

Is there any difference in *Lactobacillus* abundance between age groups during ovarian stimulation in fresh *in vitro* fertilization (IVF) cycles?

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ABSTRACT

Aim To investigate an influence of age and body mass index (BMI) as determinants of infertility treatment success *in vitro* fertilization (IVF) procedures confirmed so far, on the amount of *Lactobacillus* spp. (LS) and *Gardnerella vaginalis* (GV) in the endometrium in fresh IVF cycles at the time of embryo transfer.

Methods This a prospective single-centre study included patients who underwent fresh embryo transfer (ET) in a private IVF Centre. A catheter tip for embryo transfer was used for collecting samples from the endometrial cavity. Real time polymerase chain reaction (RT-PCR) was used to determine LS and GV quantity. Patients were divided into two age groups: <35 years and ≥35 years. Normal BMI group included patients with 18.-24.9 kg/m², and the increased BMI group patients with BMI ≥ 25kg/m².

Results Tips for embryo transfer catheters were positive on LS in 55 (96.4%) patients. The median of quantification cycle of RT-PCR (Cq RT-PCR) for LS was 33, and 31 for the younger and older groups, respectively. Twenty-one (36.8%) patients tested positive for GV. The median of Cq RT-PCR for GV was 33 and 34 for the younger and older groups, respectively. There were 36 patients with normal and 18 with increased BMI. BMI showed no correlation with LS amount in the endometrial cavity.

Conclusion Aging affects the endometrial microbiome resulting in an increase of *Lactobacillus* number, but not the number of GV.

Keywords: assisted reproductive technologies, endometrium, human reproduction, reproductive tract bacteria

INTRODUCTION

The latest scientific reports suggest endometrial microbiome has a unique bacterial composition that may play an important role in reproduction and pathological conditions (1–4). Different bacterial compositions are found in anatomical niches despite the anatomical connection between the vagina and the endometrium (5). The composition of the female genital tract microbiota is influenced by numerous factors, including age, pH in

the vagina, hormonal secretions, the menstrual cycle, contraceptives, antibiotic use, and sexual activity (6,7). *Lactobacillus* spp. (LS), the dominant bacterium in the vagina of women during pregnancy (8,9), can bind to the surface of vaginal epithelial cells to prevent the attachment of other microorganisms in the vagina. It cannot only produce lactic acid by decomposing glycogen in the vagina to maintain a stable pH, but also kill intracellular microorganisms by inducing the autophagy of vaginal epithelial cells (10). The incidence of infertility has increased during past years, and it is widely recognized as a world health problem. One of seven couples cannot get pregnant despite having regular unprotected sex throughout a year (11). Maternal age, endometrial microbiome, and body mass index (BMI) are factors that can affect *in vitro* fertilization (IVF) outcomes.

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The endometrial microbiome is less explored than the vaginal one. Different studies reported the presence of LS and *Gardnerella vaginalis* (GV) in the endometrial cavity (2,11). The dominance of LS in the endometrial cavity has been associated with a higher pregnancy rate (12,13) and a lower one with miscarriage rate (14). GV is more abundant in patients with chronic endometritis (15), but GV did not adversely affect the implantation process (16).

Overweight and obesity are more and more common in infertile couples (17). Pre-pregnancy BMI is a potentially modifiable and preventable lifestyle-related factor associated with neonatal outcomes (18).

A significant number of studies explored the endometrial microbiome in IVF cycles, and they focused on identifying all bacteria in this anatomical environment, as well as all species of *Lactobacillus* (2,5). The question arises as to whether the presence or quantity of bacteria plays a vital role in the function of the endometrium and whether other factors such as ovarian stimulation, body mass index, age of life and others could affect the microbiome, not only its diversity but also the abundance of bacteria in the endometrial cavity (2,5,19,20).

To our knowledge, this is the first study in the Balkan region to explore the role of *Lactobacillus* spp. and *Gardnerella vaginalis* in the endometrial cavity in IVF cycles. It is also one of the rare studies that compare BMI and age with the amount of bacteria in fresh IVF cycles.

The aim of this study was to investigate an influence of age and BMI as determinants of infertility treatment success *in vitro* fertilization procedures so far confirmed on the amount of LS and GV in the endometrium in fresh IVF cycles at the time of embryo transfer.

PATIENTS AND METHODS

Patients and study design

This prospective single-centre study included 57 patients who underwent fresh embryo transfer (ET) in a private IVF Centre during the period February to December 2023. After the embryo transfer, the catheter tip was used for sample collection, and real-time polymerase chain reaction (RT PCR) was used to determine the quantity of LS and GV.

The data for BMI value were collected from patients' medical history.

The patients were divided into two age groups: <35 years and ≥ 35 years.

BMI groups were formed according to the World Health Organization's recommendation (21). Due to the limited number of obese patients (BMI ≥ 30 kg/m²) in our study, overweight (BMI ≥ 25 kg/m²) and obese patients were

combined into one group for statistical analysis. The "Normal BMI Group" includes patients with BMI between 18.5 and 24.9 kg/m², and the "Increased BMI Group" includes patients with BMI ≥ 25 kg/m².

All patients gave their consent to participate in the study.

The study was approved by the Ethical Board of Institute for Human Reproduction "Dr. Balić" and research was performed in accordance with the Declaration of Helsinki.

Methods

After placing the patient in the gynaecological position, the vaginal speculum was inserted. A double-lumen catheter was used for embryo transfer (Guardia™ Access Embryo Transfer Catheter, Cook Incorporates, USA). The outer catheter was placed through the cervical canal. Then, the inner catheter was placed through the outer catheter in the endometrial cavity. Each embryo transfer was performed under ultrasound control. Embryo transfer was performed on the third or the fifth day of *embryo growth*. After the embryo transfer was completed, every catheter was checked for retention of embryos. The tip of the catheter was immersed in an ASAN transport medium (ASAN Pharm. Co. LTD., South Korea). Maintaining the optimal temperature as recommended by the manufacturer, the media were transported to the laboratory for sample analysis.

QIAamp DNA Mini Kit was used for DNA isolation (Qiagen, Hilden, Germany). DNA isolation was performed manually as recommended by the manufacturer. Real-Time PCR Kit for quantitative detection of LS and GV (Sacace Biotechnologies S.r.l., Como, Italy) was used for the detection of LS, and it was done on the apparatus (Applied Biosystem 7500, Foster City, California, USA). Forty cycles of amplification were done and the quantification cycle (Cq) of RT PCR (RT-PCR) was determined. Cq RT-PCR are inversely proportional to the amount of target nucleic acid that is in the sample. Lower Cq RT-PCR indicate high amounts of the target sequence and higher Cq RT-PCR mean lower amounts of target nucleic acid. The target region was 16S rRNA.

Statistical analysis

For statistical analysis, Mann-Whitney U, Pearson correlation coefficient, and regression analysis were used. Significance was set at $p < 0.05$.

RESULTS

Embryo transfer of fresh embryos was performed in 57 patients (Table 1); clinical pregnancy was confirmed among the 17 (29.8%) women.

The tips for embryo transfer catheters were positive on LS in 55 (96.4%) patients (Table 2). The median of CqRT-PCR for LS was 33 and 31 for the younger and

Table 1. Demographic and clinical characteristics of patients

Variable	Total	<35 years	≥ 35 years
No (%) of patients (years)	57	30 (52.6)	27 (47.4)
Arithmetic mean ±SD (min.-max.)			
Age (years)	34.5±5.9 (19-45)	29.8±3.1 (19-34)	39.7±3.0 (36-45)
BMI (kg/m ²)	24.4±4.1 (16.9-41.1)	24.6±3.1 (19.4-34.4)	24.29±5.0 (16.9±41.1)
No (%) of patients			
BMI (kg/m²)			
≤18.5	1 (1.8)	0	1 (3.7)
18.5-24.9	38 (66.7)	20 (66.7)	18 (66.7)
25.0-29.9	13 (22.8)	8 (26.7)	5 (18.5)
≥30.0	5 (8.8)	2 (6.7)	3 (11.1)
Previous birth			
No	53 (93)	29 (96.7)	24 (88.9)
Yes	4 (7)	1 (3.3)	3 (11.1)
Cause of infertility			
Tubal factor	5 (8.8)	3 (10)	2 (7.4)
PCOS	9 (15.8)	6 (20)	3 (11.1)
Male factor	20 (35.1)	11 (36.7)	9 (33.3)
Other cause	23 (40.4)	10 (33.3)	13 (48.1)
Day of embryo transfer			
Day 3	34 (59.6)	13 (43.3)	21 (77.8)
Day 5	23 (40.4)	17 (56.7)	6 (22.2)
Number of transferred embryos			
1	28 (49.1)	21 (30)	7 (25.9)
2	29 (50.9)	9 (70)	20 (25.9)

SD, standard deviation PCOS, polycystic ovary syndrome

older age groups, respectively. The quantity of LS increased with age, and the amount of LS was significantly higher in the older group ($p=0.048$) (Table 2).

Twenty-one (36.8%) patients tested positive for GV. The median of Cq RT-PCR for GV was 33 and 34 for the younger and older groups, respectively ($p=0.275$) (Table 2).

One patient had a BMI <18.5 kg/m² and she was excluded from statistical analysis. There were 36 patients with normal and 18 with increased BMI. There was no statistical difference in LS quantity and BMI ($p=0.593$).

The LS quantity showed no statistically significant difference by age ($p=0.135$) and BMI ($p=0.405$).

DISCUSSION

Initial scientific reports about the endometrial cavity as a sterile anatomical niche have been abandoned for a long time, and it has been demonstrated that the endometrial microbiome is a unique ecosystem composed of different bacteria (2,3). The LS are considered the most abundant bacteria in the endometrial cavity (3,5). A few studies explored the mechanism wherein LS could influence endometrial receptivity (22,23).

Aim of this study was to investigate an influence of age and BMI on the amount of LS and GV in the endometrium in fresh IVF cycles at the time of embryo transfer.

Table 2. Amount of *Lactobacillus* spp. and *Gardnerella vaginalis* in the endometrial cavity in fresh *in vitro* fertilization (IVF) cycles

Bacteria (No of positive samples)	Cq RT PCR in age groups		P
	<35 years	≥ 35 years	
	Median (min-max) (No of patients)	Median (min-max) (No of patients)	
<i>Lactobacillus</i> spp. (55/57)	33 (30-37) (28)	31 (28-36) (27)	0.048
<i>Gardnerella vaginalis</i> (21/57)	33 (32-38) (10)	34 (31-36) (11)	0.275

Cq RT PCR quantification cycle in real-time polymerase chain reaction

An endometrium is primarily colonized with LS, then with *Gardnerella* spp., *Bifidobacterium* spp., *Streptococcus* spp. and *Prevotella* spp. (24). In 96.7% of our patients LS was found in the endometrium, which correlates with the results of other research (92%) (24). Some authors reported LS were presented in more than 80% of patients (2).

An important factor for the success of the IVF procedure is maternal age (25). The percentage of women deferring childbearing until their late 30s and early 40s has greatly increased globally. Reportedly, older age was accompanied by a decrease in *Lactobacillus*-dominant microbiota (20). Surprisingly, in our study, using the transfer of fresh embryos, the amount of LS in the endometrium increased in older age. The abundance of the LS in the vaginal microbiota depends on a hormone level (26). Thus, an increase of estrogen level in the pregnancy affects the amount of the LS (27,28); supraphysiological serum levels of estradiol during controlled ovarian stimulation could affect different outcome. An increased abundance of lactate-producing *Lactobacillus* can create an acidic environment that can affect implantation (22). Historically, lactate has been perceived as an end point of glucose metabolism, but today lactic acid is considered as a new signalling molecule in early pregnancy that could play a role in trophoblast invasion and angiogenesis (23). Lactate may be considered as a component of a feedback loop where blastocyst-derived lactate promotes transcription of vascular endothelial growth factor in the surrounding tissue, which in turn has a direct effect on both uterine remodelling, immune regulation, and survival and blastocyst function (23). In summary, a lower pH number could interfere blastocyst to produce lactate, which could cause implantation failure. Endometrial polyps reduce pH in the endometrial cavity which could be one of the non-mechanical causes of subfertility (29).

The increase in the abundance of LS may represent a physiological phenomenon that positively modulates endometrial receptivity, which is certainly reduced due to older age. *Lactobacillus* domination in endometrial cavity has been linked with better implantation and pregnancy rate (24). The other study found no benefit from *Lactobacillus* dominance in the endometrial cavity (30). Previous studies did not investigate the number of bacteria; we used RT PCR to measure the quantity of bacteria. The composition and function of the endometrial microbiome will be a research subject in the next period, and further research is needed to resolve these scientific questions.

GV was the second most common bacteria in the endometrial cavity (5). *Gardnerella vaginalis* was present in patients with reproductive failure (31). Every third patient in our study was positive for GV in the endometrium; the amount of GV was higher in the younger age group (no statistically significant).

Some studies found that obese women are more likely to have adverse vaginal microbiomes; less likely to have *Lactobacillus*-dominance, and more likely to have microbiome diversity and bacterial vaginosis (32). Observing the two BMI groups in our study, a significant correlation with LS quantity was not found.

The limitation of this study is the sample size, especially in the patients with positive GV. A larger sample should be tested to determine the stability of GV in the endometrial cavity.

The advantage of this research is that we used the quantity value of LS and GV in the endometrial cavity.

In conclusion, every IVF procedure aims to make a live-birth baby. The mother's age is a proven factor affecting the IVF outcome, and younger mothers have better outcomes. The microbiome's composition, bacterial diversity, and the number of some bacteria could modify the success of the IVF procedure. We concluded that the *Lactobacillus* number in the endometrium shows dynamics over time, and that older patients have a significantly higher number of LS in the endometrium. Considering the benefits provided by LS in the vagina and gut, we think that an increase of LS abundance in older age could represent a positive compensatory mechanism for better reproductive outcomes despite reduced receptivity, probably through lactic acid production, and this could also be the reason for decreasing the number of *Gardnerella vaginalis* in older patients.

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TRANSPARENCY DECLARATION

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