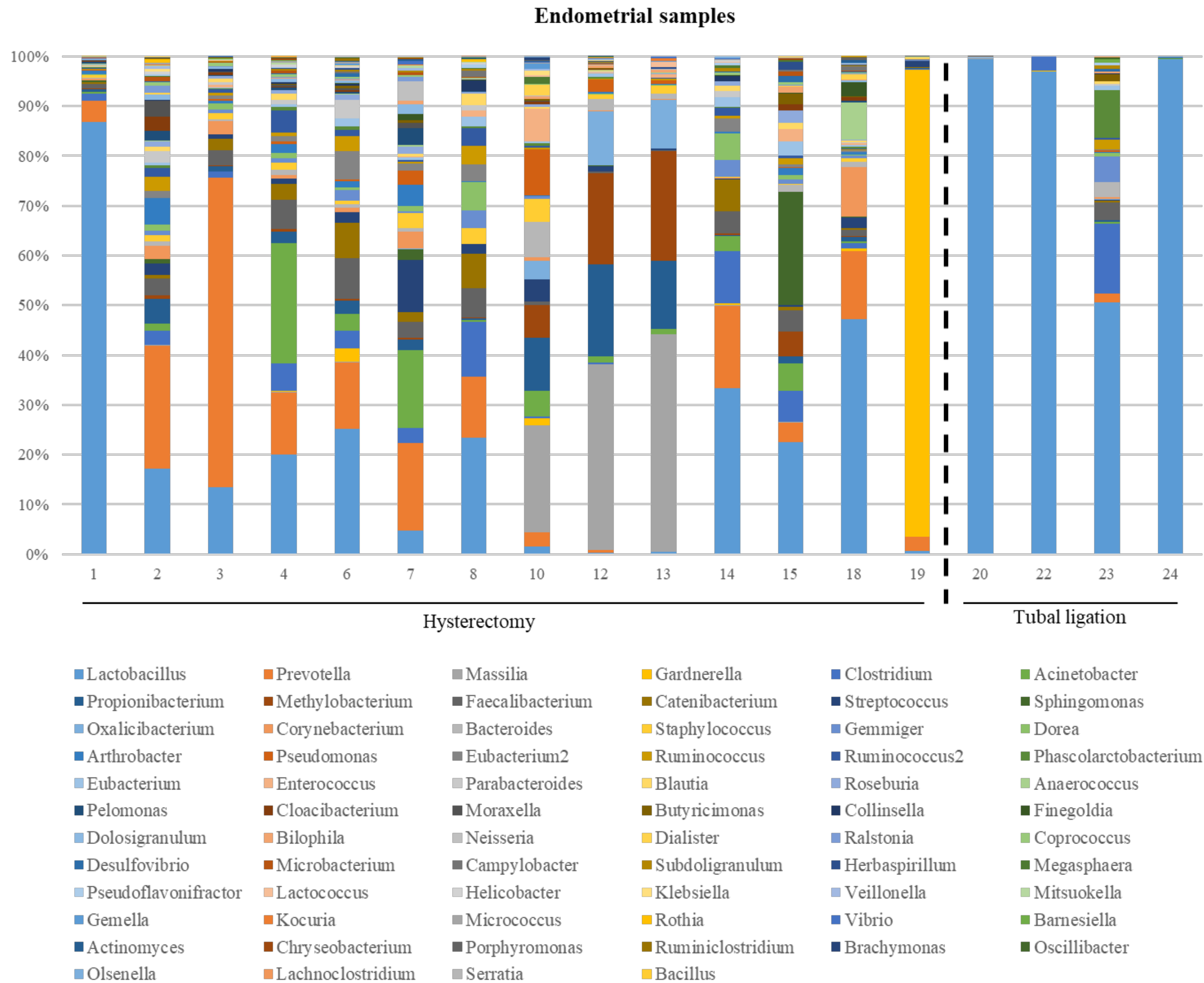
Figure 2. Most abundant bacteria (with relative abundances higher than 1%) detected in FT samples. Total laparoscopic hysterectomy with bilateral salpingo-oophorectomy or laparoscopic tubal ligation methods were applied for sampling.

Since the fertile women undergoing tubal ligation had no pathology associated and women undergoing hysterectomy were diagnosed with benign uterine fibroids, a comparative microbiome analysis was performed to detect potential influence, if any, of the uterine fibroids on microbial microenvironment in the tubes. Neither significant differences in microbial diversity nor differentially abundance analysis was identified between the groups (Supplementary Table 5).

3.4 Microbial profiles of endometrial samples

The microbiome composition in the endometrial samples was heterogeneous between the samples, where *Lactobacillus* showed the highest average abundance (relative abundance=23.0

251 [6.89;49.8]), followed by *Prevotella* (relative abundance=4.13 [0.85;13.7]), *Faecalibacterium* (relative
252 abundance=2.18 [0.24;4.12]), and *Clostridum* (relative abundance=2.08 [0.32;5.06]) were the most
253 abundant microbes in the samples (Figure 3, Supplementary Table4).



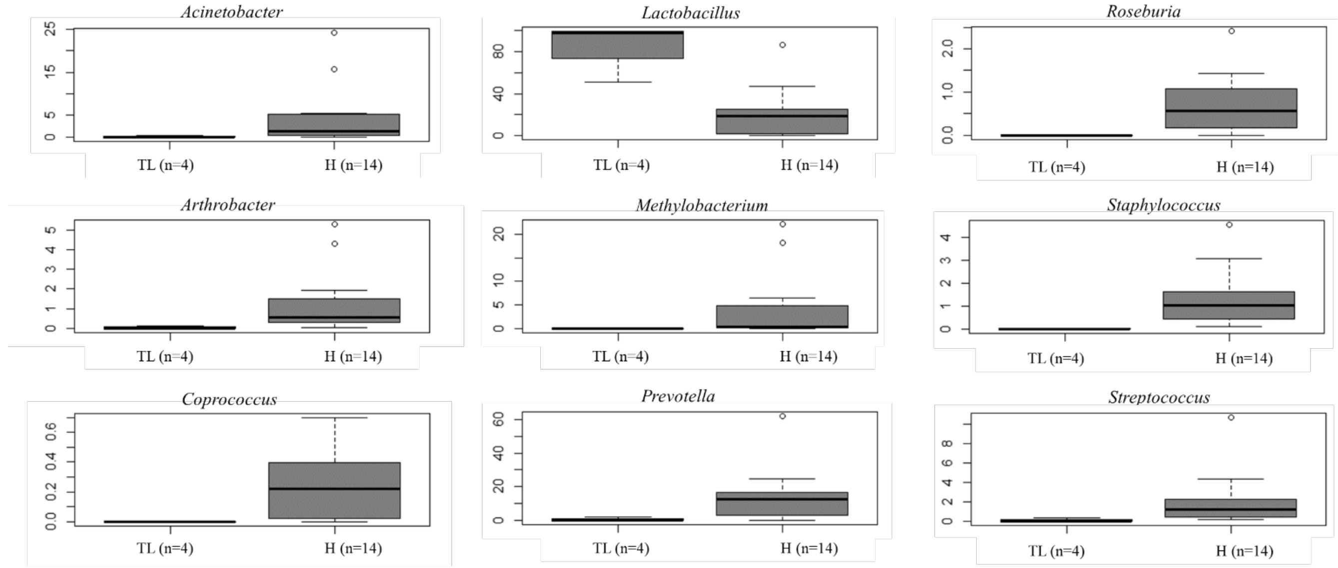
254

255 **Figure 3**. Most abundant bacteria (with relative abundances higher than 1%) detected in the
256 endometrial samples. Total laparoscopic hysterectomy with bilateral salpingo-oophorectomy or
257 laparoscopic tubal ligation methods were applied for sampling.

258

259 Hysterectomy procedure where the sample was obtained directly from the uterus was applied
260 for fertile women presenting benign uterine condition (although during sampling the fibroid area was
261 avoided), while tubal ligation procedure was performed for sterilization purposes (absence of the
262 disease) and endometrial biopsy was obtained transcervically. Therefore, we aimed to compare whether
263 the uterine microenvironment could be influenced by the fibroids and whether the sampling method
264 via cervix (high bacterial contamination risk) could have an impact on the microbial composition in
265 endometrial samples. When comparing the microbiome of the two sampling techniques, 20 bacteria
266 presented significantly different abundance (Supplementary Table 6). When applying the multiple
267 testing correction, nine bacteria remained as marginally different between the groups, where
268 *Lactobacillus* were more abundant while *Acinetobacter, Arthrobacter, Coprococcus,*

269 *Methylobacterium, Prevotella, Roseburia, Staphylococcus, Streptococcus* were less abundant in fertile
270 women with samples obtained transcervically (Figure 4, Supplementary Table 6).



271

272 **Figure 4**. Relative abundance of nine bacteria between samples obtained (H) directly from uterus
273 (fertile women with fibroids) and (TL) transcervically when undergoing tubal ligation (fertile women
274 without the disease). After multiple testing correction adjustment, the difference remained marginal
275 (FDR=0.083).

276

277 **3.5 Microbiome composition between endometrial and FT samples**

278 When comparing microbial composition between the endometrium and FT, the endometrial
279 samples from tubal ligation group were not included due to the significant microbiome differences
280 which refer to the possible vaginal/cervical contamination (high *Lactobacillus* abundance). Thus, 16
281 FT samples and 14 endometrial samples were compared. A big part of the detected taxa was shared
282 between the both sites (60 bacteria), while 17 bacterial genera were found exclusively in FT samples
283 and 10 could be considered as endometrial-specific (Figure 5, Table 2).

284 Of these detected genera (Supplementary Table 7), the relative abundance of 11 bacteria was
285 significantly different when compared uterine and FT samples. Specifically, *Gardnerella* (p=0.002;
286 FDR=0.042), *Klebsiella* (p=0.004; FDR=0.042), *Olsenella* (p=0.004; FDR=0.042), *Oscillibacter*
287 (=0.004; FDR=0.042) and *Veillonella* (p=0.004; FDR=0.042) were more prevalent in endometrium,
288 while *Enhydrobacter* (p=0.001; FDR=0.042), *Granulicatella* (p=0.001; FDR=0.042), *Haemophilus*
289 (p=0.003; FDR=0.042), *Rhizobium* (p=0.003; FDR=0.042), *Alistipes* (p=0.006; FDR=0.048) and
290 *Paracoccus* (p=0.006; FDR=0.048) were more abundant in FT samples.

291

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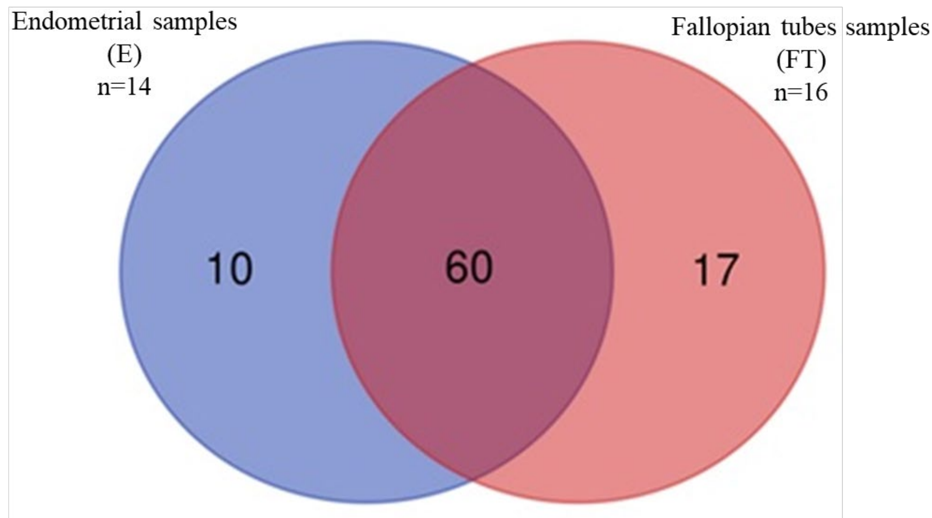


293 **Figure 5.** Venn diagram illustrating the bacteria present in the upper reproductive tract.

294 **Table 2.** Microbial composition of the endometrial and FT samples. The asterisks represent the
295 differentially abundant microbial taxa between uterine and Fallopian tubes samples analyzed by non-
296 paramatric Mann-Whitney *U* test ($p<0.05$). P-values were adjusted using the method of Benjamini and
297 Hochberg to apply the multiple testing correction (False Discovery Rate, FDR).

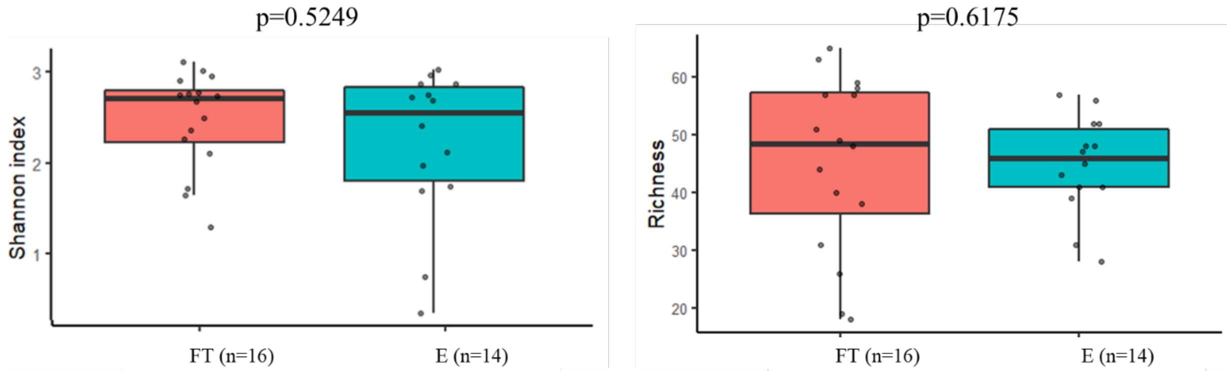
298

Fallopian tubes	Fallopian tubes and Uterus			Uterus
Aeromonas	*Acinetobacter*	*Actinomyces*	*Anaerococcus*	*Barnesiella*
*Alistipes**	*Arthrobacter*	*Bacillus*	*Bacteroides*	*Brachymonas*
Bifidobacterium	*Bilophila*	*Blautia*	*Butyricimonas*	*Chryseobacterium*
Brachyspira	*Campylobacter*	*Catenibacterium*	*Cloacibacterium*	*Gardnerella**
Brevundimonas	*Clostridium*	*Collinsella*	*Coprococcus*	*Klebsiella**
Burkholderia	*Corynebacterium*	*Desulfovibrio*	*Dialister*	*Olsenella**
Comamonas	*Dolosigranulum*	*Dorea*	*Enterococcus*	*Oscillibacter**
*Enhydrobacter**	*Eubacterium*	*Eubacterium2*	*Faecalibacterium*	*Serratia*
Flavonifractor	*Finegoldia*	*Gemella*	*Gemmiger*	*Veillonella**
Fusobacterium	*Helicobacter*	*Herbaspirillum*	*Kocuria*	*Vibrio*
*Granulicatella**	*Lachnoclostridium*	*Lactobacillus*	*Lactococcus*	
*Haemophilus**	*Massilia*	*Megasphaera*	*Methylobacterium*	
*Paracoccus**	*Microbacterium*	*Micrococcus*	*Mitsuokella*	
Parasutterella	*Moraxella*	*Neisseria*	*Oxalicibacterium*	
*Rhizobium**	*Parabacteroides*	*Pelomonas*	*Phascolarctobacterium*	
Shewanella	*Porphyromonas*	*Prevotella*	*Propionibacterium*	
Sutterella	*Pseudoflavonifractor*	*Pseudomonas*	*Ralstonia*	
	Roseburia	*Rothia*	*Ruminiclostridium*	
	Ruminococcus	*Ruminococcus2*	*Sphingomonas*	

Staphylococcus	*Streptococcus*	*Subdoligranulum*

299

300 Concerning microbiome diversity, no significant differences were detected between the
301 endometrial and FT samples in alpha-diversity metrics (i.e., Shannon, OTUs number [richness]),
302 (Figure 6). Beta-diversity represented by PCoA blot based on Bray-Curtis distances did not show any
303 significant dissimilarities between the microbiome composition of both types of samples (Figure 7).



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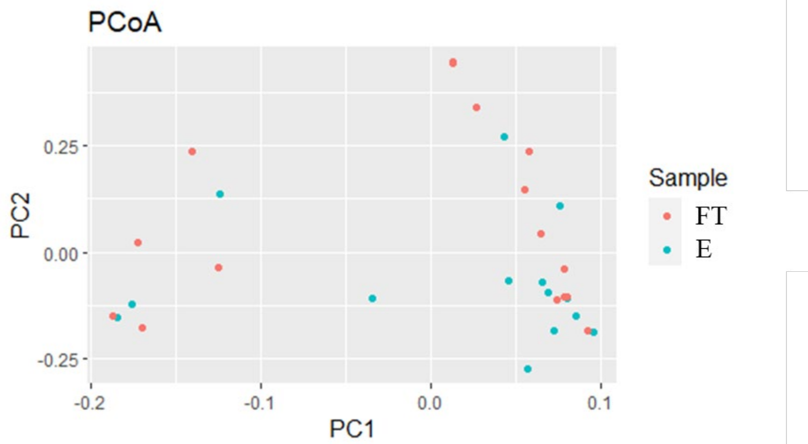
305 **Figure 6.** Alpha-diversity metrics (i.e., Shannon, OTUs number [richness]) of Fallopian tubes (FT)
306 and endometrial samples (E).



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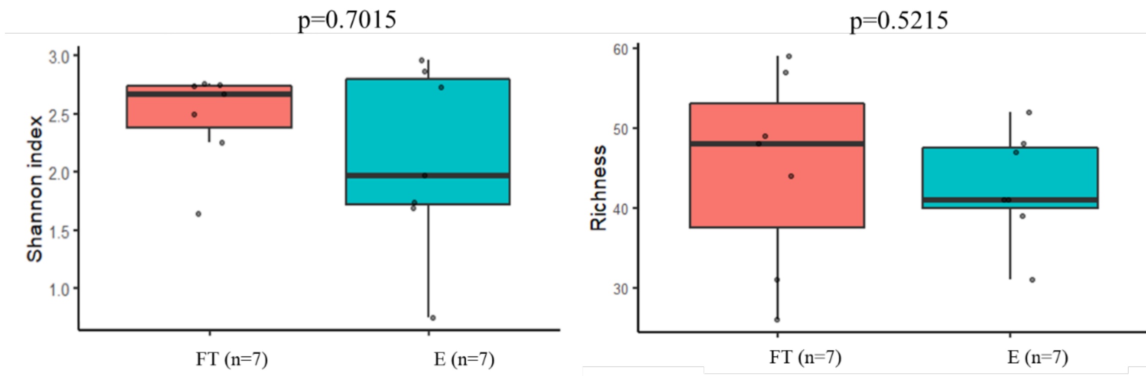
308

309 **Figure 7.** Beta-diversity represented by principal coordinate analysis (PCoA) based on Bray-Curtis
310 distances (PERMANOVA, p=0.389) between endometrial (E) and Fallopian tube (FT) samples.

311 **3.6 Sensitivity analysis with patients with both, endometrial and FT samples**

312 A sensitivity analysis was performed only with patients from hysterectomy (n=7) whose samples
313 were valid for both tissues (endometrium and FT) (Figure 1, Supplementary Table 3), avoiding thus
314 the possible contamination effect from cervical bacteria.

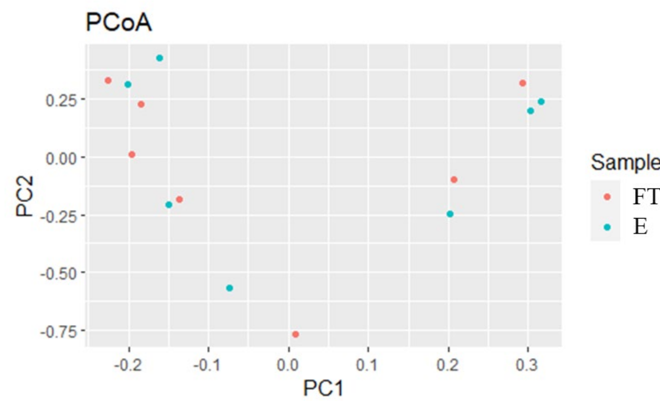
315 First, a microbial diversity comparison between the microbiomes from the endometrial and FT samples
316 were performed. Microbiome alpha- (Figure 8) and beta- (Figure 9) diversities were not significantly
317 different between the two tissue types (p >0,05).

318


319

320 **Figure 8.** Alpha-diversity metrics (i.e., Shannon, OTUs number [richness]) of Fallopian tubes (FT)
321 and endometrial (E) samples when the restricted group of patients with paired samples was selected.

322

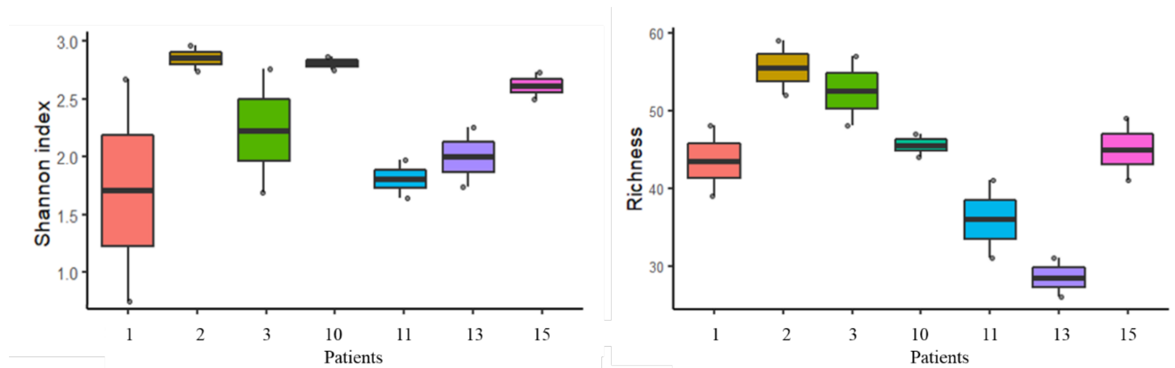


323

324 **Figure 9.** Beta-diversity represented by principal coordinate analysis (PCoA) based on Bray-Curtis
325 distances of patients with paired samples (PERMANOVA, p=0.706).

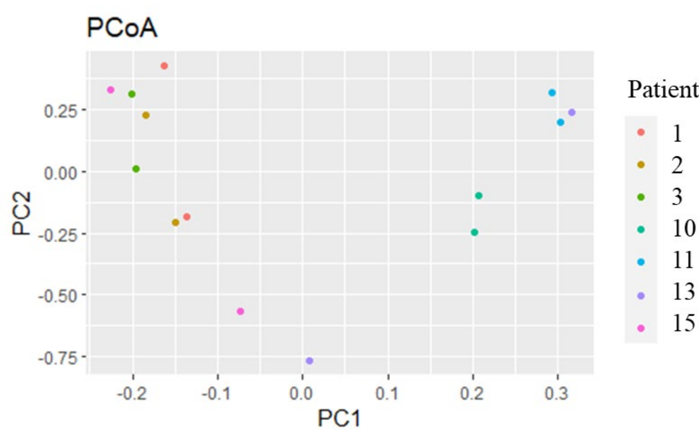
326 Among this more restricted subset of samples, the statistical differences in the relative
327 abundances of the 11 bacteria (*Gardnerella, Klebsiella, Olsenella, Oscillibacter, Veillonella,*
328 *Enhydrobacter, Granulicatella, Haemophilus, Rhizobium, Alistipes and Paracoccus*) found in the
329 previous analysis when compared all endometrial and FT samples were lost after adjusting for the
330 multiple testing correction (False Discovery Rate, FDR) (Supplementary Table 8).

331 As a next step, we performed an additional comparison taking into account each pair of the
332 tissue samples corresponding to their respective patient. Alpha-diversity analysis did not detect any
333 statistically significant differences between paired tissue samples of each patient (Shannon diversity
334 index and OTUs number both with p >0.05) (Figure 10). However, beta-diversity analysis revealed a
335 significant dissimilarity when compared paired samples of the same women (PERMANOVA, p=0.044)
336 (Figure 11). Indicating that the microbiome within an individual even from two different tissue types,
337 from endometrium and FT, is more similar within an individual than between the same tissue type (e.g.
338 endometrium) from different individuals.



339

340 **Figure 10.** Alpha-diversity metrics (i.e., Shannon, OTUs number) of each paired of samples from the
341 same women (n=7), all values p >0.05. Each label indicates a patient (e.g. 1).



342

343 **Figure 11.** Beta-diversity represented by principal coordinate analysis (PCoA) based on Bray-Curtis
344 distances of patients with paired samples (PERMANOVA, p=0.044). Each patient is indicated with
345 one colour and each patients has two dots of the same colour which represents theirs respective
346 endometrial and Fallopian tube samples.

347

348 **4 Discussion**

349 Female upper reproductive tract is the physiological milieu where the first events of oocyte
350 fertilisation, early stages of embryo development and embryo implantation take place. Knowing the
351 detailed microenvironment in the FT and endometrium would provide us the tools to manipulate and
352 improve the *in vitro* conditions used in the assisted reproduction techniques. It is estimated that over
353 20% of couples suffer infertility worldwide and with the socioeconomic situation where the couples
354 delay family planning and have children later in life, the need for infertility treatment is continuously
355 increasing(26).

356 Since there is a growing awareness that the microbes that colonize our body are involved in
357 various pathological processes, microbiome studies of female reproductive tract are a hot topic to
358 understand their role in the first crucial events of embryo development and pregnancy establishment
359 (6). Different bacteria have been identified in the female upper reproductive tract, where bacterial
360 imbalance in the uterine cavity has been associated with implantation failure and decreased success of
361 assisted reproductive techniques, endometriosis, endometritis, polyps and endometrial cancer

362 (10,27,28). On the other hand, very few studies have analysed FT microbiome due to the clear ethical
363 and technical issues (obtaining FT sample compromises the future fertility). Altogether, there is yet no
364 consensus on the core microbial composition of the upper reproductive tract, neither in healthy nor
365 pathological conditions (10,12,29–31) and clearly more research is required.

366 The current study analysed the microbial composition of the upper reproductive tract in women
367 with confirmed fertility by studying FT and endometrial samples from women diagnosed with benign
368 uterine pathology or without the disease. Our study findings highlight the presence of a similar (>80%)
369 endogenous microbial community along both sites of the upper reproductive tract, where
370 *Lactobacillus*, *Prevotella* and *Faecalibacterium* were the most prevalent taxa. Since in humans the
371 intramural portion of the uterine tube does not allow a real physical separation between the fallopian
372 tube and uterine environments, it is reasonable to think that there is smooth communication between
373 these anatomical regions and their microbiome could be the same, which is corroborated by our results.
374 We detected 60 bacteria in common in both tissues, while 17 bacterial genera were FT-specific and 10
375 uniquely present in the endometrium. *Gardnerella*, *Klebsiella*, *Olsenella*, *Oscillibacter* and *Veillonella*
376 were statistically significantly associated with the endometrial samples, and *Enhydrobacter*,
377 *Granulicatella*, *Haemophilus*, *Rhizobium*, *Alistipes* and *Paracoccus* were related to FT samples.
378 Although the presence of these bacteria in the upper reproductive tract has been previously described
379 (8,16,32), the site specificity that is seen in our results was not reported before.

380 When comparing the FT and endometrial samples obtained from the same women, although the
381 sample size was limited, it was clear that the two distinct tissue microbiomes were more similar within
382 a person than the same tissue sample between different individuals. This data support the hypothesis
383 that each person has its own "microbial fingerprints" which microbial residents are tuned into our
384 history and the environment of our body – namely our genetics, diet and developmental history – in
385 such a way that they stick with us and help combat invaders over time (33). So, it is expected that there
386 are more similarities between the microbiome of different body sites from one individual than a specific
387 body sites from different individuals. Similar results were previously described although with a more
388 heterogenous cohort (18). Thus, we still have to establish what is the 'core' microbiome and what may
389 be healthy in one person might differ from that of the other person, adding to the complexity of
390 investigating human microbiome.

391 Our study composed of fertile women with benign uterine condition (fibroids) and women
392 without the disease opting for terminal contraceptive method (tubal ligation). That led to the two
393 different methods for obtaining study material, hysterectomy and tubal ligation. The FT samples were
394 obtained in both cohorts the same way, which allowed us to evaluate the effect of fibroid-related uterine
395 microenvironment (as the endometrial biopsies were fibroid free but their effect on endometrium could
396 be present) on FT microbiome. Our study results did not detect any association between the fibroid-
397 free endometrial microbiome from women with uterine fibroids and the microbiome of FT, indicating
398 that fibroid-related uterine environment does not seem to affect FT microenvironment.

399 In the case of endometrial samples, the sampling method differed drastically: in the
400 hysterectomy, the reproductive organs are removed, and the endometrial samples were obtained
401 directly opening the uterus under sterile conditions, while in the tubal ligation patients the endometrial
402 samples were obtained transcervically and therefore with high bacterial contamination risk from the
403 vagina/cervix. Thus, when analysing the endometrial samples from these two cohorts, we cannot clarify
404 whether the significant differences we observed in the microbial composition are due to the fibroid-
405 associated uterine microenvironment or due to the sampling method. When applying the multiple
406 testing correction, nine bacteria remained as marginally different between the groups, where

407 *Lactobacillus* was more abundant in fertile women with samples obtained transcervically while
408 *Acinetobacter, Arthrobacter, Coprococcus, Methylobacterium, Prevotella, Roseburia,*
409 *Staphylococcus, Streptococcus* were less abundant. The difference of *Lactobacillus* abundance
410 depending on the type of sampling method was previously reported, where a lower dominance is linked
411 to the surgeries with a lower contamination risk from the vagina and cervix like hysterectomy(30),
412 laparoscopy (8) and/or during caesarean section (34) in comparison to transcervical sampling methods
413 (10). In line with previous studies, the uterine samples that were collected through the cervix presented
414 a clear dominance of *Lactobacillus* (abundance of 87%), while the samples obtained at hysterectomy
415 showed higher diversity and lower prevalence of *Lactobacillus* (abundance of 21%). Based on these
416 findings, we believe that in our study the sampling method had stronger effect on the endometrial
417 microbiome than the fibroid-free uterine sample from women with this benign uterine condition.
418 Winters *et al*. reported that the endometria of women with a median age of 45, who underwent
419 hysterectomy for fibroids, were dominated by *Acinetobacter* (abundance of 60%) (30). Other studies
420 have suggested that *Acinetobacter* may be associated with a normal (or at least benign) endometrium
421 while *Methylobacterium* was associated with endometrial cancer (35). In our study, disease-free
422 endometrial samples from women with uterine fibroids showed relative abundance of *Acinetobacter*
423 (0.02 [0.00;0.09]) and *Methylobacterium* (0.00 [0.00;0.01]). These two genera, however, together with
424 *Arthrobacter, Coprococcus, Prevotella, Roseburia, Staphylococcus,* and *Streptococcus* genera
425 differentially present in endometrial samples in our study have been considered as common
426 contaminant genera (9), therefore more research is required to understand what is contamination and
427 what not, the roles of these bacteria in uterine health, and whether the microbial composition is
428 impacted by factors like uterine fibroids and other pathologies.

429 Our study is the first to analyze the endometrial and FT samples together from women with
430 confirmed fertility, nevertheless there are some limitations that should be highlighted. Firstly, the
431 relatively small sample size makes the study results preliminary that should be confirmed in a bigger
432 sample size. Secondly, the endometrial samples were obtained in different cycle phases meaning that
433 we cannot focus on endometrial receptivity phase. Thirdly, although utmost care was taken to obtain
434 fibroid-free tissue when sampling endometrial biopsies, the effect of fibroids on uterine
435 microenvironment cannot be ruled out. Thirdly, the study design lacked negative controls in the
436 sampling process, and therefore stringent decontamination tool and strict data processing methods were
437 applied.

438 In conclusion, our study results corroborate that the female upper reproductive tract harbours an
439 endogenous microbiome, although in a low microbial biomass, and in big part of the microbial profile
440 is shared between the FT and the endometrium, sharing over 80% of the detected taxa. Also, it seems
441 that the women have unique microbial profiles where two distinct tissues (FT and endometrium) share
442 more bacterial similarities than the same tissue sample (e.g. endometrium) between two individuals.
443 Unravelling the female upper reproductive microbiome is helpful in understanding the natural
444 microenvironment in the tissues where the first processes of oocyte fertilisation and embryo
445 development are taking place and could be therefore used for improving *in vitro* fertilisation and
446 embryo culture conditions in the help of treating infertile patients.

447

448 ## 5 Conflict of Interest

449 *The authors declare that the research was conducted in the absence of any commercial or financial*
450 *relationships that could be construed as a potential conflict of interest.*

451 **6 Author Contributions**

452 AC-G, PC and SA conceived the idea and designed the study. PC, MT P-S, ML S-F done provided all
453 the required documentation to the Ethics Research Committee (CEIC) of Clinical University Hospital
454 "Virgen de la Arrixaca" (HCUVA). MT P-S, ML S-F and AC-G recruited the patients. AC-G
455 performed the sample and data collection. TE was the responsible of the registration, storage and
456 processing of the samples at the Biobanco en Red de la Región de Murcia, BIOBANC-MUR. CMR
457 performed the amplification, library preparation and sequencing of the samples. IP-P, ES-E, IL-B
458 analyzed the data. SA, AC-G and IPP wrote the first manuscript draft. The manuscript was written
459 through contributions of all authors. All authors have given approval to the final version of the
460 manuscript.

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