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RESEARCH ARTICLE

Derangements of vaginal and cervical canal microbiota determined with real-time PCR in women with recurrent miscarriages

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ABSTRACT

Balanced vaginal microbiota and, as a continuum, cervical canal microbiota help prevent reproductive disorders, including recurrent miscarriage (RM). In a significant proportion of couples with RM, routine diagnostic workup cannot find any manageable cause, leading to a requirement for new diagnostic tools. In the present study, we determined the quantitative composition of the microbiota of the vagina and cervical canal, assessed by real-time polymerase chain reaction, in women with RM. It also evaluated their derangements related to the pathogenesis of RM, and thus the suitability of this test as a diagnostic tool for managing RM. Vaginal and cervical canal specimens of 25 women with RM and 25 healthy volunteers were collected. The test results revealed information about the total vaginal bacterial biomass by measuring the abundance of *Lactobacillus* spp.; other bacteria; and pathogens, including *Mycoplasma hominis*, *Mycoplasma genitalium*, *Ureaplasma (urealyticum + parvum)*, and *Candida* spp. Overall, the findings of this study implied the abundance of *Lactobacillus* spp. decreased in women with RM with an increase in the abundance of other microorganisms in accordance with the reduction in the abundance of *Lactobacillus* spp. due to aerobic vaginitis and bacterial vaginosis. Vaginal and cervical canal microbiota need to be considered during the diagnostic workup of women with RM.

IMPACT STATEMENT

- **What is already known on this subject?** Recurrent miscarriage (RM) is a well-known reproductive disorder. Its diagnostic workup is not successful in determining the underlying problem in many cases. Hence, novel diagnostic tools based on real-time polymerase chain reaction (PCR) are needed for evaluating reproductive microbiota, which are considerably reliable, to satisfy the expectations of women with RM.
- **What do the results of this study add?** Overall, the decrease in the abundance of *Lactobacillus* spp. was found to be related to RM, and the patterns of the presence of other microorganisms were in accordance with the reduction in the abundance of *Lactobacillus* spp. These findings suggested an important role of vaginal and cervical canal microbiota in the pathogenesis of RM.
- **What are the implications of these findings for clinical practice and/or further research?** Additional research is warranted to elucidate the functional impact of altered components of the microbiota of vaginal and cervical canals on the physiology of the local cervical canal and its participation in the microbiota of the endometrial cavity, especially regarding unsuccessful pregnancies as a result of the disturbed physiology of the local endometrial microenvironment. However, possible applications of real-time PCR-based tests for the screening of subclinical infections in clinical practice require the performance of further investigations in patients with RM.

KEYWORDS

Cervical canal microbiota; real-time PCR; recurrent miscarriage; vaginal microbiota

Introduction

Recurrent miscarriage (RM) is defined as the loss of three or more consecutive pregnancies; it develops in about 1–3% of couples trying to conceive (Green and O'Donoghue 2019; Woolner et al. 2020). Several well-defined factors, such as chromosomal abnormalities, uterine anatomic abnormalities, and antiphospholipid antibody syndrome, required consideration during the diagnostic workup of RM. However, the aetiology of RM remains elusive in about half of the cases, increasing depression and anxiety and lowering self-esteem (Di Prima et al. 2011; Alijotas-Reig and Garrido-Gimenez 2013). Therefore, RM is one of the most frustrating conditions, leaving a woman with RM with a burden of uncertainty requiring counselling with empathy and understanding. Unfortunately, several women cannot be satisfied with an explanation about the underlying factors responsible for their suffering (Morley et al. 2013; Tulandi and Al-Fozan 2014; Hong Li and Marren 2018; Ticconi et al. 2019; Papas and Kutteh 2020).

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Considerable debates exist on the diagnostic workup of women with RM. One of the cornerstones of the current drive towards developing new diagnostic possibilities is deciphering the effect of reproductive tract microbiota on reproductive functions. Although vaginal microbiota has been described previously, we have more detailed information on microbiota with the technological improvements in molecular biology. Since 2002, many studies have shown that the dominant microorganism of vaginal microbiota is *Lactobacillus* spp. (Reid and Burton 2002; Pavlova et al. 2002). The health of the vaginal ecosystem is mostly affected by the composition and mass of vaginal microbiota that fluctuates from time to time but still maintains its balance (Al-Nasiry et al. 2020). Vaginal lactobacilli suppress the growth and attachment of pathogens via several mechanisms, including the production of hydrogen peroxide, lactic acid, and bacteriocins, competitively reducing adhesion to the vaginal epithelium, and enhancing capabilities of the immune system (Amabebe and Anumba 2018). A healthy vaginal microbial balance is one of the key factors for developing conception and progress of pregnancy (Green and O'Donoghue 2019; Vieco-Saiz et al. 2019). Numerous studies have established that vaginal colonisation and infections have a direct impact on female reproductive function (Schoenmakers and Laven 2020). This may also be effective during pregnancy loss in women with RM. The findings of pertinent literature support that patients frequently encounter RM compared with controls when the abundance of *Mycoplasma genitalium*, aerobic lactobacilli, *Staphylococcus epidermidis*, *enterococci*, *Escherichia coli*, and *Bacteroides* species increase within vaginal microbiota (Santiago et al. 2011; Churchill et al. 2018; Kalia et al. 2020). Histological tests performed on women with RM reveal a high prevalence of infections, such as endometritis, diagnosed mainly by the presence of inflammation or plasma cells in the endometrium, although these findings have not represented adequate specificity. According to microbiological studies in fertile women, lactobacilli are dominant (>90%) in the normal uterine microbiota. The loss of this dominance may reduce the implantation rate and increase the chance of RM (Churchill et al. 2018). The screening of disadvantageous vaginal and cervical microbiota that may exert a significant influence on reproductive outcomes in patients with RM represents a challenge for physicians. The analysis of these microbiota has the potential to identify patients with RM who can benefit from the restoration of normal vaginal and cervical microbiota. Using molecular genetic methods for detecting disorders of vaginal microbiota has some crucial advantages, including its high sensitivity and capability of detecting multiple microorganisms. Aerobic vaginitis (AV) can be diagnosed with the decreased abundance of *Lactobacillus* spp. and increased abundance of facultative anaerobic microorganisms, such as members of Enterobacteriaceae, *Streptococcus* spp., and *Staphylococcus* spp. (Kaambo et al. 2018). Bacterial vaginosis (BV) is also characterised by the decreased abundance of *Lactobacillus* spp. and is also related to the increase in the abundance of several obligate anaerobic microorganisms, such as *Gardnerella vaginalis/Prevotella bivia/Porphyromonas* spp.; *Eubacterium* spp.; *Sneathia* spp./*Leptotrichia* spp./*Fusobacterium* spp.; *Megasphaera* spp./*Veillonella* spp./*Dialister* spp.; *Lachnobacterium* spp./*Clostridium* spp.; *Mobiluncus* spp./*Corynebacterium* spp.; *Peptostreptococcus* spp.; and *Atopobium vaginae* (Kaambo et al. 2018). This study aimed to evaluate the quantitative composition of the microbiota of the vagina and cervical canal, assessed by real-time PCR, in women with RM, to determine its relevance to the pathogenesis of RM, and thus its suitability as a diagnostic tool for managing RM.

Materials and methods

This was a case–control study in the outpatient gynaecology service of Samsun Training and Research Hospital in Samsun, Turkey, performed between July and December 2019. A total of 25 nonpregnant women aged 22–46 years with a confirmed diagnosis of unexplained RM (defined as the loss of ≥3 consecutive pregnancies before 20 weeks of gestation) and age-matched 25 healthy nonpregnant women aged 24–41 years with no history of miscarriage and autoimmune disease as controls were included in the study. All the control women had at least one previous successful pregnancy.

The sample size of the study groups was calculated with a calculated sample size for two proportions. For a comparison requiring sample size; 40% was considered as a relevant disease effect of RM for the proportion of decreased *Lactobacillus* spp. in vaginal/cervical microbiota, assuming a power of 0.80 and an alpha error of 0.05. The required sample size was 23, and our final sample size was 25 per group with a drop-out rate of 10%. Women were excluded from the study to enhance the expected significances, if they had used douches, vaginal medications or suppositories, feminine sprays, genital wipes, or contraceptive spermicides in the last month; if they were under treatment with systemic drugs, such as corticosteroids, antibiotics, and probiotics; if they were immunocompromised; if they were applied any intrauterine device within last 3 months; if they had an endometrial cavity–related lesions, a previous history of thrombosis, autoimmune or endocrine diseases, systemic infection, recent major surgery or trauma, alcohol consumption, and cigarette smoking, BMI more than 40, or diabetes mellitus; or if they had sexual activity for 3 days before microbiota sampling. The study participants had to be free of subjective vaginal complaints, including vaginal discharge and vulvar itching, vaginal bleeding, and clinical signs of vaginal infection, and they had to have a microscopically normal vaginal flora with direct microscopy.

Patients with RM were enrolled in the study at least 3 months later after their last miscarriage experience. For all study participants, the medical history and clinical and laboratory variables were recorded. Using a vaginal examination, we assessed vaginal secretions clinically according to the following specifications: quantity, consistency, colour, and odour to exclude patients with overt vaginal infections. During a vaginal examination from the back vaginal vault, a specimen of vaginal secretion was collected from participants with a dry sterile swab for microbiological evaluation after excess cervical mucus was removed with a cotton swab in the middle of the second half of the natural menstrual cycle

in both case groups. Later, the cervix was washed with a sterile sodium chloride solution, and a probe was inserted into the cervical canal to the depth of up to 1.5 cm; the probe was retrieved carefully to avoid contact with the vaginal walls. The participants were previously asked for abstinence from sex for at least 3 days before sample collection. All samples were analysed using a Femoflor 16 Real-Time PCR Detection Kit (DNA-Technology Research & Production, LLC, Moscow, Russia) to assess the state of vaginal and cervical microbiota. The vaginal and cervical specimens were then transferred to the tubes containing a 'DNA-Technology' Prep-Rapid DNA Extraction Kit (P-001/1EU) solution. Originally the system Femoflor 16 was developed for estimating the condition of the vagina; in the present study, it was also used for assessing the microbiota of the cervical canal. The specifications of Femoflor 16 real-time PCR test according to the producer were as the follows: it quantitatively assessed the total bacterial mass of vaginal microbiota including details about *Lactobacillus* spp.; *Enterobacterium* spp.; *Streptococcus* spp.; *Staphylococcus* spp.; *Gardnerella vaginalis/Prevotella bivia/Porphyromonas* spp.; *Eubacterium* spp.; *Sneathia* spp./*Leptotrichia* spp./*Fusobacterium* spp.; *Megasphaera* spp./*Veillonella* spp./*Dialister* spp.; *Lachnobacterium* spp./*Clostridium* spp.; *Mobiluncus* spp./*Corynebacterium* spp.; *Peptostreptococcus* spp.; *Atopobium vaginae*; *Mycoplasma hominis*; *Mycoplasma genitalium*; *Ureaplasma* (*urealyticum* + *parvum*); and *Candida* spp. The diagnostic sensitivity and specificity of Femoflor for detecting these microorganisms were 97%, according to the documentation provided by the manufacturer (Femoflor 16 Real-time PCR Detection Kit, www.dna-technology.ru) (Vlasova et al. 2016).

Ethics approval and consent to participate

All patients with RM and controls gave written informed consent for the investigation and conditions of enrolment. The study and its consent procedure were approved by the Clinical Research Ethics Committee of the Samsun Training and Research Hospital (Registered as KAEK-2019/2/17). The study was conducted in adherence to the Declaration of Helsinki 2013.

Statistical analysis

The IBM SPSS Statistics for Windows, Version 23.0 (IBM Corp., NY, USA) was used for data analyses. The data were presented as median with the interquartile range or percentage as appropriate. Numeric variables were analysed with the Mann–Whitney test after the Kolmogorov–Smirnoff normality test. The rates of parameters were compared with the chi-square test. A *p*-value $<.05$ was chosen for the acceptance of significances.

Results

The study was completed with 25 women with RM and 25 healthy women with normal pregnancy outcomes. Table 1 presents the age and rates of gravidity, parity, live birth, and miscarriage of the participants. No significant difference was found between healthy women and women with RM in terms of the median age [32 (25.7–37.0) and 35 (29.7–38.2), respectively; $p > .05$)]. The gravidity and miscarriage numbers of women with RM were significantly higher than those of healthy women with normal pregnancy, but their parity and live birth numbers were significantly lower than those of the controls ($p < .05$). Figure 1 presents the state of *Lactobacillus* spp. in vaginal and cervical specimens obtained from healthy women and women with RM. The rates of the decreased abundance of *Lactobacillus* spp. in the vaginal and cervical specimens were significantly higher in women with RM compared with healthy women (52 *vs*. 16 and 60 *vs*. 20%, respectively; $p < .05$).

Figure 2 shows the state of *Gardnerella vaginalis/Prevotella bivia/Porphyromonas* spp. in vaginal and cervical specimens

Table 1. Demographic features of patients with recurrent miscarriage and controls.

	RM ($n = 25$)	Controls ($n = 25$)
Age (year)	32 (25.7–37)	35 (29.7–38.2)
Gravidity	4 (3–5)[a]	2 (2–3)
Parity	0 (0–1)[b]	2 (2–3)
Live birth	0 (0–1)[c]	2 (2–3)
Miscarriage	3 (3–4)[d]	0 (0–0)

Data are presented as median (interquartile range).
[a,b,c,d]$p < .05$.

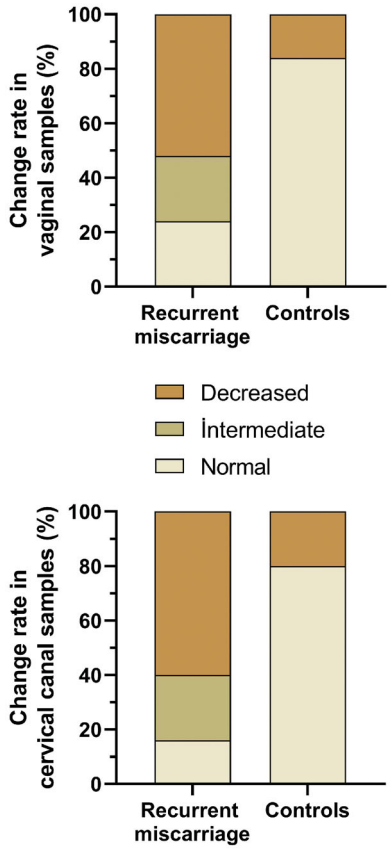

Figure 1. Microbiota including *Lactobacillus* spp. of vaginal and cervical canal specimens obtained from healthy women and women with RM. Normal, intermediate, and decreased prevalence rates *Lactobacillus spp.* are presented as percentages and analysed with the chi-square test. The rates of the decreased abundance of *Lactobacillus* spp. were significantly higher in the vaginal and cervical canal specimens obtained from women with RM ($p < .05$).

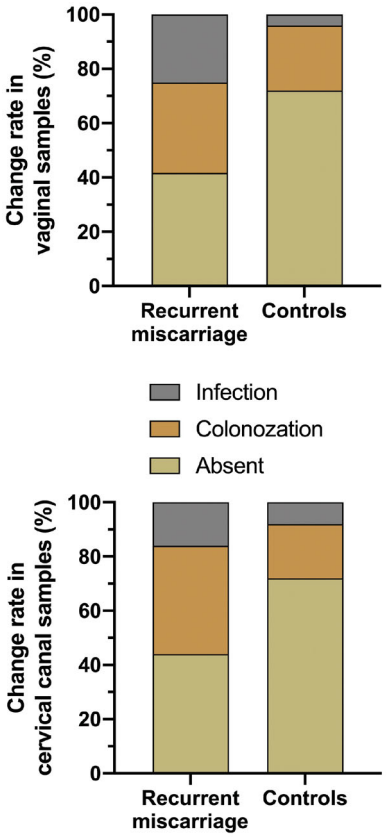

Figure 2. Microbiota including *Gardnerella vaginalis/Prevotella bivia/Porphyromonas* spp. in vaginal and cervical canal specimens obtained from healthy women and women with RM. Rates of absence, colonisation, and infection with *Gardnerella vaginalis/Prevotella bivia/Porphyromonas* spp. are presented as percentages and analysed with the chi-square test. The rate of infection with these microorganisms was significantly higher in the vaginal specimens obtained from patients with RM ($p < .05$).

obtained from healthy women and women with RM. The rate of infection with these microorganisms in the vaginal specimens was significantly higher in RM women than in healthy women (25 *vs.* 4%; $p < .05$). The rate of infection with these microorganisms in the cervical specimens was higher in RM women than in healthy women, but the difference did not reach statistical significance (16 *vs.* 8%; $p > .05$).

Table 2 includes the state of *Enterobacterium* spp., *Streptococcus* spp., *Staphylococcus* spp., *Eubacterium* spp., *Sneathia* spp./Leptotrihia spp./*Fusobacterium* spp., *Megasphaera* spp./Veilonella spp./*Dialister* spp., *Lachnobacterium* spp./*Clostridium* spp., *Mobiluncus* spp./*Corynebacterium* spp., *Peptostreptococcus* spp., and *Atopobium vaginae* in vaginal and cervical specimens obtained from healthy women and women with RM. Analyses of data regarding facultative anaerobic microorganisms related to AV revealed the following: (1) The rate of 1–10 and 10–100% total microorganisms detected (TMD) of *Enterobacterium* spp. in the vaginal and cervical specimens were significantly higher in women with RM compared with healthy women (76 *vs.* 28 and 72 *vs.* 28%, respectively; $p < .05$). (2) The rates of 1–10 and 10–100% TMD of *Streptococcus* spp. in the vaginal and cervical specimens obtained from healthy women and women with RM were found to be similar (*Streptococcus* spp.: 4 *vs.* 4 and 4 *vs.* 8%, respectively; $p > .05$). (3) The rates of 1–10 and 10–100% TMD of *Staphylococcus* spp. in the cervical specimens were significantly higher in women with RM compared with healthy women (20 *vs.* 12%; $p < .05$). However, the rates of 1–10 and 10–100% TMD of *Staphylococcus* spp. in the vaginal specimens obtained from healthy women and women with RM were found to be similar (0 *vs.* 0%, respectively; $p > .05$).

Analyses of data regarding obligate anaerobic microorganisms related to BV revealed the following. The rates of 1–10 and 10–100% TMD of *Eubacterium* spp. in the vaginal specimens were significantly higher in women with RM than in healthy women (40 *vs.* 8%; $p < .05$). However, the rates of 1–10 and 10–100% TMD of *Eubacterium* spp. in the cervical specimens obtained from healthy women and women with RM were found to be similar (28 *vs.* 8%, respectively; $p > .05$). The rates of 1–10 and 10–100% TMD of *Megasphaera* spp./Veilonella spp./*Dialister* spp. in the vaginal and cervical specimens were significantly higher in women with RM than in healthy women (16 *vs.* 0 and 12 *vs.* 8%, respectively; $p < .05$). The rates of 1–10 and 10–100% TMD of *Sneathia* spp./Leptotrihia spp./*Fusobacterium* spp., and *Lachnobacterium* spp./*Clostridium* spp. in the vaginal and cervical specimens obtained from healthy women and women with RM were found to be similar (*Sneathia* spp./Leptotrihia spp./*Fusobacterium* spp.: 20 *vs.* 0 and 16 *vs.* 0%, respectively; *Lachnobacterium* spp./*Clostridium* spp.: 8 *vs.* 0 and 0 *vs.* 0%, respectively; $p > .05$). The rates of 1–10 and 10–100% TMD of *Peptostreptococcus* spp. in the vaginal and cervical specimens were significantly higher in women with RM than in healthy women (16 *vs.* 0 and 8 *vs.* 0%, respectively; $p < .05$). The rates of 1–10 and 10–100% TMD of *Mobiluncus* spp./*Corynebacterium* spp. and *Atopobium vaginae* in the vaginal and cervical specimens obtained from healthy women and women with RM were found to be similar (*Mobiluncus* spp./*Corynebacterium* spp.: 8 *vs.* 0 and 0 *vs.* 4%, respectively; *Atopobium vaginae*: 16 *vs.* 4 and 8 *vs.* 0%, respectively; $p > .05$).

Figure 3 shows the state of *Candida* spp., *Mycoplasma hominis*, and *Ureaplasma* (*urealyticum* + *parvum*) in vaginal and cervical canal specimens obtained from healthy women and women with RM. The infection rates of *Candida* spp., *Mycoplasma hominis*, and *Ureaplasma* (*urealyticum* + *parvum*) in the vaginal and cervical specimens obtained from healthy women and women with RM were found to be similar (*Candida* spp.: 16 *vs.* 16 and 20 *vs.* 12%, respectively; *Mycoplasma hominis*: 16 *vs.* 0 and 8 *vs.* 0%, respectively; *Ureaplasma* (*urealyticum* + *parvum*): 28 *vs.* 4 and 12 *vs.* 0%, respectively; $p > .05$).

Mycoplasma genitalium could not be detected in vaginal and cervical canal specimens in both healthy women and women with RM.

Discussion

In this study, we analysed the composition of microbiota of the vagina and cervical canal of healthy women and women with RM. The real-time PCR test, including a panel of microorganisms related to vaginal infections, was used to determine its ability as a diagnostic test within the workup of women

Table 2. Microbiota data including *Enterobacterium* spp., *Streptococcus* spp., *Staphylococcus* spp., *Eubacterium* spp., *Sneathia* spp./*Leptotrihia* spp./*Fusobacterium* spp., *Megasphaera* spp./*Veilonella* spp./*Dialister* spp., *Lachnobacterium* spp./*Clostridium* spp., *Mobiluncus* spp./*Corynebacterium* spp., *Peptostreptococcus* spp., and *Atopobium vaginae* in vaginal and cervical specimens obtained from healthy women and women with RM.

Microorganisms			No detection	0–0.1%	0.1–1%	1–10%	10–100%
Facultative anaerobic microorganisms related to aerobic vaginitis							
Enterobacteriaceae (%)	Vaginal	RM	20	4	0	12	64
		Controls	44	24	4	4	24
	Cervical	RM	20	0	8	0	72
		Controls	52	12	8	4	24
Streptococcus spp. (%)	Vaginal	RM	76	16	4	4	0
		Controls	88	4	4	0	4
	Cervical	RM	84	12	0	4	0
		Controls	84	8	0	0	8
Staphylococcus spp. (%)	Vaginal	RM	68	32	0	0	0
		Controls	88	12	0	0	0
	Cervical	RM	52	24	4	12	8
		Controls	88	0	0	4	8
Obligate anaerobic microorganisms related to the bacterial vaginosis							
Eubacterium spp. (%)	Vaginal	RM	24	24	12	20	20
		Controls	56	24	12	8	0
	Cervical	RM	28	16	28	16	12
		Controls	68	12	12	8	0
Sneathia spp.–*Leptotrichia* spp.–*Fusobacterium* spp. (%)	Vaginal	RM	68	12	0	4	16
		Controls	92	4	4	0	0
	Cervical	RM	72	8	4	8	8
		Controls	96	0	4	0	0
Megasphaera spp.–*Veibnella* spp.–*Dialister* spp. (%)	Vaginal	RM	36	32	16	8	8
		Controls	80	16	4	0	0
	Cervical	RM	36	28	24	8	4
		Controls	80	4	8	8	0
Lachnobacterium spp. + *Clostridium* spp. (%)	Vaginal	RM	60	24	8	4	4
		Controls	80	12	8	0	0
	Cervical	RM	68	24	8	0	0
		Controls	80	20	0	0	0
Mobiluncus spp. + *Corynebacterium* spp. (%)	Vaginal	RM	64	16	12	8	0
		Controls	80	20	0	0	0
	Cervical	RM	72	12	16	0	0
		Controls	88	0	8	4	0
Peptostreptococcus spp. (%)	Vaginal	RM	56	16	12	16	0
		Controls	88	12	0	0	0
	Cervical	RM	68	12	12	8	0
		Controls	100	0	0	0	0
Atopobium vaginae (%)	Vaginal	RM	8	64	12	12	4
		Controls	32	64	0	4	0
	Cervical	RM	16	68	8	4	4
		Controls	32	64	4	0	0

Rates of no detection and 0–0.1, 0.1–1, 1–10, and 10–100% total microorganisms detected (TMD) with studied microorganisms are presented and analysed with the chi-square test. RM, Recurrent miscarriage. For *Enterobacterium* spp., the rates of 1–10 and 10–100% TMD were significantly higher in the vaginal and cervical specimens obtained from women with RM ($p < .05$). For *Staphylococcus* spp., the rates of 1–10 and 10–100% TMD were significantly higher in the cervical specimens obtained from women with RM ($p < .05$). For *Megasphaera* spp./*Veilonella* spp./*Dialister* spp., the rates of 1–10 and 10–100% TMD were significantly higher in the vaginal and cervical specimens obtained from women with RM ($p < .05$). For *Peptostreptococcus* spp., the rates of 1–10 and 10–100% TMD were significantly higher in the vaginal and cervical specimens obtained from women with RM ($p < .05$).

with RM. The study groups were found as comparable regarding their age; however, women with RM had higher gravidity and number of miscarriages, but lower parity and number of live births. Women with RM had a lower abundance of *Lactobacillus* spp. in the vagina and cervical canal compared with healthy women. This could be interpreted as related to the increased rate of AV or BV in women with RM. Analyses of data regarding facultative anaerobic microorganisms related to AV revealed that the abundance of members of Enterobacteriaceae and *Staphylococcus* spp., but not *Streptococcus* spp., increased in women with RM. Analyses of data regarding obligate anaerobic microorganisms related to BV showed that the abundance of *Gardnerella vaginalis*/*Prevotella bivia*/*Porphyromonas* spp., *Eubacterium* spp., *Megasphaera* spp.–Veibnella spp.–*Dialister* spp., and *Peptostreptococcus* spp., but not of other studied microorganisms, increased in women with RM. Overall, the rates of colonisation and infection with *Candida* spp., *Mycoplasma hominis*, and *Ureaplasma* (*urealyticum* + *parvum*) in vaginal and cervical specimens were found to be similar in healthy women and women with RM.

When the culture-negative microorganisms are the focus for diagnostic purposes, molecular diagnostic tests, such as PCR-based tests, are more cost-effective compared with culture-based methods. The number of PCR-based tests for microbiota has increased recently. Accordingly, molecular methods provide a useful perspective on microbial community composition and function, but they are not a substitute for cultivation-based studies when cultural methods are more feasible and cheaper.

The evidence of the microorganisms within the test panel, used in our study, except *Mycoplasma genitalium* was observed in vaginal and cervical canal specimens from both healthy women and women with RM. Overall, the decrease in

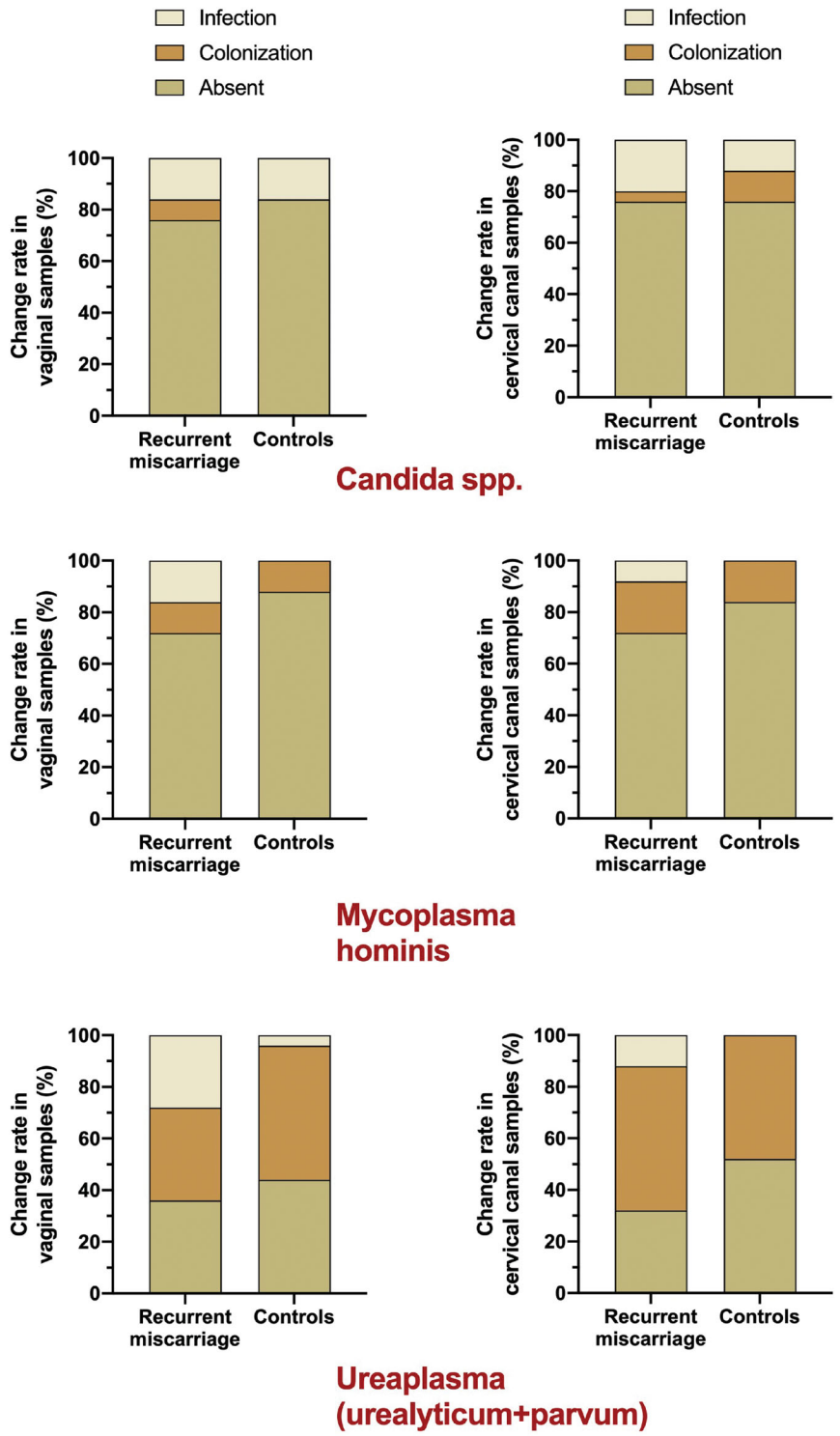

Figure 3. Microbiota data including *Candida* spp., *Mycoplasma hominis*, and *Ureaplasma* (*urealyticum* + *parvum*) in vaginal and cervical specimens obtained from healthy women and women with RM. Rates of absence, colonisation, and infection with these microorganisms are presented and analysed with the chi-square test. Regarding *Candida* spp., *Mycoplasma hominis*, and *Ureaplasma* (*urealyticum* + *parvum*), these rates in vaginal and cervical specimens obtained from the study groups were comparable ($p > .05$).

the abundance of *Lactobacillus* spp. was found to be related to RM, and this decrease affected the presence of other microorganisms accordingly. These findings implied an important role of vaginal and cervical canal microbiota in the pathogenesis of RM.

The current researches on the microbiome aimed to fill in the missing details in the pathophysiology of reproductive disorders; the composition of vaginal, cervical, and uterine microbiota might play important roles during the course of these disorders. The amount of bacterial biomass gradually decreased from vaginal to endometrial samples (Moreno et al. 2016; Chen et al. 2017; Li et al. 2018; Bardos et al. 2019). Several factors, such as hormonal cyclic changes that occur during women's reproductive period, lactation,

diabetes mellitus, stress, sexual activity, vaginal douching, and use of oral contraceptives and other hormonal drugs, can alter the dynamic composition of vaginal and cervical canal microbiota. Vaginal microbiota may also be affected by adverse conditions, such as non-specific inflammatory processes related to the growth of opportunistic and pathogenic microorganisms (Lamont 2015; Curty et al. 2019). *Lactobacillus*-dominated microbiota in the vagina can be one of the reliable markers of the healthy female reproductive tract. After puberty, important changes occur in the vaginal epithelium, including maturation, proliferation, and accumulation of glycogen under the influence of increased oestrogen. Glycogen is catabolized to smaller polymers in the vaginal epithelium; then, vaginal *Lactobacillus* species further metabolise these polymers to D-lactic acid that protects against infections (Amabebe and Anumba 2018). *Lactobacillus* species also produce antimicrobial peptides (bacteriocins), which inhibit the growth and establishment of pathogenic microorganisms (Vieco-Saiz et al. 2019). Besides lactic acid from lactobacilli in the vaginal microenvironment, other products, such as hydrogen peroxide, inhibit the growth of potential pathogens (Kaur et al. 2020).

Vaginal microbiota can show a wide range of gynaecologic infectious diseases, such as BV, which can lead to the acquisition and transmission of sexually transmitted infections, preterm birth, and pelvic inflammatory disease. However, many asymptomatic healthy women also have a rich diversity in their vaginal microbiota. More detailed management strategies are needed to improve the detection of women requiring drug use, promote health, and prevent and optimally treat diseases in women with the disturbed microbiota of the reproductive tract (Lewis et al. 2017; Coleman and Gaydos 2018). The vaginal microbiota is one of the fundamental influencers of women's sexual and reproductive health. Altered microbial composition of the vaginal microbiota, particularly with a significant decrease in the abundance of *Lactobacillus* species, might be implicated in several gynaecologic and obstetric disorders. Despite the dominance of *Lactobacillus* in most vaginal microbiotas from puberty to menopause, the composition of any given vaginal microbiota is highly individualised (Song et al. 2020). Overall, the vaginal microbiota of most women remains relatively stable throughout the menstrual cycle, with slight variations in diversity and only modest fluctuations in species richness. Vaginal microbiota exhibits more diversity from one woman to another woman compared with fluctuations in one woman (Chaban et al. 2014). Apart from host genetics and infections, changes in diet, lifestyle, and hygiene status can influence this delicate state of equilibrium. More importantly, the gonadal hormones strongly influence the overall composition and function of vaginal microbiota (Kaur et al. 2020).

A healthy vaginal microbiota is considered to be essential for preventing genital infections due to its function as an important first line of defense of the body. Any deviation in this microbial composition can result in several genital conditions, including BV and AV. BV is the most frequent cause of vaginal discharge in women of reproductive age, defined as a rapid shift in the composition of the vaginal microbiota from a *Lactobacillus*-dominated to a polymicrobial microbiota, compromising a mixture of diverse anaerobes; however, the condition remains asymptomatic, at least, in the half of the cases (Coleman and Gaydos 2018; McKinnon et al. 2019). Despite the administration of several drugs against BV, more than 50% of women experience recurrent episodes of BV. In addition, more than 85% of BV cases are asymptomatic despite significantly elevated genital inflammation (Happel et al. 2020). However, a vast body of evidence shows that the pathogenic effects of BV are not confined to the lower genital tract. BV is strongly associated with reproductive failure, notably late foetal loss and preterm birth (Leitich and Kaider 2003; Leitich and Kiss 2007). In accordance with the results of published studies, our study showed that the rate of microbiota disruptions supporting AV and BV was higher in women with RM compared with normal women.

Since its first introduction in 2002, AV has gained considerable attention because of prominent inflammatory changes and the requirement of different clinical management. Similar to BV, AV has also decreased abundance of *Lactobacillus* spp. and colonisation of mainly aerobic enteric commensals or pathogens, including Group B *Streptococcus* (*S. agalactiae*), *Enterococcus faecalis*, *Escherichia coli*, and *S. aureus* (Kaambo et al. 2018).

With the help of wet mount microscopy, the decrease in the abundance of *Lactobacillus* spp. can be used to diagnose both BV and AV. When direct microscopy reveals cocci or coarse bacilli, parabasal epithelial cells, and leukocytes, AV is diagnosed. The diagnosis of AV is also possible with the molecular diagnostic methods and microscopic criteria graded on a quantitative scale (Kaambo et al. 2018). The set of microorganisms used in this study, included *Enterobacterium* spp., *Streptococcus* spp., *Staphylococcus* spp., *Eubacterium* spp., *Sneathia* spp./Leptotrihia spp./*Fusobacterium* spp., *Megasphaera* spp./Veilonella spp./*Dialister* spp., *Lachnobacterium* spp./*Clostridium* spp., *Mobiluncus* spp./*Corynebacterium* spp., *Peptostreptococcus* spp., and *Atopobium vaginae* in vaginal and cervical specimens. Among these microorganisms, considerably higher rates of potential agents of AV were noted, including especially members of Enterobacteriaceae, *Eubacterium* spp., *Fusobacterium* spp., and *Peptostreptococcus* spp. in accordance with the literature (Oerlemans et al. 2020). According to the literature, *Prevotella* is presumably an organism, which may be associated with AV more closely than with BV (Oerlemans et al. 2020). In addition, AV is more dangerous in pregnancy and is related to the development of human papilloma virus (HPV) induced cervical cancer (Kaambo and Africa 2017; Curty et al. 2019; Usyk et al. 2020).

Recent findings of the pertinent literature increased the awareness of the role of cervicovaginal microbiota in pregnancy outcomes (Gerson et al. 2020). Our study contributed significantly to the existing literature by showing the link of a nonoptimal vaginal and cervical canal microbiota to RM with the real-time PCR of vaginal and cervical canal specimens. The microbiota of the reproductive tract has been inferred from the vaginal bacterial communities as an essential component of the human microbiota. The cervical canal and endometrial cavity have microbiota with the potential to influence the functions of reproductive organs (Baker et al. 2018; D'Ippolito et al. 2018; Bardos et al. 2019). Despite being

adjacent to the bacterially colonised vagina, the cervical mucous controls uterine microbiota. Some studies found that *Lactobacillus* was more prominent in women with endometrial polyps or chronic endometritis (Fang et al. 2016).

Stout et al. characterised vaginal microorganisms in a large, longitudinal cohort of pregnant women for assessing the characteristics of the microbial community leading to subsequent preterm birth (Stout et al. 2017). They concluded that the vaginal bacterial community revealed the stability of richness and diversity during pregnancy in term-delivered women, but not in preterm-delivered women. They suggested that the microbial changes from the first trimester to the second trimester need to be further studied. In another study, DiGiulio et al. investigated human indigenous microbial communities (microbiota), using a case–control cohort of 40 women, to characterise weekly variations in the vaginal, gut, and oral microbiota during and after pregnancy. They found that microbiota diversity remained relatively stable at each body site during pregnancy. They noted that if the vaginal microbial community was altered, it could lead to preterm birth (DiGiulio et al. 2015).

Chronic cervicitis develops because of the changes in the cervicovaginal bacterial microbiota and adverse conditions to the functioning of the immune system. This may progress to pelvic inflammatory disease and BV, contributing to persistent HPV infection and cervical cancer. Derangements of vaginal microbiota may ease the progress to carcinogenesis by modulating host mechanisms related to immune response alterations and end with DNA damage, or by directly eliciting tissue damage, thus facilitating infection by oncoviruses (Curty et al. 2019; Klein et al. 2019). In a study conducted to evaluate the course of pregnancy and delivery in women with a high risk of chronic infection and examine the effect of the presence of certain microorganisms in the genital microbiota, this condition was associated with pregnancy loss, premature rupture of membranes, and premature birth (Barinov et al. 2020). The presence of chronic inflammation in women of reproductive age generally predisposes to the reduction in non-specific immune defense. Physiological immune adaptations in pregnancy may further contribute to reactivation and long-term persistence of chronic infection, leading to pregnancy-related complications (Racicot and Mor 2017; Yan et al. 2018). Barinov et al. suggested the timely identification of women with infections and/or inflammatory conditions in pregnancy to prevent potential complications associated with microbial persistence (Barinov et al. 2020). Churchill et al. investigated the microbiota in the endometrial fluid collected in the natural cycle of women with RM during the window of implantation. They noted that the endometrial microbiota was highly variable in women with RM, with half of them having an abnormal profile (Churchill et al. 2018). Fang et al. conducted a study to characterise different bacteria populations in the vagina and uterus of women using 16S rRNA sequencing to assess the differences in the overall bacterial community and determine the relationship of vaginal and uterine microbiota with endometrial polyp and chronic endometritis (Fang et al. 2016). They concluded that diverse bacterial species were found in the uterine cavity of both healthy women and patients with an endometrial polyp, indicating that the uterine cavity might have a rich and unique network of microbiota. However, few differences in the composition and diversity of the microorganisms of the endometrial cavity were found between patients with endometrial polyps with and without chronic endometritis. As another area of research gaining popularity to develop new management modalities, chronic endometritis seems to play an important role in the pathogenesis of RM, since it is still poorly considered in most diagnostic workups of RM. Recent studies support the concept that CE needs to be considered a cause of RM. Since adequate management of chronic endometritis in women with RM can lead to an improvement in the rate of successful pregnancy outcomes (Cicinelli et al. 2014), the clear detection of this condition can be beneficial at least in a subset of these patients (Ticconi et al. 2019). Overall, the current literature and the findings of our study revealed derangement occurring in the microbiota of the reproductive system from the vagina to the cervical canal and endometrial cavity and resulting in chronic cervicitis and chronic endometritis, and, finally, RM.

Regarding the limitations of our study, we needed to consider the possibility of contamination during cervical canal sampling, although, before collecting the endocervical specimens, we sterilised the vagina following surgical demands after exposing the cervix using a vaginal speculum and finishing vaginal sampling. Therefore, we needed to keep in mind the possibility of subclinical cervicitis caused by these pathogens, which adversely contributed to the composition of endocervical microbiota. In this study, we found similar detection rates of some microorganisms (*Peptostreptococcus* spp. and *Mycoplasma hominis*) due to the evaluation of the rates of microbiological categories (rates of no detection and 0–0.1, 0.1–1, 1–10, and 10–100% TMD or rates of absence, colonisation, and infection) for individual microorganisms with chi-square tests. Not performing the vaginal culture in the participants was another limitation of our study, although no overt vaginal infection was found in the participants with direct microscopy of vaginal smear.

In conclusion, the findings of this study support a decrease in the abundance of *Lactobacillus* spp. and, in accordance, an increase in the abundance of microorganisms related to AV and BV revealed that, in the diagnostic workup of women with RM, the real-time PCR-based molecular microbiological test can help detect AV and BV requiring treatment to increase successful pregnancy outcomes. Additional research is warranted to elucidate the functional impact of dysbiotic microbiota or specific bacterial species of the cervical canal on the physiology of the local cervical canal and their participation in the microbiota of the endometrial cavity, especially regarding unsuccessful pregnancies because of disturbed physiology of the local endometrial microenvironment. However, possible applications of real-time PCR-based tests for the screening of subclinical infections in clinical practice require the performance of further investigations in patients with RM.

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