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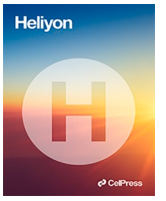

Research article

Correlation between hysteroscopic features and specific microbial species in women with chronic endometritis

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ABSTRACT

Objective and rationale: Chronic endometritis (CE) has recently been associated with unexplained infertility and recurrent miscarriages. The current gold standard for CE detection is histopathological examination. However, office hysteroscopy and endometrial cultures are also significant, due to the possible link between CE and various microorganisms. Bacterial colonization of the endometrium has been associated with reduced success rates of in vitro fertilisation embryo transfer. Few studies have tried to correlate CE hysteroscopy findings with pathogenic microorganisms. This prospective cohort study sought to establish whether hysteroscopic diagnostic lesions correlate with specific microbial species.

Methods: The study encompassed women undergoing diagnostic tests for a range of subfertility health issues. 189 women completed the standard office diagnostic hysteroscopy (DH). 181 had also endometrial samples taken for microbial culture investigation. Correlation analysis (χ^2 and Fisher's exact test) between hysteroscopic findings suggestive of CE and endometrial cultures was carried out. Logistic regression models were also fitted to measure whether a positive endometrial culture could affect CE conditions.

Results: A significant association of *E. coli* was observed between the hysteroscopically characterized CE + group with focal hyperplasia, when compared to the non–CE group. Logistic regression analysis revealed that women positive for *E. coli* were 4.423 times more likely to have focal endometrial hyperplasia. No other significant correlations were identified between DH and positive endometrial cultures.

Conclusions: The presence of *E. coli* in the endometrium was significantly correlated with focal hyperplasia findings from hysteroscopy, emphasizing the importance of microbial cultures in the diagnosis and targeted treatment of CE in women with subfertility.

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1. Introduction

In recent years, research studies have demonstrated that chronic endometritis (CE) is associated with cases of unexplained infertility and recurrent miscarriages (RM) [1,2]. Clinically, recurrent miscarriages (RM) are characterized as experiencing three or more miscarriages prior to the 20th week of pregnancy [3]. CE is considered the long–term persistent inflammation of the endometrial stroma, characterized by the infiltration of neutrophils and plasma cells, generally caused by bacteria, viruses and other pathogens [3–5]. Most women with CE remain primarily asymptomatic [1,5], with approximately 10 % exhibiting symptoms such as chronic pelvic pain, abnormal uterine bleeding (AUB), dyspareunia, and leucorrhea [6,7].

Several diagnostic methods have been proposed to detect CE, with the current gold standard being the histopathological examination in order to identify infiltration of the endometrial mucosa by plasma cells [6–9]. Additionally, immunohistochemistry (IHC) using the $CD138^{+}$ (syndecan–1) monoclonal antibody has been used [10]. Specifically, $CD138^{+}$ IHC has demonstrated greater diagnostic accuracy and sensitivity in identifying plasma cells in endometrial samples compared to the histological investigation, with lower intra– and inter–observable variability [11].

Furthermore, microbial cultures of endometrial samples could be utilized since the onset of CE has been linked to a plethora of microorganisms, including Gram (−) negative, such as Escherichia coli and Klebsiella spp., Gram (+) positive, e.g., Streptococcus species and *Enterococcus faecalis*, other microorganisms like Mycoplasma spp, Ureaplasma spp. and Gardnerella vaginalis, and intracellular bacteria, like *Chlamydia trachomatis* [5,12]. Research has shown that preexisting microbial invasion [5] or colonization of the endometrium with Gram (−) bacteria was associated with a decrease in the success rates of in vitro fertilisation embryo transfer (IVF–ET) [4,13]. Microbial culture, still, is the only approach capable of providing objective information for targeted antibiotic therapy [2]. Furthermore, the emergence of cutting–edge molecular microbiology techniques for the identification and characterization of microorganisms in CE could potentially evolve into the next gold standard method [2], but always in conjunction with the culture of endometrial samples. Indeed, the genital tract and gut microbiome play a pivotal role in infertility by influencing physiology, metabolism, nutrition, and immune functions. In recent years, several studies and meta–analyses have provided evidence for the significant implications of microbiota imbalances for a plethora of fertility conditions, such as CE, Polycystic Ovary Syndrome (PCOS), and altered immune responses (including B cells, NK cells, TH_1/TH_2 imbalances), resulting in recurrent reproductive failures, endocrine–related infertility, or reduced endometrial receptivity [14–16].

Likewise, fluid diagnostic hysteroscopy (DH) has been used for diagnosing CE, which comprises a minimally invasive procedure used for detecting intrauterine aberrations [1,6,7], with potential high diagnostic accuracy [9]. DH is an important routine medical technique for women with reproductive issues, such as RM or repeated implantation failures in IVF, allowing for a detailed examination of the cervical canal and the endometrial cavity to obtain focused endometrial samples for histological analysis [6,7]. Although DH has been proposed as a possible diagnostic tool for the identification of CE in infertility–related issues [17,18], however, the absence of an established or validated standard consensus for hysteroscopic features renders its applicability controversial [7–9,19]. The following three major criteria have been proposed for the diagnosis of CE: a) micropolyps (<1 mm); b) endometrial stromal edema; c) diffused or focal superficial hyperemia [2,7,20]. Although several studies have correlated CE lesions with the intrauterine microbiome [17,21,20], to our knowledge, none have investigated the possibility that specific lesions could be correlated to a certain microbial species.

1.1. The main aim of the clinical study was

- To determine whether detected DH endometrial lesions (e.g., micropolyps, etc.) correlated with a specific microbial species (e.g., *Enterococcus faecalis*, etc.).

2. Materials and methods

2.1. Study duration and locations

This prospective cohort study was carried out at Locus Medicus S.A. diagnostic center, Athens, Greece, from 2019 to 2023. The study was approved by the Scientific Committee of LETO GENERAL, MATERNITY AND GYNECOLOGY CLINIC S.A. (ref. no: 01A).

2.2. Study population – inclusion and exclusion criteria

The study population included women who visited Locus Medicus S.A., after complaining of infertility, habitual abortions, menometrorrhagia, endometrial polyps suspected by transvaginal ultrasound, endometriosis, PCOS, and ovarian insufficiency. All women were offered an office DH [according to the Science Interest Group of the European Society for Gynaecological Endoscopy (ESGE), office indicates the setting and model of care, and diagnostic the type of hysteroscopy] [22]. A total of 287 women were included from all age groups and for all clinical trials. All subjects were asked to sign a written informed consent form, and immediately after, they were given information about the clinical trial process, duration, potential risks and benefits. From the initial 287 women, 87 did not meet the inclusion criteria, and 6 refused to participate. Exclusion criteria were similar to the ones described by others [3,6,8,23]. Briefly, they included: a) Uterine anatomical abnormalities (e.g., septum). b) Clinical or ultrasound diagnosis of endometriosis. c) Current autoimmune condition. d) Suspected or recent endometrial infection. e) Recent antibiotic treatment for microbial infection (<3 months). f) Previous Dilation and Curettage (D&C) within the preceding 3 months. g) Previous surgery for myoma, endometrial

polyps and/or endometriosis within the preceding 3 months. h) History of oral contraceptive use (<3 months). i) Hormonal replacement therapy within the past 3 months. j) Unwilling to participate. From the 194 women that started the diagnostic hysteroscopy, 5 did not complete the procedure, due to pain or discomfort, and therefore excluded from the study. From the 189 that completed the DH, only 181 had endometrial aspiration (lavage) and vaginal swabs taken for further microbial culture investigation (Fig. 1). Women identified with CE were given antibiotic therapy. Following the therapy, all women were asked to repeat DH and microbial cultures, and the possible pregnancy status (IVF or natural) was recorded.

2.3. Experimental methods

A detailed ultrasound scan of the uterus and ovaries, using a vaginal probe, prior the hysteroscopy helped to identify the flexion of the uterine corpus (ante – retroverted). Oral administration of Hyoscine (Scopolamine) butylbromide 10 mg 2 h before the hysteroscopy, decreased pain and the consequences of the vagal effect. In parallel, administration of non–Steroidal Anti–Inflammatory Drugs (NSAIDs), such as Indomethacin 75 mg orally aided in alleviating pain in those women with a lower limit of acceptable discomfort [24]. Following, the subjects with empty urine bladder, were placed in gynecological position in order to start the hysteroscopy

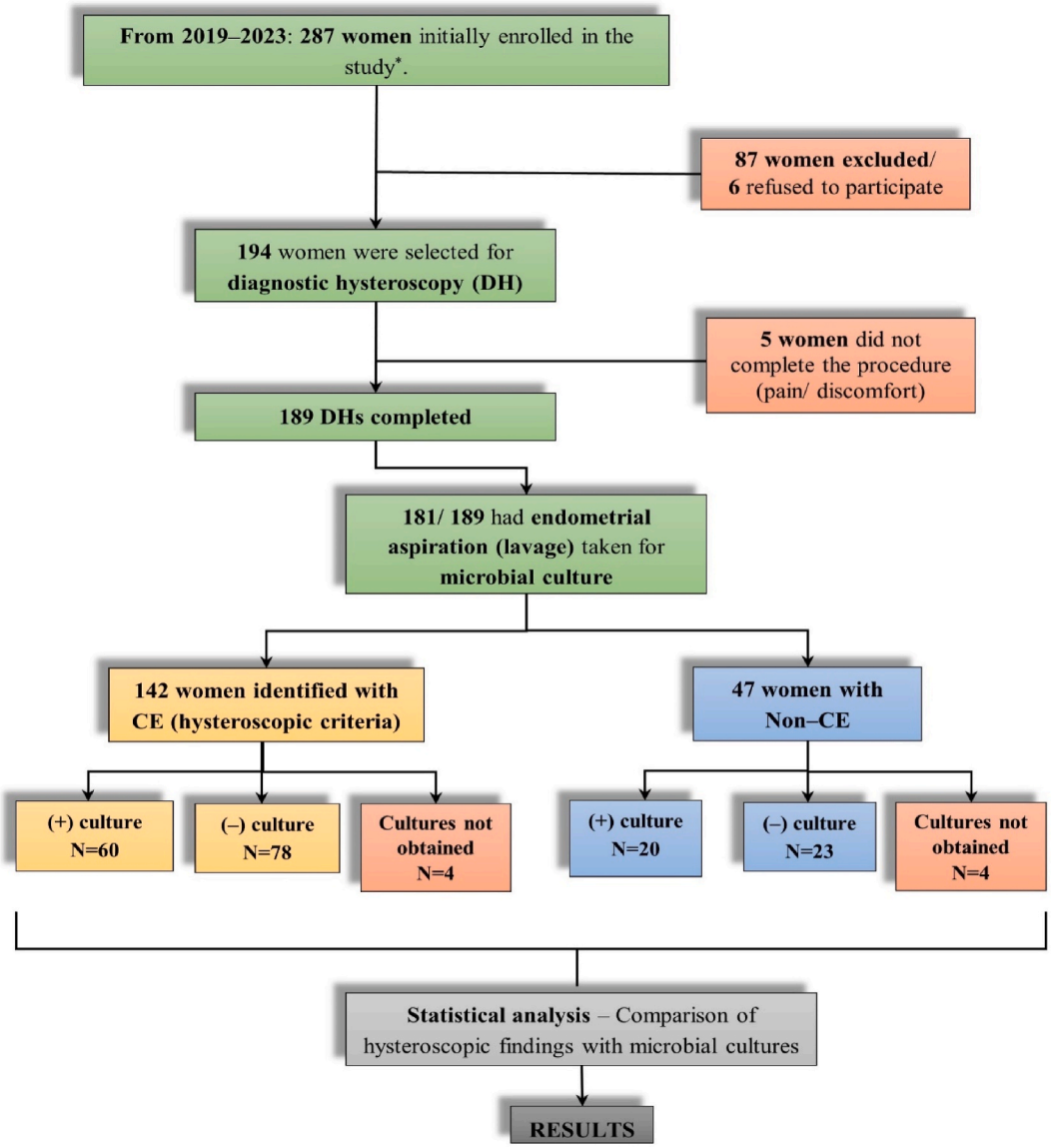

Fig. 1. Flowchart of the Chronic Endometritis protocol followed. *Women complaining for subfertility, habitual abortions, menometrorrhagia, recurrent infection of the lower genital tract, endometrial polyps (ultrasound finding), endometriosis, PCOS, genetic factor, ovarian insufficiency, were offered a diagnostic hysteroscopy.

procedure. 181 vaginal swabs were taken prior to hysteroscopy, in order to discriminate vaginal pathogens, from endometrial infectious agents.

2.3.1. Office hysteroscopy

A vaginal speculum was used, and the cervix was fully exposed. Meticulous clearance of the vaginal mucus was accomplished with normal saline (0.9 % NaCl) soaked cotton swabs, in order to minimize any risk of contamination from sampling. The use of betadine was avoided, because it could enter the cervical canal and give false negative results to the microbial cultures of the endometrial tissue or the fluid obtained at the end of each hysteroscopy. The use of tenaculum to grasp the cervix was also excluded, in order to provide

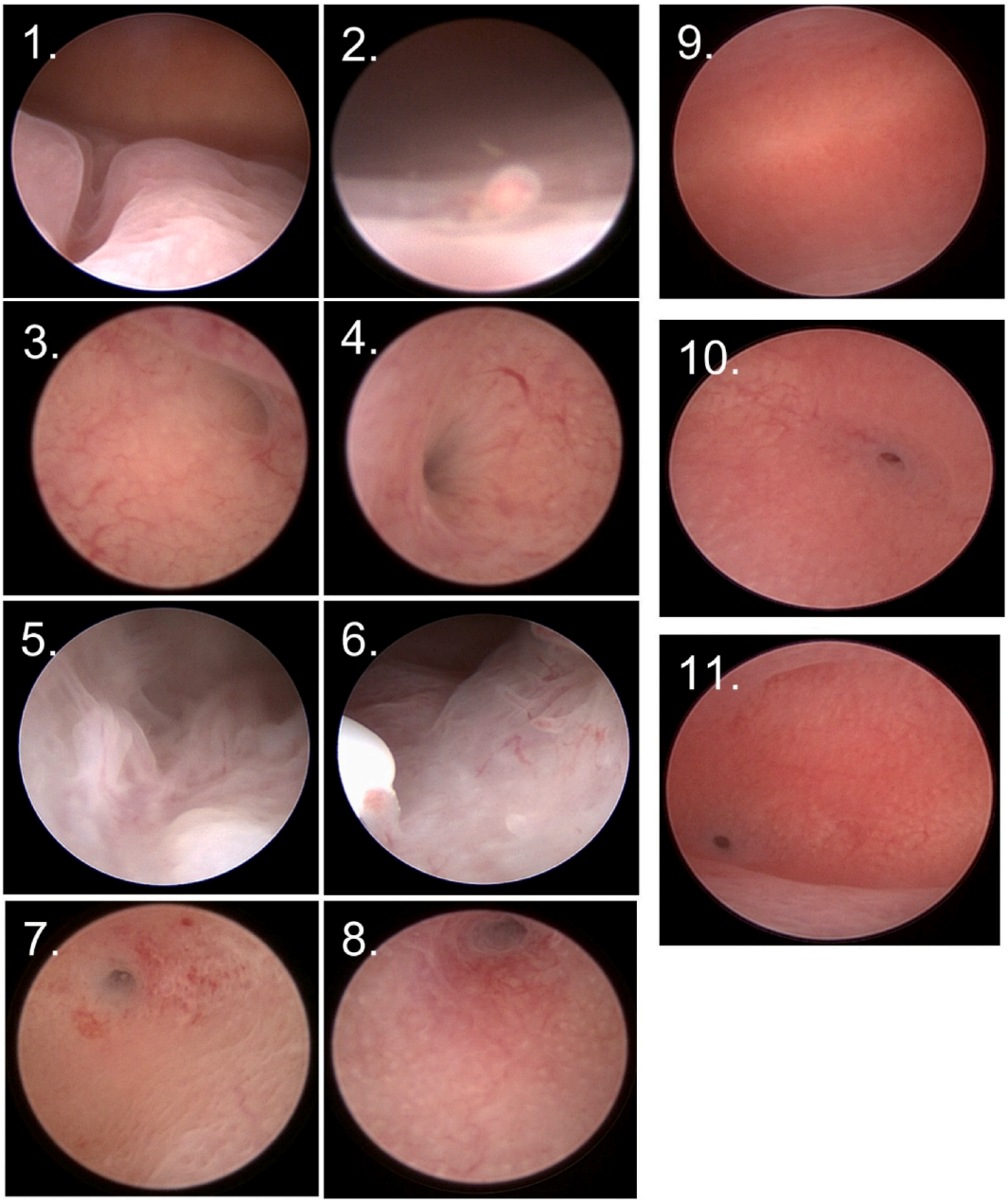

Fig. 2. Hysteroscopic findings. 1–8: Hysteroscopic findings in cases of chronic endometritis, from 4 women with positive endometrial cultures (*E. coli*). 1. Focal hyperplasia of the endometrium (posterior wall). 2. Micropolyp (posterior wall of the endometrium). 3. Left tubal ostium, focal hyperaemia. 4. Right tubal ostium, focal hyperaemia. 5. Hyperplasia (with micropolyps) on the endocervical glandular epithelium. 6. Micropolyps on the left–posterior wall of the endometrial cavity. 7. Left tubal ostium, hyperaemia. Micropolyp is visible in the tubal opening. 8. Right tubal ostium, hyperaemia. 9–10. Normal hysteroscopic images. 9. Overview of endometrial cavity. 10. Left tubal ostium. 11. Right tubal ostium.

less discomfort to the patients.

Office hysteroscopy was performed using a standardized approach [24–26], using a lens–based 2.9 mm OD (30^0), 24 cm length, continuous–flow mini–telescope (CAMPO TROPHYSCOPE®, HOPKINS®, Karl Storz, Tuttlingen, Germany). All hysteroscopies were performed in the follicular phase of cycle (day 7–13) by the same physician (author VK). Intrauterine pressure was generated by a simple drip from a bag suspended 1 m above the patient. A 300W light source with a xenon bulb, a high–definition digital camera (Karl Storz) and a video color screen were used (TELE PACK®, Karl Storz). All hysteroscopic procedures were digitally recorded.

Normal saline was preferred as a distention medium, instead of gas (CO_2), as the later may lead to pain after the procedure, due to the passage of CO_2 through the fallopian tubes [27]. After the endoscope had been inserted approximately 2 cm beyond the external ostium, the speculum was carefully removed from the vagina (anterior valve first), which gave the operator greater feasibility in passing the hysteroscope further inside the cervical canal, and through the internal cervical ostium, and into the uterine cavity. To minimize discomfort, the lowest possible intrauterine pressure of the distending medium (80–100 mm Hg) was used, in the shortest time possible for the endometrial cavity to be evaluated.

Advancing the hysteroscope through the internal cervical orifice, the narrowest portion of the endocervical canal, required smooth adjustments of the hysteroscope axis (anterior–posterior) and rotation of the fiber optic cable, as previously described by well experienced endoscopists [24]. The patient was advised to continuously express verbally the level of pain, throughout the procedure, in order to lessen the patient's discomfort, and the potential of a vagal reaction. The endocervical canal, uterine cavity, both the anterior and posterior uterine walls, tubal orifices, and endometrium were thoroughly examined, as described by Moreno et al. [2], and the findings recorded. The procedure was considered complete only when the entire uterine cavity and both tubal ostia were visualized.

Uterine anomalies (i.e., endometrial polyps, adhesions, submucous myoma, or septa) were diagnosed according to the American Society of Reproductive Medicine classification [28]. Diagnosis of Chronic Endometritis (CE) was based upon previously published criteria [7,9,29–35]: a) mucosal stromal edema, b) focal or diffuse superficial hyperaemia (included "strawberry aspect": hyperemic spots interrupted by white/pale focal areas), c) micro-polyps of <1 mm in size. We also took into consideration d) endometrial focal hyperplasia, and e) intrauterine adhesions (IUA) (Fig. 2), which are also observed at office hysteroscopy in cases of CE (confirmed by culture and/or histology).

After each hysteroscopy procedure was completed, an aspiration cannula (Ainsegrey, R. IMOS, Italy) with 1.8 mm external diameter, attached to a syringe filled with 3–5 ml sterile normal saline, was carefully inserted, through the cervix, into the endometrial cavity, taking care not to touch the vaginal walls, and the fluid was repeatedly (3–4 times) infused and aspirated (endometrial lavage). The endometrial aspirate was placed into a sterile pot and given to the laboratory for microbial cultures.

2.3.2. Microbiological analysis – bacterial cultures

Endometrial and vaginal samples were processed in accordance with the current microbiological culture standards [36]. Briefly, vaginal swabs and endometrial lavage samples were sent to the laboratory immediately after hysteroscopy. The professionals processing all samples for microbial analysis were blind to the hysteroscopy results (CE or non–CE), in order to eliminate bias, and vice versa. One half of the sample was kept in Nutrient Broth (Bioprepare®, Attica, Greece) and incubated at 37^0C for 24 h. The other half of endometrial fluid was first centrifuged at 2500 rpm for 7 min, and then:

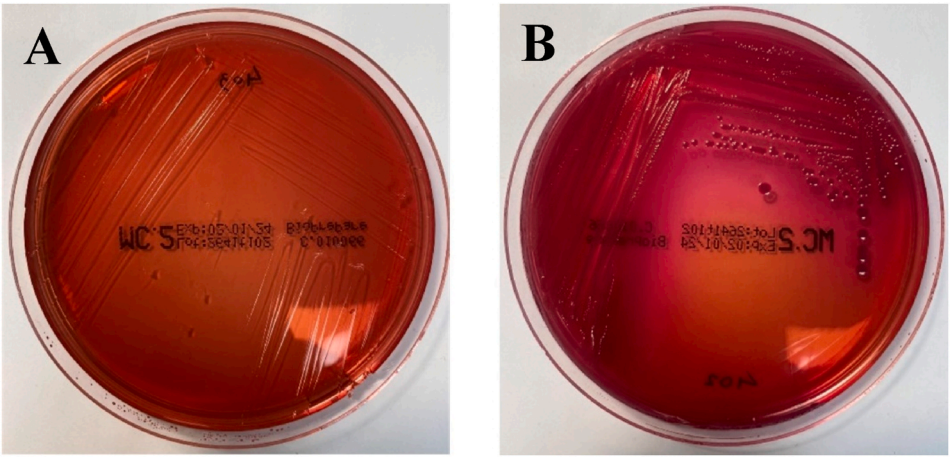

Fig. 3. Endometrial bacterial culture. A. Left image shows a negative endometrial microbial culture. B. Right image shows a *E. coli* positive endometrial culture. Microorganism identification was carried out according to the API System test kits (BioMérieux, France). Culture media: MacConkey N°2 (Bioprepare®, Athens, Greece).

- One part of the sediment was incubated aerobically onto culture media (media A, B, C and D below), at 37^0C for 24 h, for the isolation of Gram (+) and Gram (−) bacteria; and for the isolation of microorganisms that could grow more efficiently under CO_2 conditions (media D and E).
- The second part was incubated anaerobically (media F) onto culture media at 37^0C for 48 h, for the isolation of anerobic microorganisms.

Specifically, the following culture media were used:

A. Blood agar (Bioprepare®, Athens, Greece): For the aerobic cultivation and isolation of Gram (+) and Gram (−) bacteria. This is a general–purpose medium supporting growth of a wide range of microorganisms. It is often used in combination with a selective agar to differentiate bacterial species (e.g., Streptococcus spp.).
B. Mac Conkey N^{o}2 (Bioprepare®, Athens, Greece): For the isolation and differentiation of Gram (−) bacilli, Enterobacteriaceae, like Escherichia coli, and Pseudomonas spp. This media inhibited the growth of Gram (+) bacteria (except from Enterococcus spp) (Fig. 3).
C. Sabouraud Dextrose agar (SDA) w/Chloramphenicol & Gentamycin (Bioprepare®, Athens, Greece) was used to isolate types of fungi and filamentous bacteria.
D. Detection of Mycoplasma and Ureaplasma species required separate inoculation onto specialized media. Therefore, the Mycoplasma Agar–A7 (Bioprepare®, Athens, Greece) and Mycoplasma broth (MYCOPLASMA U–A, Bioprepare®, Athens, Greece) were used and the samples were incubated using a standardized procedure. The incubation was carried out in a chamber at 36^0C–37^0C aerobically (for the broth), and at 36^0C–37^0C, with 5%–7% CO_2 conditions (for the A7 agar), for 48–72 h.
E. Chocolate agar (Bioprepare®, Athens, Greece): This nonselective, enriched growth medium was used for the isolation of microorganisms that grow better under 5%–7% CO_2 conditions. Another variant of the medium, the Thayer–Martin agar, that contained a mixture of antibiotics was used for the isolation of Neisseria gonorrhoeae.
F. CDC Blood Agar (Bioprepare®, Athens, Greece) culture media was used for the isolation of anaerobic bacteria, with the simultaneous inoculation of 5 μg metronidazole antibiotic disc. The inoculated CDC blood agar plates were then placed inside specialized bags and sealed (GenBag, BioMérieux, France), and incubated under anaerobic conditions, at 37^0C for 48 h.

The next day, regardless of the first culture outcome, the other half of the sample that was kept in Nutrient Broth (Bioprepare®, Athens, Greece) was centrifuged, and the sediment was cultured, as previously described, in all media, under aerobic and anaerobic conditions (termed the 2nd culture).

In case of a positive culture, microorganism identification to the species level [i.e., Gram (+), Gram (−) bacteria and yeasts] was carried out with the API System test kits (BioMérieux, France). The system offered a large and robust database accessible through the Internet–based APIWEB™ (BioMérieux, France) service.

Evaluation of the in vitro susceptibility of the microorganism to antimicrobial agents (antibiotic susceptibility testing) was performed using the commonly used Kirby–Bauer method, with BioRad paper disks (BioRad, France) placed on the surface of a Mueller–Hinton Agar. This method was based on a standardized procedures and adopted as consensual standard by the CLSI [37]. These paper disks, soaked with a defined concentration of antimicrobial agent, were deposited on the surface of the Mueller–Hinton Agar plate, that previously was spread evenly across the whole plate with a concentrated, 0.5 McFarland standard scale inoculum of the bacteria cultured in a broth. Then, the agar plates were incubated for 24 h to allow for the bacteria to grow. After incubation, the Mueller–Hinton Agar plates were examined for the presence of clear zones on the agar around the antibiotic disks, indicating inhibition of the bacterial growth (zone of inhibition). Subsequently, the dimensions of the zones were measured and compared to established tables/values (for the various antimicrobial agents tested), to ascertain the clinical susceptibility category (i.e., resistant, intermediate, susceptible), always according to the diameter of the inhibitions zone and the bacterial strain isolated.

2.4. Statistical analysis

Correlations between hysteroscopic findings suggestive of CE and endometrial cultures, as well as between endometrial and vaginal

Table 1
Baseline demographic and clinical characteristics of the CE and control groups of women in the study.

CLINICAL	CE + group (n = 142)	Non–CE Group (n = 47)	P value
Age (Years)	37.36 ± 4.79 (22–49)	37.32 ± 3.87 (27–44)	N.S.
Infertility Duration (Years)	3.25 ± 2.73 (0.05–14.00)	3.18 ± 2.73 (0.05–11.00)	N.S.
MEDICAL HISTORY			
Primary Infertility	79.86 %	68.09 %	
Secondary Infertility	12.95 %	23.40 %	
IUI	23.74 %	23.40 %	
IVF	38.93 %	25.53 %	
Miscarriages (1–5)	23.02 %	27.66 %	

Note: Data are expressed as Mean ± SD. In parentheses: Min and max values are given in terms of an interval. IUI, Intrauterine Insemination; IVF, In vitro fertilisation.

cultures (categorical variable), were evaluated using contingency tables, chi–square (χ^2) test and Fisher's exact test. It was calculated that for the total number of subjects that completed the DH (189), for a confidence interval (CI) 95 %, a margin of error of 5 %, an estimated effect size between 20% and 50 % and an estimated power of at least 0.80 and above, the sample size required would be between 120 and 385. A p value < 0.05 was considered statistically significant. The aforementioned tests were performed using SPSS (SPSS Version 25; IBM Corp, Armonk, NY). Logistic regression models (using the R version 4.3.1; The R Foundation for Statistical Computing) were fitted to measure the extent in which a positive endometrial culture could affect CE conditions. Model parameters were calculated using the maximum likelihood method [38]. Overall model evaluation was carried out using likelihood ratio tests (test statistic comparison to χ^2 distribution, with degrees of freedom dependent on the number of predictor variables).

3. Results

189 women completed a diagnostic hysteroscopy, 181 of which had a vaginal swab and an endometrial lavage taken for microbial investigation (culture). Distribution analysis showed that all participants' age ranged between 22 and 49 years. The mean ± SD (Standard Deviation) of age was 37.35 ± 4.55 years (range = 22–49). Table 1 shows the baseline demographic and other infertility characteristics of CE and non–CE groups.

Out of the 189 women, 142 were identified as CE positive at hysteroscopy (142/189 = 75.13 %), whereas 47 women were identified as CE negative (47/189 = 24.87 %). From the 142 women identified with CE, 60 had a positive endometrial culture (CE^+ culture group; 60/142 = 42.25 %), whereas the rest were negative (CE^- culture group; 78/142 = 54.93 %), with 2.82 % (4/142) of cultures not obtained. From the non–CE group, 20 had a positive endometrial culture (non–CE^+ culture group; 20/47 = 42.55 %), whereas the rest were negative (non–CE^- culture group; 23/47 = 48.94 %), with 8.51 % (4/47) of cultures not obtained. The prevalence of CE in women with specific hysteroscopic features is given in Table 2.

Frequency analysis revealed that the bacteria identified in the positive cultures (regardless of the CE status) were:

1. Gram (+) bacteria at 58.75 % (47/80), with most frequent: *Enterococcus faecalis* at 32.50 % (26/80) and Staphylococcus spp. (*Staphylococcus aureus*, S. haemolyticus, and S. epidermitis) at 21.25 % (17/80).
2. Gram (−) bacteria at 38.75 % (31/80), with most frequent: *E. coli* at 22.50 % (18/80), *Proteus mirabilis* at 6.25 % (5/80) and *Enterobacter cloacae* at 5.00 % (4/80).

Further analysis of the frequency of the bacteria identified, in relation to the CE status (CE + group vs non–CE group) is given in Table 3.

The Pearson's χ^2 analysis (in conjunction with Fisher's exact test) for the observed counts of the endoscopic findings and the bacterial species showed:

1. A near significant correlation between the Enterobacteriaceae spp. investigated and the endometrial adhesions finding at hysteroscopy ($\chi^2 = 3.830$, df = 1, p = 0.072).
2. A significant statistical difference between the frequencies of Enterobacteriaceae spp. and women with hyperplasia (8/28 = 28.60 %) vs women with no hyperplasia (14/153 = 9.2 %) ($\chi^2 = 8.361$, df = 1, p = 0.009). Further χ^2 analysis, on the specific Enterobacteriaceae spp. revealed a significant association ($\chi^2 = 8.383$, df = 1, p = 0.010) of *E. coli* in women with focal endometrial hyperplasia (7/28 = 25.00 %) vs women with no hyperplasia (11/153 = 7.20 %) (Table 4).

The findings indicated that it was more likely that *E. coli* was associated with focal endometrial hyperplasia identified using hysteroscopy. Furthermore, logistic regression analysis revealed that women tested positive for *E. coli* had odds of having focal hyperplasia increased by 4.423 times [β = 1.487, z = 2.756, odds ratio = 4.423, 95 % CI (1.481–12.628), p = 0.006] (Table 5).

3. From all women tested, only 26 vaginal cultures were positive (26/171 = 15.20 %). For the CE + group, only 11 were found with positive vaginal cultures (11/76 = 14.50 %). Furthermore, from all *E. coli* positive endometrial cultures (18), only 4 had in concordance vaginal cultures (4/18 = 22.22 %) tested positive. However, one was identified as a Streptococcus spp., one was identified as Mycoplasma/Ureaplasma, one was identified as Candida spp., and the last one was positive for Mycoplasma/Ureaplasma and Candida spp., simultaneously. Furthermore, 3 out of the 7 *E. coli* positive endometrial cultures in the focal hyperplasia group had positive vaginal cultures; however, none of them was *E. coli*.

Table 2
Frequency analysis of specific hysteroscopic features for the CE group.

	CE + group (n = 142)	
Diagnostic Hysteroscopy features	n	%
Hyperemia	135	95.07
Diffused	46	32.39
Focal	89	62.68
Micropolyps	59	41.55
Endometrial Adhesions	14	9.86
Focal Hyperplasia	28	19.72

Table 3
Frequency analysis of the most common bacteria endometrial culture results in relation to the CE group. Percentages (%) in the table are given in relation to the group. Blue area indicates Gram (+) bacteria. Orange area indicates Gram (−) bacteria. Note the positive cultures in the non–CE group.

	CE+ group (n=138)		non–CE group (n=43)		*P* value
Bacteria	**n**	**%**	**n**	**%**	
Staphylococcus aureus	4	2.9	0	0.0	*.57*
Staphylococcus haemolyticus	8	5.8	2	4.7	*1.00*
Staphylococcus epidermidis	1	0.7	2	4.7	*.14*
Streptococcus agalactiae	4	2.9	0	0.0	*.57*
Enterococcus faecalis	20	14.5	6	14.0	*.93*
Escherichia coli	**12**	**8.7**	**6**	**14.0**	*.38*
Enterobacter cloacae	3	2.2	1	2.3	*1.00*

*p≤0.05, **p≤0.01.

Table 4
Contingency table analysis of hysteroscopic finding Hyperplasia and Enterobacteriaceae spp., *E. coli* and *Enterobacter cloacae*.

Hyperplasia					
Escherichia coli	Absent	Present	Total	χ^2	P value
Negative	142	21	163		
Positive	11	7	18		
Total	153	28	181		
%	7.2 %	25.0 %		8.383	0.01**
Enterobacter cloacae	Absent	Present	Total	χ^2	P value
Negative	150	27	177		
Positive	3	1	4		
Total	153	28	181		
%	2.0 %	3.6 %		0.284	0.493

*p ≤ 0.05, **p ≤ 0.01.

Table 5
Logistic regression analysis of bacteria for the Hyperplasia DH feature.

Bacteria	β	OR	95 % CI	p
Gram (−)	0.816	2.261	0.852–5.614	0.086
Streptococcus/Enterococcus	0.644	1.904	0.616–5.366	0.236
Staphylococcus	−0.742	0.476	0.025–2.654	0.489
Enterobacteriaceae[†]	1.471	4.352	1.450–12.353	0.006 **
[†]Further analysis of Enterobacteriaceae group				
Escherichia coli	1.487	4.423	1.481–12.628	0.006 **
Enterobacter cloacae	0.840	2.317	0.112–19.114	0.476

CI: Confidence interval; OR: Odd Radio. *p ≤ 0.05, **p ≤ 0.01.

4. There was no statistically significant correlation ($\chi^2 = 0.122$, df = 1, p = 0.727) of the positive endometrial cultures, between the CE (60/138 = 43.48 %) and the non–CE groups (20/43 = 46.51 %).
5. There were no statistically significant correlations of other hysteroscopic findings vs positive endometrial cultures.
6. There were no significant correlations between the vaginal cultures (data not shown) and endometrial cultures.

4. Discussion

The present prospective cohort study utilized the standardized office hysteroscopy technique to investigate the correlation of CE anomalies with certain bacteria spp. Using this technique, 142/189 women were identified with CE, and *E. coli* was significantly

associated with focal endometrial hyperplasia (Fig. 2. Image 1), despite the fact that it was not the dominant bacterial spp. in the positive endometrial culture subgroup (*Enterococcus faecalis* prevailed at 32.50 %).

In recent years, efforts have been made to correlate hysteroscopic findings in women with CE to bacteria spp., so that they might perform as prognostic factors for treatment outcomes without the need for repeated endometrial biopsies or cultures [3,39–42]. In the present study, we identified a higher percentage of women with CE (75.13 %) at hysteroscopy, when most studies have shown a lower prevalence range (9.23 %–57.80 %) [2,6,19,40,41,43–46]. This finding could be attributed to different diagnostic methodologies used by each group. However, the group by Cicinelli et al. [30], in a controlled clinical study, identified 200/211 cases with CE using fluid hysteroscopy, a percentage of 94.80 %, that was confirmed and correlated with the histological findings; a higher percentage than our findings. Moreno et al. [2], also found a high percentage of CE based on hysteroscopic findings (96.92 %). Nevertheless, our results confirmed previous observations that CE has a high prevalence in women suffering from subfertility [2,40]. One possible explanation for this high percentage in our study could be the uniqueness of women that visit Locus Medicus S.A. diagnostic centre, which mainly focused on cases of subfertility, with the majority of them having a long–lasting infertility history. Additionally, another possible reason could be the fact that with the new criteria for identifying CE, as described by others [7,23], and with the use of fluid DH (normal saline) versus CO_2, the hysteroscopic abnormalities of CE were more easily recognizable [6,8]. Although there is a tendency for DH to overdiagnose cases of CE in the literature, results by Kitaya and Yasuo [46] suggested that CE could be a more common pathological condition than before thought. Therefore, we need to keep in mind that methodological differences (sampling collection, laboratory examination and findings evaluation), population sample characteristics and ethnic differences might account for the different results observed.

Previous studies have already correlated CE with bacteriological findings, with the most common bacteria being *E. coli*, *Enterococcus faecalis*, Streptococci, Staphylococci, Mycoplasmas, Ureaplasmas, *Chlamydia trachomatis* etc. [2,6,44,47]. However, to the best of our knowledge, this is the first study to have identified and associated a specific bacteria species (*E. coli*) to a specific hysteroscopic finding (focal endometrial hyperplasia) (Fig. 2, Image 1), particularly within the CE + group. Cicinelli et al. [6], investigated the correlation of CE–positive and negative women at hysteroscopy with positive endometrial and vaginal cultures. They found correlations for many common infectious agents from the endometrium of CE patients, and in another study, they found 26.60 % of the CE cases were positive for *E. coli* and *Enterococcus faecalis* [40]; however, they did not correlate the specific hysteroscopic findings to a bacteria spp. In our study, it is very interesting that, although *Enterococcus faecalis* was the more dominant microorganism, instead, *E. coli* was found to be associated with focal endometrial hyperplasia. Moreover, the odds ratio implied that the presence of *E. coli* increased the likelihood of focal endometrial hyperplasia by more than fourfold, suggesting a potential pathogenic role for this bacterium in CE–associated focal hyperplasia. This association is particularly noteworthy since, in animal studies, *E. coli* has been linked with multiple areas of hyperplasia [48]. Also, in male subjects with benign prostatic hyperplasia (in biopsy), *E. coli* is the most commonly isolated microorganism [49]. As a result, our findings provide evidence of the possible association of hysteroscopic features with certain bacteria species, an observation that could be particularly helpful in optimizing antibiotic treatment.

Indeed, as Cicinelli et al., stated in their discussion, in order to simplify the therapeutic procedures for CE, most physicians will subscribe broad–spectrum antibiotics (e.g., doxycycline, ciprofloxacin) that, in general, target *Chlamydia trachomatis* and N. gonorrhoeae [6]. In concordance with the findings by others [2,3], our result indicated that such antibiotic treatment will not be effective on the common bacteria (e.g., *Enterococcus faecalis*) which prevail in cases of CE, since these are frequently resistant. It is more probable that it will lead to increased antibiotic resistance, recurrent chronic endometritis, and persistent subfertility [43,50]. Therefore, when hysteroscopy is indicative of CE, the identification of pathogenic bacteria inside the endometrial cavity could be accomplished by microbial culture. This standardized procedure, as described above, could aid in more targeted antibiotic therapy, and it will help to improve the reproductive outcome in women with recurrent miscarriages and repeated implantation failures in IVF [3,34]. There is evidence that focused antibiotic therapy seems to be associated with an improved reproductive outcome [3]. In our study, a follow–up was carried out on 11 women that had completed the targeted antibiotic therapy (data not shown). It was very exciting and promising, that 8/11 women had a successful pregnancy (7 after IVF, one after natural conception), and only 3 had a failed IVF attempt. However, due to the small size of the follow–up group, these observations need to be confirmed by a larger cohort. Therefore, from our observations, we believe that combining fluid mini–hysteroscopy (inflammation) and endometrial cultures (infectious agent), allows for a more reliable diagnosis of CE; should be performed routinely; and provides certain information that will guide the gynaecologist to the most effective therapy.

Another significant observation in our study was the lack of correlation between *E. coli* positive endometrial cultures and vaginal cultures. This result, along with the variation in microbial species detected in the vaginal cultures of women with *E. coli* positive endometrial cultures, indicated that the endometrial environment might have a distinct microbial profile, separate from the vaginal environmental milieu, as suggested by the literature [51–53].

The contamination risk of the endometrial samples with vaginal flora during hysteroscopy was of outmost importance for the correct interpretation of culture results. In one study [6], 32.60 % of the CE cases had the same infectious microorganisms found in both endometrial and vaginal cultures. Bacteria colonizing the vagina and cervix could be transmitted into the endometrium, thus producing false positive endometrial cultures. In our study, we were able to fully minimize that risk and exclude contamination of the endometrial samples, by performing DH with the help of a vaginal speculum (at the beginning of the procedure) without a tenaculum [54–56], and with great care to avoid any contact between the endoscope and the vaginal walls [40]. Tien CT et al. [56], used betadine–soaked cotton swabs to disinfect the vagina and cervix before hysteroscopy, to overcome possible contamination of the endometrial samples. In the present study, the use of betadine was avoided because it could enter the cervical canal and give false negative results to the microbial cultures of the endometrial fluid obtained at the end of each hysteroscopy. Instead, meticulous clearance of the vaginal mucus was accomplished with cotton swabs soaked with sterile normal saline prior to and after insertion of the

hysteroscope in the endometrial cavity in order to minimize any risk of contamination from sampling.

Another interesting finding in our study was the six *E. coli* positive endometrial cultures in the non–CE group. We believe these are probable initial or recent infections, or infections with lower microbial concentration, without yet the inflammation having developed any hysteroscopically observable findings inside the endometrial cavity (e.g., focal hyperplasia, micropolyps, hyperemia etc.). Lastly, we did not observe any statistically significant associations of hysteroscopically diagnosed CE abnormalities with other bacteria spp., such as Streptococcus spp., Mycoplasmas and Ureaplasmas, consistent with Liu et al. [41], but not in concordance with other studies [3,7].

Our study has several strengths, such as the association of *E. coli* with focal hyperplasia, which provides depth and specificity to the research, potentially highlighting a significant clinical correlation. Significant associations between certain microbial species and hysteroscopic findings that could have possible clinical implications might be the path for future research. By associating specific microbes with certain endometrial features at hysteroscopy, this study offers the option of targeted antibiotic therapy to improve fertility treatment results in women with CE. Furthermore, the inclusion of women with various reproductive health issues (infertility, endometrial polyps, PCOS, menometrorrhagia, etc.) ensured that the findings may be applicable across a broad spectrum of endometrial health conditions. The use of a detailed and analytical methodology of the endometrial microbial culture procedures, focused on a subgroup of CE–causing bacteria, such as Mycoplasmas, Ureaplasmas, and Gardnerella vaginalis, that are usually not culturable under standard laboratory conditions, eliminated any false–negative results and/or contamination bias. Lastly, the present study findings could be applied, both in office and outpatient settings, without the need for intravenous sedation and with minimal financial burden, to both the patients and the health care facility centres (public or private).

On the other hand, the present study has limitations. The sample size may need to be larger to represent the broader population, potentially affecting the external validity of the findings. However, most studies investigating associations between CE and pathogenic microorganisms have recruited approximately the same, or, in some cases, even a lower number of subjects [2,23,30,34,41,43,44,46]. While office hysteroscopy is a standardized method in the field, inherent biases or inaccuracies may affect the procedure, leading to potential misclassification or under–detection of certain conditions. For example, the physician's hysteroscopic expertise level can impact the precision of diagnosing CE [23]. However, all hysteroscopies were performed by author VK, a gynaecologist–endoscopist with a special interest in managing women with subfertility and CE.

Bacterial culture still stands as one of the most important tools in the diagnosis of CE. This method allows the identification of microorganisms and enables the prescription of precise, targeted therapy [57]. However, some authors [2] have raised concerns about the routine use of endometrial culture. First, it takes a long time to accomplish; second, there is a risk of contamination from vaginal bacteria; and third, not all microorganisms responsible for CE can be cultured in the microbial laboratory. Although all the above could be considered as limitations, nevertheless, our method for microbial culture of endometrial fluid aspirate provided definite results in 48–72 h; we were able to culture an extensive range of bacteria responsible for CE using our standardized method of microbial investigation; and the method used for endometrial sampling almost eliminated the risk of contamination.

In our study, we chose office hysteroscopy, a less invasive method than biopsy, with well–defined diagnostic criteria for CE in literature, to diagnose CE and correlate with specific microbial species. On the other hand, since histologic identification and confirmation of CE remains the gold standard to date [23], this could be considered a limitation. Nevertheless, endometrial stromal plasma cells can frequently be missed at microscopy, especially when using hematoxylin and eosin (H&E) staining, because they are morphologically almost identical to monocytes and fibroblast cells [45,58]. Although the accepted gold standard for diagnosis of CE is the presence of endometrial stromal plasma cells (ESPCs), their histological identification is sometimes hindered by infiltration of mononuclear cells, mitosis and stromal cells proliferation, plasmacytoid appearance of stromal cells (fibroblasts and mononuclear cells) or late secretory phase decidual transformation of the endometrium [59]. Moreover, the identification of these cells depends on the experience of the histopathologist performing the microscopic analysis of the specimens. Cicinelli et al. [29,39], reported that the presence of endometrial micropolyps at hysteroscopy suggests the existence of CE. Interestingly, they obtained a positive diagnostic correlation of 93.4 % with the pathology findings, following their criteria of hysteroscopic diagnosis. Another study even reported higher reliability of hysteroscopy identifying CE cases (positive predictive value – PPV = 98.1 %) [60]. These findings have been replicated by others [61] with an 86.5 % correlation of hysteroscopic vs histological diagnosis. As a result, Puente et al., in their review on chronic endometritis concluded that hysteroscopy could be considered a gold standard tool for diagnosing of CE, considering its high correlation with histological findings [59].

Furthermore, in recent years, molecular diagnostic methods have been used, based on Real Time (RT) polymerase chain reaction (PCR) and Nucleic Acid Amplification Tests (NAATs), to investigate the microbiological aetiology of pathological conditions, such as CE [2,62]. It is widely accepted [63] that the application of molecular microbiology techniques, particularly with the aid of PCR, has revolutionized the detection and characterization of microorganisms across various medical fields. A recent study, by Moreno et al. [2], conducted a comparative analysis between molecular microbiology and three classical methods for diagnosing CE: histology, hysteroscopy and microbial culture, on 113 infertile women. Endometrial samples were screened with RT–PCR for nine CE pathogens. The authors concluded that RT–PCR showed similar results to all three classic diagnostic methods for CE. Another step forward, is the recent implementation of Next Generation Sequencing (NGS) techniques used to screen the microbiome of human tissue, such as the endometrium. The microbiota plays a crucial role in numerous vital functions within the human body [9,15–17]. A recent study by Liu et al. [41], has used NGS to investigate the microbiota of CE and non–CE patients. They found that the median relative abundance of Lactobacilli on endometrial specimens in the CE group was 42.7 times lower than the control group. Indeed, the recent literature suggests a low abundance of Firmicutes and Lactobacillus and a high abundance of Proteobacteria (e.g., *E. coli* and *Enterococcus faecalis*), Bacteroides spp., Prevotella, and Actinobacteria (e.g., Gardnerella vaginalis), when compared to controls [42,52,64]. On the other hand, another NGS study has provided information that non–Lactobacilli are also present in healthy fertile women's

endometrium [5]. Consequently, these methods could identify which bacteria exhibit higher abundance in the endometrium of women with CE compared to the endometrium of fertile women [41,64]. These findings support the usefulness of molecular methods in aiding the diagnosis of CE–causing pathogens. Nonetheless, these methods are rarely applied in a clinical practice setting, primarily due to the expensive equipment, specialized laboratory personnel expertise requirements, and, eventually, potential treatment delays. Moreover, it is known that molecular methods do not discriminate between viable and non–viable bacterial DNA [2], which is crucial information when trying to identify the etiological agent of endometrial inflammation. Therefore, we cannot underestimate the validity of the classical microbial methods used in our study, and as such, being inexpensive, fast and non–labour intensive compared to the RT–PCR techniques, they could accompany an office hysteroscopy without any major financial burden to the patients.

5. Conclusion

In conclusion, this study sheds light on the intricate relationship between the endometrial microbial environment and CE, emphasizing the need for a comprehensive approach to understanding, diagnosing, and treating this condition. The significant findings related to endometrial infection with *E. coli* and its association with focal endometrial hyperplasia (as a specific hysteroscopic feature), in particular, open new avenues for targeted research and interventions. The findings highlight the importance of investigating endometrial microbiota in women diagnosed with CE at hysteroscopy. Our study, for the first time, showed that focal endometrial hyperplasia correlates in a statistically significant manner with specific microbiota, and therefore, we believe that other researchers could, in the future, design multicenter studies to further elucidate any possible relations between distinct hysteroscopic features with isolated or families of bacteria, and to determine the causative nature of these microbes in CE pathology. Moreover, in clinical practice, when performing an office/outpatient hysteroscopy and microbial cultures of the endometrial cavity aspirate are technically unavailable, specific hysteroscopic findings, like focal endometrial hyperplasia, can lead to the use of specific antibiotics (as β–lactams, e.g. penicillin, ampicillin, cephalosporins), known to be effective against Enterobacteriaceae like *E. coli*, without further delay of the infertility treatment, and this would undoubtedly lower the anxiety of the patient waiting for IVF. While we acknowledge the limitations of this study, nevertheless, we believe that since, up to date, treatment of CE depends upon expensive molecular microbiology methods (microbiome), the information provided herein could be especially valuable for specialists, as classical microbial cultures, being cost–effective and more accessible to accomplish, would be most widely utilized worldwide for detecting common bacteria in routine clinical practice. This includes understanding whether these bacteria are merely opportunistic or play a significant role in the initiation or progression of CE. Finally, targeted antibiotic treatment appears to be associated with an improved reproductive outcome.

Ethics statement

This study was performed in accordance with the principles outlined in the ethical standards of the "A Guide for Research Ethics Committees for Biological Research (REC)" (particularly section 1) issued by the National Commission for Bioethics and Technoethics, in Greece, and the 1964 Declaration of Helsinki and its later amendments on ethical standards.

This study was reviewed and approved by the Scientific Committee of LETO GENERAL, MATERNITY AND GYNECOLOGY CLINIC S.A., with the approval number: 01A.

All participants/patients (or their proxies/legal guardians) provided informed consent to participate in the study.

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Data availability statement

Data from this study has not been deposited into a publicly available repository. Data will be made available upon request.

CRediT authorship contribution statement

Vassilis Kapetanios: Writing – review & editing, Validation, Supervision, Resources, Methodology, Investigation, Data curation, Conceptualization. **Maria Lampraki:** Writing – review & editing, Resources, Project administration, Methodology, Data curation. **Georgios Georgoulias:** Writing – review & editing, Supervision, Methodology, Data curation. **Stavros Kasdaglis:** Writing – review & editing, Resources, Methodology, Data curation, Dr. **Stylianos Kliafas:** Writing – review & editing, Visualization, Validation, Software, Methodology, Investigation, Formal analysis, Data curation. **Nikolaos Gkavra:** Writing – review & editing, Visualization, Validation, Software, Methodology, Investigation, Formal analysis, Data curation, Dr. **Maria Xountasi:** Writing – review & editing, Methodology. **Vassilis Tsilivakos:** Writing – review & editing, Validation, Conceptualization. **Michail Leventopoulos:** Writing – review & editing, Writing – original draft, Visualization, Validation, Software, Resources, Investigation, Formal analysis, Data curation, Dr.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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