ORIGINAL ARTICLE

WILEY Reproductive Medicine and Biology

A pilot study and case reports on endometrial microbiota and pregnancy outcome: An analysis using 16S rRNA gene sequencing among IVF patients, and trial therapeutic intervention for dysbiotic endometrium

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Abstract

Purpose: The present study aimed to analyze the pregnancy outcomes of IVF patients presenting *Lactobacillus*-dominated microbiota (LDM) or non-*Lactobacillus*-dominated microbiota (NLDM) of their endometrium and to report cases who were treated for NLDM concurrently with antibiotics and prebiotic/probiotic supplements in a Japanese infertile population.

Methods: Ninety-two IVF patients were recruited from August 2017 to March 2018. Endometrial fluid samples for sequencing were collected using an IUI catheter. The bacterial status of the endometrium and the pregnancy outcomes were analyzed. For cases with NLDM, antibiotics and prebiotics/probiotics were administered according to their individual microbial conditions.

Results: Forty-seven cases (51.1%) presented LDM and 45 cases (48.9%) presented NLDM at initial analysis. Nine Patients with NLDM were treated by antibiotics and prebiotics/probiotics, and successfully became *Lactobacillus*-dominant. Pregnancy rates by single vitrified-warmed blastocyst transfers were higher in the LDM group (58.9% per patient and 36.3% per FBT) than in the NLDM group (47.2% per patient and 34.7% per FBT) but not significantly different.

Conclusion: The results of this study could not necessarily prove the clear benefit of establishing *Lactobacillus*-dominated endometrium in terms of pregnancy outcome, but there is significance in searching for endometrial microbial status of infertile patients and recovering *Lactobacillus*-dominated endometrium might benefit implantation.

KEYWORDS
dysbiosis, endometrium, infertility, prebiotics, probiotics

Kyono and Hashimoto contributed equally to this study.

1 | INTRODUCTION

The uterine cavity had been considered to be sterile until recent studies using next-generation sequencing of the 16S rRNA gene revealed the existence of an endometrial microbiota represented by *Lactobacillus* and other bacteria.[1-3] *Lactobacillus* species generally dominate the vagina of healthy asymptomatic women, and they presumably play key roles in preventing bacterial vaginosis and other urogenital diseases by lowering the environmental pH through lactic acid production.[4] Also, several reports have suggested that human uterine microbiota is related to implantation success.[5] Moreno et al defined the bacterial status of the endometrium as *Lactobacillus*-dominated microbiota (>90% *Lactobacillus* spp) or non-*Lactobacillus*-dominated microbiota (<90% *Lactobacillus* spp with >10% of other bacteria), based on the composition of the microbiota in the endometrial fluid comprised of 191 operational taxonomic units (OTU). The presence of non-*Lactobacillus*-dominated microbiota (NLDM) was associated with significant decrease in implantation, pregnancy, ongoing pregnancy, and live birth rates.[5]
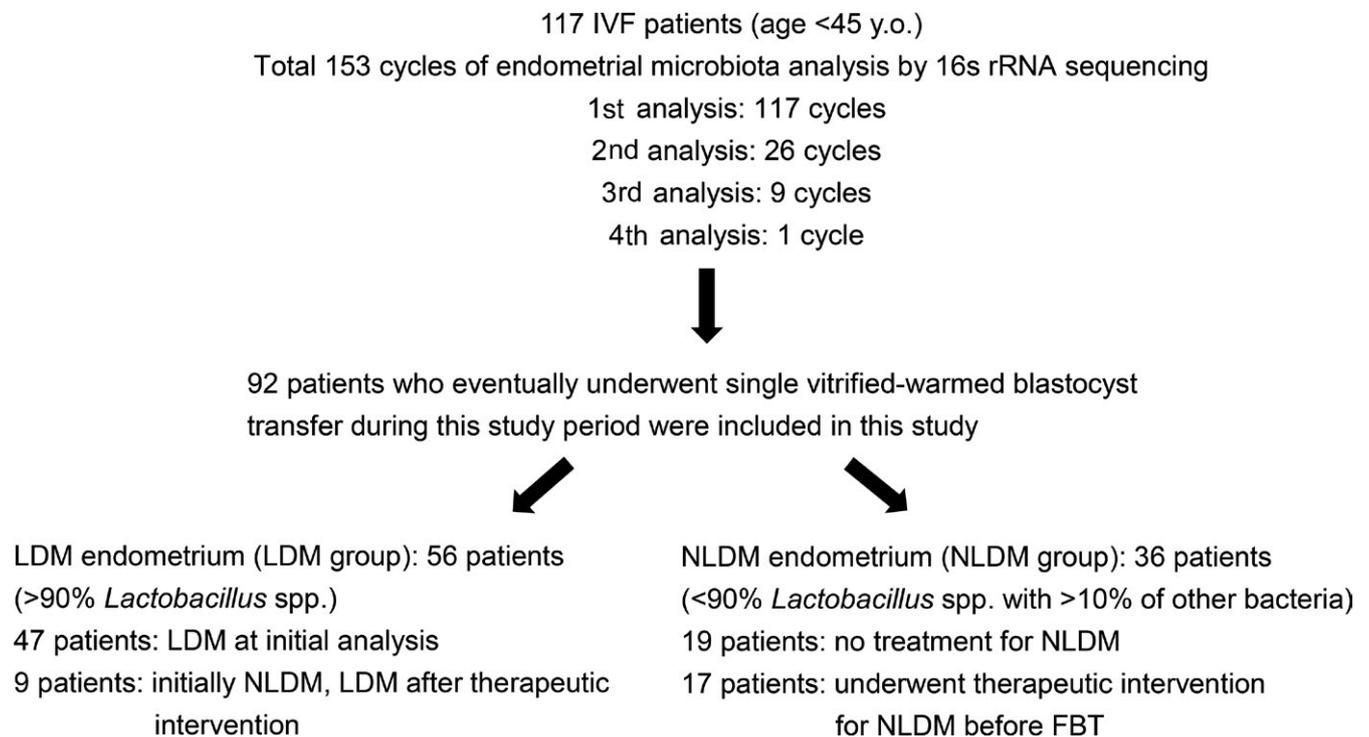
We have started our study on uterine microbiota by sequencing from August 2017 and found that the percentage of endometrial *Lactobacilli* in IVF patients was significantly lower than that of non-IVF patients and healthy volunteers.[6] Also, the median percentages of endometrial *Lactobacilli* in the pregnant cases were more than 90%, which may implicate the possibility that *Lactobacillus* dominancy in the human endometrium might benefit embryo implantation.[6] To what extent the human uterine microbiomes are involved in implantation failure is still not clear, and the treatment strategy for dysbiotic endometrium is still not established. This present study aimed to analyze the pregnancy outcomes of IVF patients presenting *Lactobacillus*-dominated microbiota (LDM) or non-*Lactobacillus*-dominated microbiota (NLDM) of their endometrium and to report cases who were successfully treated for NLDM with antibiotics and prebiotic/probiotic supplements in a Japanese infertile population.

2 | MATERIALS AND METHODS

2.1 | Patients and samples

A total of 117 IVF patients younger than 45 years old agreed to undergo endometrial microbiome analysis in our center from August 2017 to March 2018. Twenty-six patients underwent second analysis, nine patients underwent third analysis, and one patient underwent fourth analysis during the study period; a total of 153 cycles of endometrial microbiome analysis by sequencing were performed (Figure 1). Among the 117 patients, 92 patients who underwent frozen-thawed blastocyst transfer after their endometrial microbiome analysis during the study period were eligible for this study. Last follow-up date was June 30, 2018. All patients were routinely examined by vaginal ultrasound in a sterile condition to confirm the

117 IVF patients (age <45 y.o.)
Total 153 cycles of endometrial microbiota analysis by 16s rRNA sequencing
1st analysis: 117 cycles
2nd analysis: 26 cycles
3rd analysis: 9 cycles
4th analysis: 1 cycle

92 patients who eventually underwent single vitrified-warmed blastocyst transfer during this study period were included in this study

LDM endometrium (LDM group): 56 patients
(>90% *Lactobacillus* spp.)
47 patients: LDM at initial analysis
9 patients: initially NLDM, LDM after therapeutic intervention

NLDM endometrium (NLDM group): 36 patients
(<90% *Lactobacillus* spp. with >10% of other bacteria)
19 patients: no treatment for NLDM
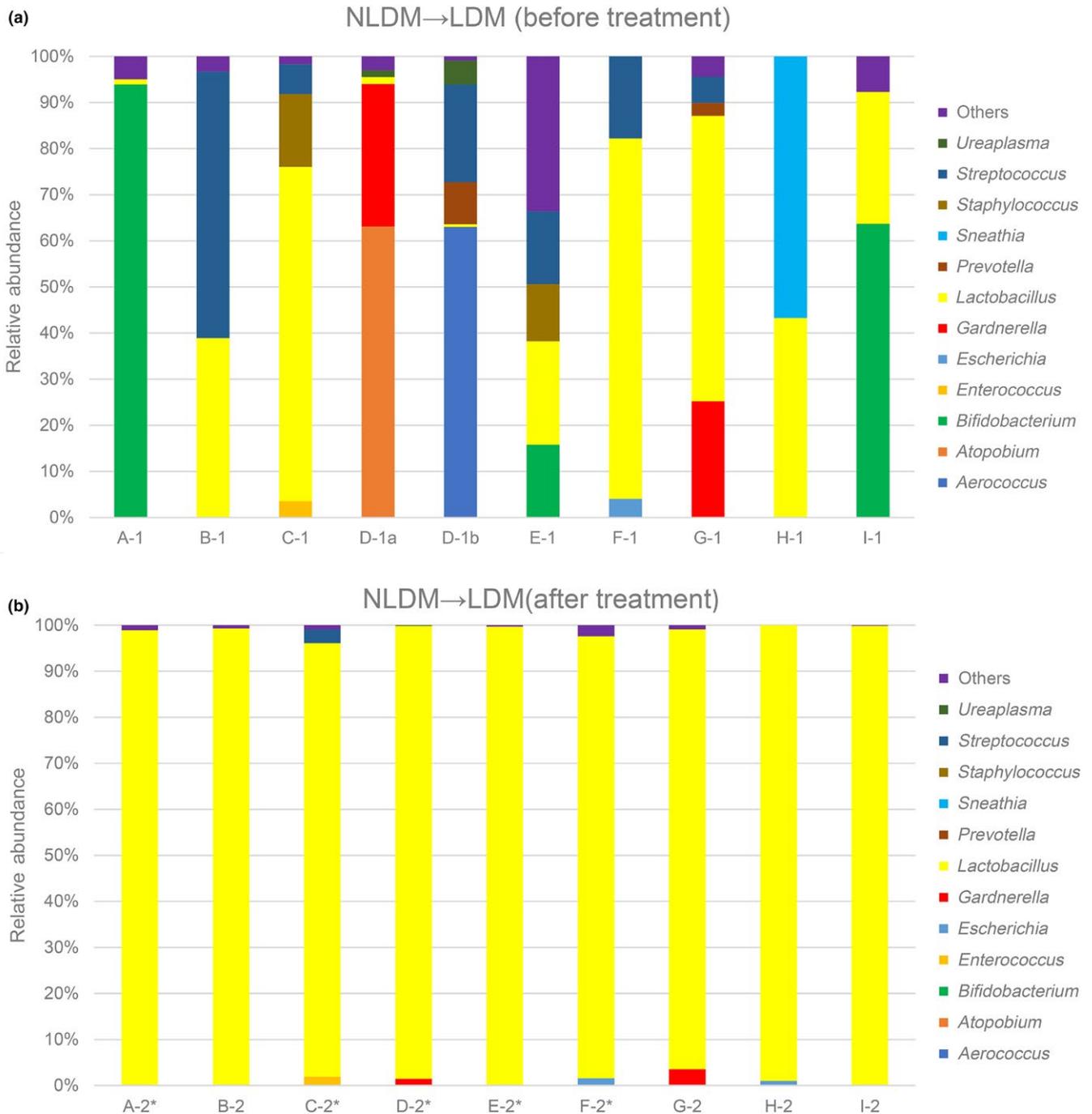17 patients: underwent therapeutic intervention for NLDM before FBT

FIGURE 1 Details of patients included in this study. A total of 117 IVF patients were analyzed from August 2017 to March 2018. Ninety-two patients who eventually underwent frozen-thawed blastocyst transfers were eligible for this study. LDM, *Lactobacillus*-dominated microbiota; NLDM, non-*Lactobacillus*-dominated microbiota; FBT, frozen-thawed blastocyst transfer

menstrual phase of the patient. The participants had no complaints suggestive of vaginitis or endometritis.

This study was approved by the Institutional Review Board of Kyono ART Clinic Takanawa on July 29, 2017. All the patients involved in this study have allowed us to use their medical record data for research in an unidentifiable manner. Written, informed consent was obtained from all patients prior to sample collection.

2.2 | Sample collection and microbiome analysis

Samples from the endometrium were taken from the participants as described previously.[6] Briefly, after cleaning the mucous around the cervical os and the uterine cervix, endometrial fluid (EF) specimens were carefully aspirated with a Kitazato IUI catheter (Kitazato Corporation, Japan) with utmost care not to touch the vaginal wall.

FIGURE 2 A, The endometrial microbiomes of the NLDM patients before treatment. Those patients became LDM after therapeutic interventions. B, The endometrial microbiomes of the LDM patients who presented NLDM before treatment. *Patients achieved pregnancy in the following FBT. The letters below the graph show each participant. The same letter belongs to the same patient

These were put into a 1-mL MMB collection tube (DNA Genotek Inc, Ottawa, ON, Canada) and were sent to Varinos Inc, Tokyo, Japan, for microbiome analysis. The bacterial profiles and percentage of *Lactobacilli* in the endometrium of the patients were provided by the endometrial flora test (Varinos Inc).

The patient profiles, bacterial status, percentage of *Lactobacilli* in endometrium of the patients, and pregnancy outcomes were analyzed. Clinical pregnancy was defined as confirmed gestational sac in the uterine cavity by ultrasound analysis.

2.3 | Trial treatment strategies for dysbiotic endometrium

As there was no standard protocol for the treatment of dysbiotic endometrium, our trial strategy was based on the combination of antibiotics, prebiotics, and/or probiotics (Table S1a,b).

In our study, after the administration of antibiotics for a week, patients were administered oral tablets of enteric bovine LF "Lactoferrin GX®" (NRL Pharma, Kawasaki, Japan) 300 mg/d according to the manufacturer's instructions and was continued consecutively. Lactoferrin (LF) is an iron-binding glycoprotein contained in human external secretions such as breast milk and is one of the prebiotics reported to work for various infectious diseases.[7] Otsuki et al reported that oral/vaginal administration of LF was effective in preventing preterm delivery for patients with a history of multiple miscarriages or early preterm delivery due to refractory bacterial vaginitis, by increasing *Lactobacilli* in their vaginal bacterial flora.[7,8]

As for probiotics, either Florgynal® Tampon Probiotique by Saforelle (Laboratoires IPRAD, Paris, France) or mediGYNE® by Saforelle (Laboratoires IPRAD, Paris, France) was administered according to the manufacturer's instructions; probiotic tampons were used from day 3 of menstruation 3 times/day during the menstrual period (for 3-4 days), and probiotic vaginal suppositories were used (1 capsule/d) after menstruation and were continued for a month.

Because the result of the endometrial microbiome analysis was known 4 weeks after the date of sample collection, there was a time lag of about a month between the date of analysis and the date of treatment started.

2.4 | Statistical analysis

Statistical analysis (using StatMate V software; Tokyo, Japan) was performed by using t test, Mann-Whitney U test, chi-square analysis, or Fisher's extract test where appropriate. A P value of <0.05 was considered statistically significant.

3 | RESULTS

3.1 | Patient profiles

The basal characteristics of the 92 IVF patients were analyzed based on the time of initial analysis. The average age of the 92 IVF cases was 36.97 ± 4.11 years old (26-44); BMI was 20.36 ± 2.68; 58 cases (63.0%) were multigravida; and 25 cases (27.2%) were multipara. All cases were Asian (90 Japanese, one Korean, and one Chinese). The past histories of failed embryo transfers were 2.47 ± 2.58 cycles.

Using the definition by Moreno et al,[5] 47 cases (51.1%) presented LDM (>90% *Lactobacillus* spp) and 45 cases (48.9%) presented NLDM (<90% *Lactobacillus* spp with >10% of other bacteria) at initial analysis (Figure 1). Nine patients had treatment of NLDM; they initially showed NLDM in their first endometrial microbial analysis but have come to show LDM after the therapeutic interventions against NLDM (Figure 2A,B). Six patients showed LDM in their second analysis, and three patients finally became

TABLE 1 Characteristics and pregnancy outcome of two groups (LDM at first analysis vs NLDM→LDM)

	LDM at first analysis	NLDM→LDM	P value
No. of patients	47	9	—
No. of FBT cycles	76	15	—
Age (y): mean ± SD	36.45 ± 3.87	35.11 ± 3.55	NS
BMI: mean ± SD	20.22 ± 2.45	19.12 ± 1.22	NS
Duration of infertility (mo): mean ± SD	21.51 ± 22.53	19.56 ± 19.57	NS
Previous ET: mean ± SD	2.68 ± 2.74	4.56 ± 3.75	NS
Multigravida patients: N (%)	28 (59.6)	4 (44.4)	NS
Multipara patients: N (%)	9 (19.1)	2 (22.2)	NS
Follow-up period[a] (mo): mean ± SD	6.74 ± 1.75	5.89 ± 0.93	NS
% of endometrial LB: median ± SD	99.00 ± 1.81	98.9 ± 2.09	NS
Pregnancy rate per FBT: N (%)	28 (36.8)	5 (33.3)	NS
Pregnancy rate per patient: N (%)	28 (59.6)	5 (55.6)	NS
Miscarriage rate: N (%)	6 (21.4)	2 (40.0)	NS

ET, embryo transfer; FBT, frozen-thawed blastocyst transfer; LB, *Lactobacillus*; LDM, *Lactobacillus*-dominated microbiota; NLDM, non-*Lactobacillus*-dominated microbiota.
[a]Until 2018.6.30.

Lactobacilli-dominant in their third analysis. Frozen-thawed blastocyst transfer (FBT) was performed after confirming *Lactobacilli*-dominant endometrial status for those nine patients (Figure 2A,B, Table 1). For those nine patients, the latest results of the endometrial microbiome analysis were used for analyzing the percentage of endometrial *Lactobacilli*. There was no difference in the background between the cases with LDM at initial analysis (47 cases) and cases who became NLDM→LDM after therapeutic interventions (nine cases; Table 1).

There was no difference between the LDM patients (56 cases) and NLDM patients (36 cases) in terms of BMI, follow-up period, and gravidity, although there were significant differences in age, parity, duration of infertility, and numbers of previous failed transfer cycles between the two groups (Table 2).

The EF specimens were collected either in follicular phase, ovulation phase, or luteal phase of the menstrual cycles, as there was intercyclic stability of the endometrial microbiome in our previous study.[6] There was no statistical difference in the timing of sampling between LDM and NLDM (Table 2).

3.2 | Endometrial microbial results and bacterial communities in the endometrium of the NLDM patients

The median percentage of endometrial *Lactobacilli* in LDM and NLDM groups were 98.95% ± 1.84% and 14.53% ± 33.07%, respectively (*P* < 0.001, Mann-Whitney test; Table 2). As shown in Figures 3 and 4, the major taxonomies in the EF specimens were *Atopobium*, *Bifidobacterium*, *Gardnerella*, *Megasphaera*, *Sneathia*, *Prevotella*, *Staphylococcus*, *Streptococcus*, etc.

3.3 | Pregnancy outcome of the patients and endometrial microbial results

Single vitrified-warmed blastocyst transfers were performed in all cases. Pregnancy rates were higher in the LDM group (58.9% per patient and 36.3% per FBT) compared to that of the NLDM group (47.2% per patient and 34.7% per FBT), but this was not significantly different (Table 2). Miscarriage rate was 24.2% in LDM pregnancies and 17.6% in NLDM pregnancies, which was also not significantly different (Table 2).

Five out of the nine patients who became NLDM→LDM after treatment (Table S1a) achieved pregnancies (3 ongoing and 2 miscarriages; Figure 2B, Table 1), with the pregnancy rates of 55.6% (5/9) per patient and 33.3% (5/15) per FBT, and the miscarriage rate of 40% (2/5). We compared the pregnancy outcomes between the cases with LDM at initial analysis and cases who became NLDM→LDM after therapeutic interventions, but found no significant difference between those two groups (Table 1).

Nineteen patients in the NLDM group selected to undergo FBT without therapeutic interventions against NLDM for various reasons; that is, the patient urged us to go on to her next FBT; the

TABLE 2 Characteristics and pregnancy outcome of two groups (LDM vs NLDM)

	LDM	NLDM	*P* value
No. of patients	56	36	—
No. of FBT cycles	91	49	—
Age (y): mean ± SD	36.23 ± 3.82	38.11 ± 4.33	<0.05[a]
BMI: mean ± SD	20.04 ± 2.30	20.87 ± 3.17	NS
Duration of infertility (mo): mean ± SD	21.19 ± 21.90	11.53 ± 11.18	<0.01[a]
Previous ET: mean ± SD	2.98 ± 2.96	1.67 ± 1.94	<0.05[a]
Multigravida patients: N (%)	32 (54.2)	26 (72.2)	NS
Multipara patients: N (%)	11 (18.6)	14 (38.9)	<0.05[b]
Follow-up period[d] (mo): mean ± SD	6.61 ± 1.67	6.43 ± 1.48	NS
Sampling timing: follicular phase: N (%)	13 (23.2)	10 (26.3)	NS
Ovulation phase: N (%)	5 (8.9)	4 (10.5)	
Luteal phase: N (%)	38 (67.9)	24 (63.2)	
% of endometrial LB: median ± SD	98.95 ± 1.84	14.53 ± 33.07	<0.001[c]
Pregnancy rate per FBT: N (%)	33 (36.3)	17 (34.7)	NS
Pregnancy rate per patient: N (%)	33 (58.9)	17 (47.2)[e]	NS
Miscarriage rate: N (%)	8 (24.2)	3 (17.6)	NS

ET, embryo transfer; FBT, frozen-thawed blastocyst transfer; LB, *Lactobacillus*; LDM, *Lactobacillus*-dominated microbiota; NLDM, non-*Lactobacillus*-dominated microbiota.
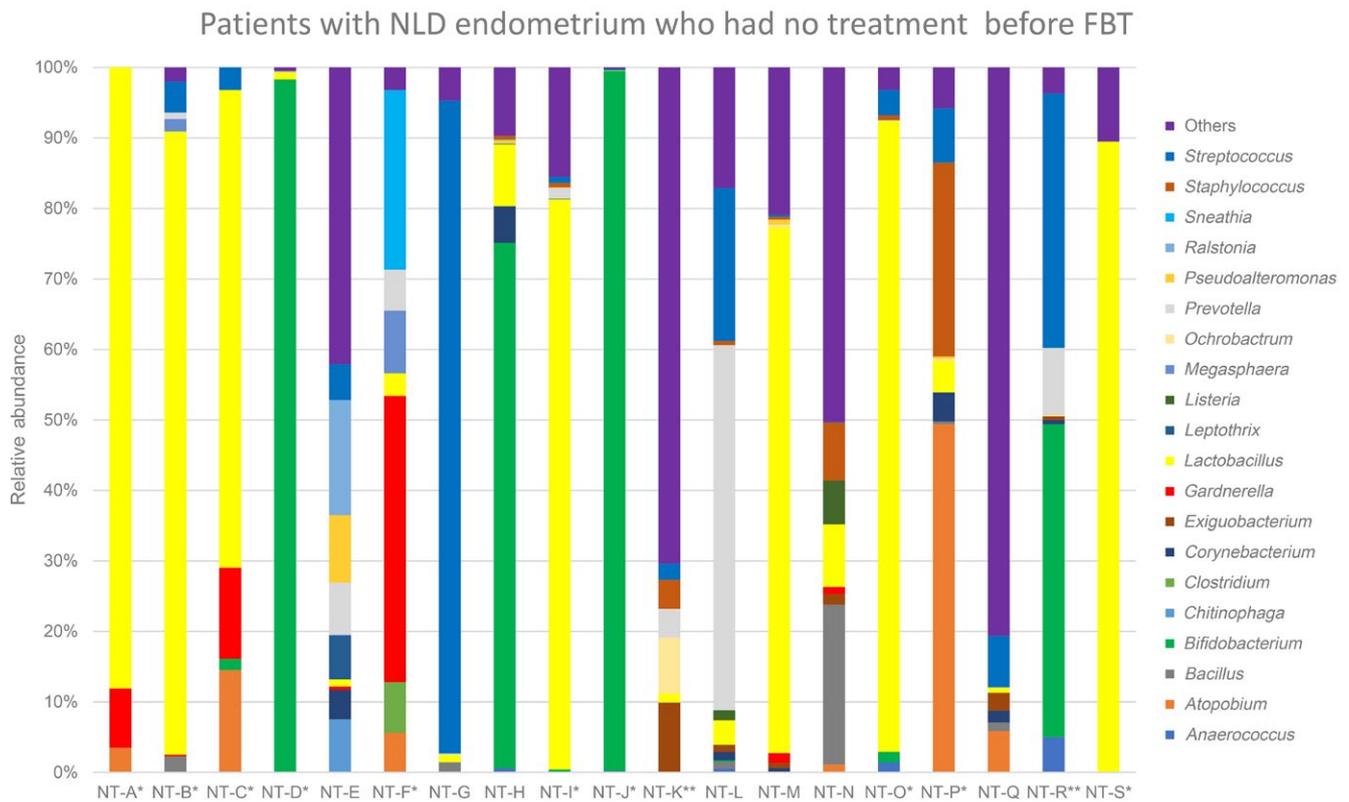
[a]*t* test.
[b]Chi-square test.
[c]Mann-Whitney *U* test.
[d]Until 2018.6.30.
[e]One patient had two pregnancies.

FIGURE 3 The endometrial microbiomes of the NLDM patients who had no interventions before FBT. *Patients achieved pregnancy in the following FBT. **Patients achieved pregnancy but ended up in early miscarriages in the following FBT. The letters below the graph show each participant

percentage and the type of major taxonomy seemed to be tolerable for embryo transfer; for some patients, EF was collected at the time of mock ET trial just before FBT; and pregnancy was achieved before confirmation of the microbial result (Figures 1 and 3). Twelve patients achieved pregnancy in this no-treatment NLDM group, with the pregnancy rates of 63.2% (12/19) per patient and 57.1% (12/21) per FBT, and the miscarriage rate of 16.7% (2/12). The good prognosis of this no-treatment NLDM group may be attributed to the following two factors; five patients with the percentage of endometrial *Lactobacilli* more than 80% achieved successful pregnancy, and two patients with dominant *Bifidobacterium* (>90%) in the endometrium also achieved pregnancy (Figure 3). When those seven patients were excluded from this no-treatment NLDM group, the pregnancy rates of the no-treatment NLDM group were 41.7% (5/12) per patient and 35.7% (5/14) per FBT, and the miscarriage rate was 40% (2/5), respectively.

Thus, we reclassified the 92 participants according to the endometrial bacterial status of ≥80% *Lactobacillus* spp or <80% *Lactobacillus* spp and reanalyzed the pregnancy outcomes (Table S2). Pregnancy rate was higher in the ≥80% *Lactobacillus* group (61.3% per patient) compared to that of the <80% *Lactobacillus* group (40.0% per patient; $P = 0.05$), although there still were differences in the background (parity, duration of infertility, and numbers of previous failed transfer cycles) of the two groups (Table S2).

Seventeen patients in the NLDM group underwent FBT after therapeutic interventions for NLDM as described above (Table S1b), and they preferred to undergo embryo transfers without reanalyzing their endometrial microbiota (Figures 1 and 4). Four patients achieved five pregnancies (4 ongoing and 1 miscarriage).

3.4 | Case reports on NLDM patients who successfully achieved LDM endometrium by trial strategies for dysbiotic endometrium

Below are the three representative cases who successfully achieved *Lactobacillus*-dominated endometrium after therapeutic interventions with antibiotics and prebiotics/probiotics (Table S1a).

3.4.1 | Case 1

Thirty-eight years old, G0P0, recurrent implantation failure of 11 failed embryo transfers with unknown reason. Her first endometrial microbial analysis by sequencing resulted in NLDM, with dominant *Streptococcus* spp (Figure 2A, B-1). LF 300 mg/d per os was started after administration of amoxicillin 750 mg/d per os for 7 days. Nineteen days after the initiation of prebiotics (56 days from her first endometrial microbial analysis), second endometrial microbial analysis was performed, resulting in LDM (Figure 2B, B-2 and 5).
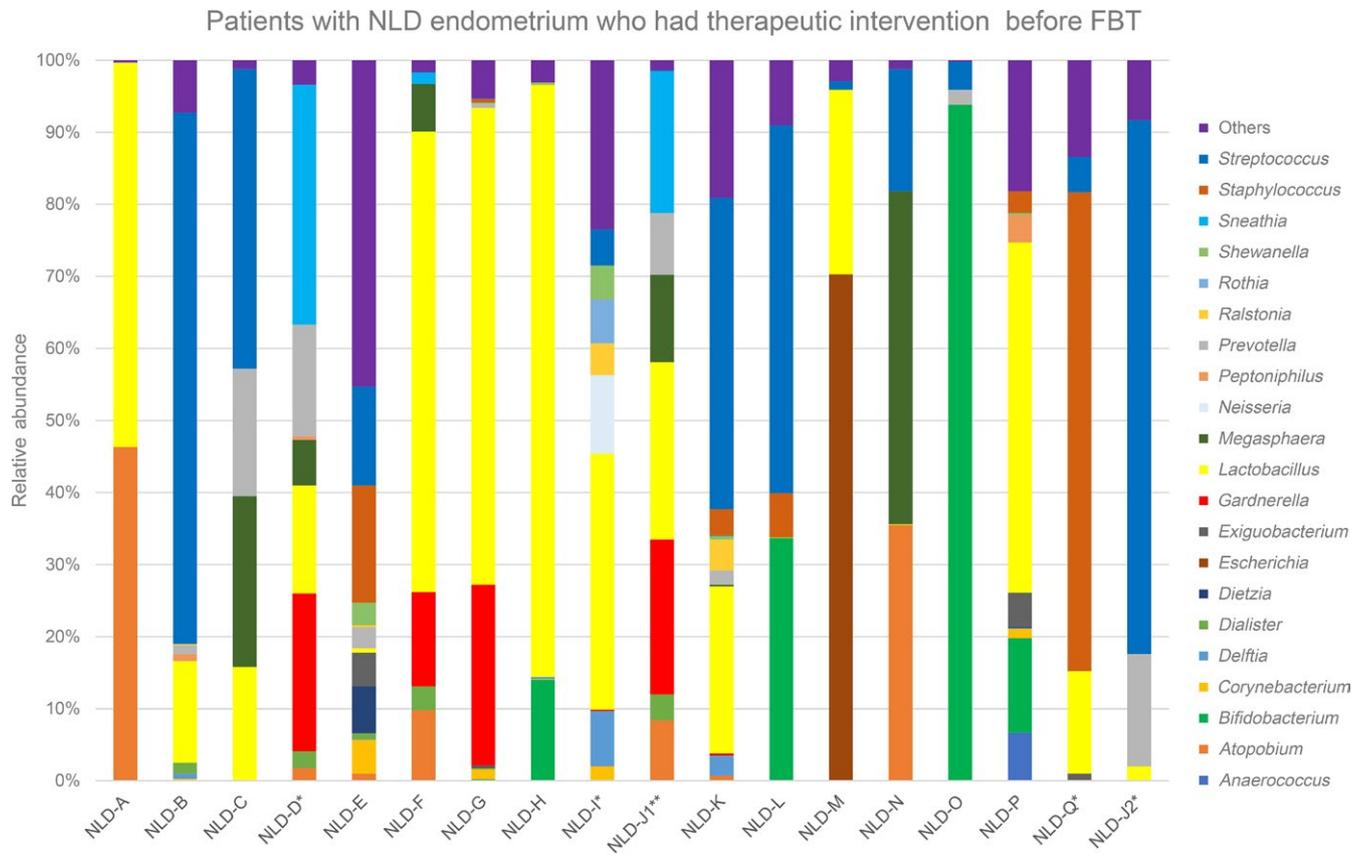
FIGURE 4 The endometrial microbiomes of the NLDM patients who had therapeutic interventions before FBT. *Patients achieved pregnancy in the following FBT. **Patients achieved pregnancy but ended up in early miscarriages in the following FBT. The letters below the graph show each participant. NLD-L is identical to NT-G in Figure 3; this patient underwent two FBTs during the study period, first FBT was after the initial endometrial microbiome analysis (without treatment), and second FBT was after the reanalysis (with treatment intervention). NLD-J1 is identical to NLD-J2
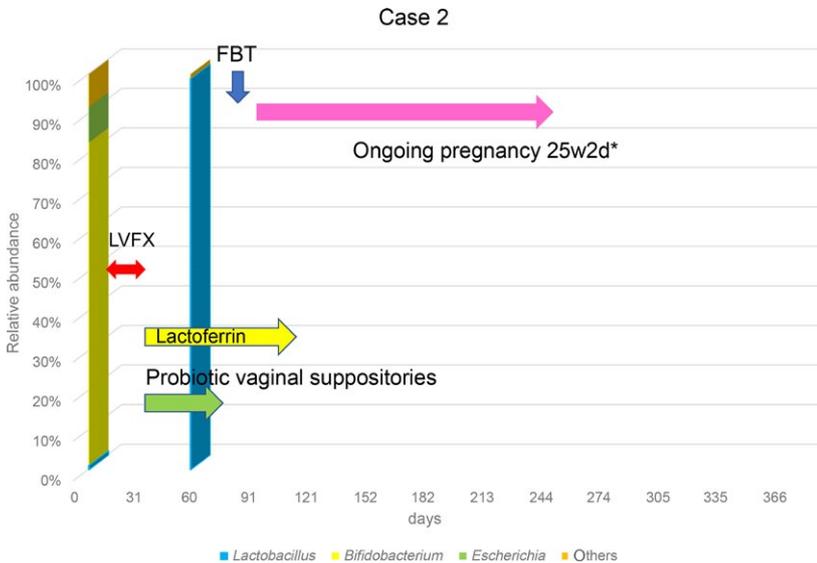
3.4.2 | Case 2

Thirty-three years old, G1P1, male factor, hoping for a second child. She had two failed FBT cycles prior to her initial endometrial microbiome analysis. Her first endometrial microbial analysis by sequencing resulted in NLDM, with dominant *Bifidobacterium* (Figure 2A, A-1). Although we explained to her that even though the result was classified as NLDM, *Bifidobacterium* dominancy may not have an adverse effect for implantation, the patient selected to have intervention for her endometrial microbes before her next
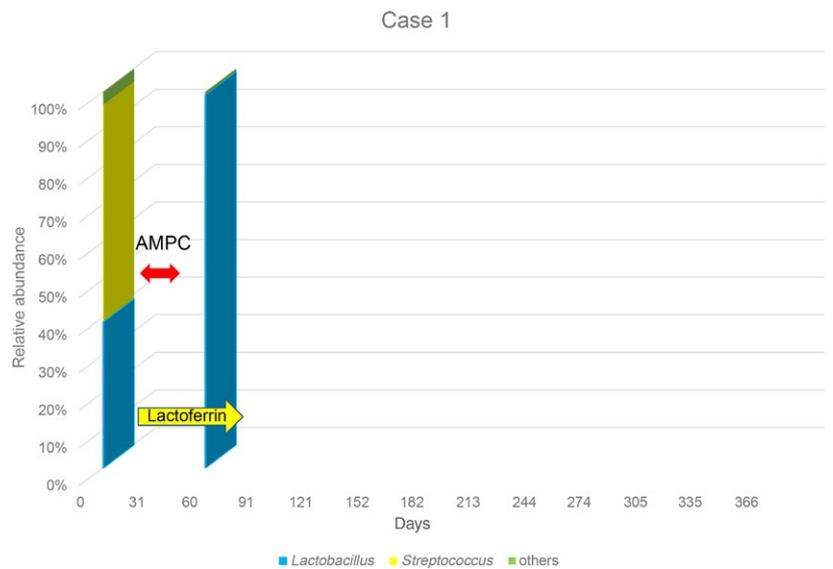
FIGURE 5 Course of treatment in Case 1 (identical to Case B in Figure 2). AMPC, amoxicillin

FBT attempt. After administration of levofloxacin 500 mg/d for 7 days per os, LF 300 mg/d per os and probiotic vaginal suppositories (1 capsule/d) were started. The reason why levofloxacin was selected was that it was one of the broad-spectrum antibiotics available at our clinic as in-house prescriptions, and this patient had not been prescribed it before. Twenty-four days after the initiation of prebiotics and probiotics, second endometrial microbial analysis was performed, resulting in LDM (Figure 2B, A-2). She had a successful ongoing pregnancy in her next FBT (Figure 6). The probiotic vaginal suppositories were discontinued after her pregnancy was confirmed by positive serum β-hCG. We recommended that she continue LF through her pregnancy.

3.4.3 | Case 3

Thirty-one years old, G2P0, primary infertility with a male factor. She had four FBT cycles prior to her initial endometrial microbiome analysis and had early miscarriage 7 months before. Her first endometrial microbial analysis by sequencing resulted in NLDM, with dominant *Atopobium* and *Gardnerella* (Figure 2A, D-1A). LF 300 mg/d was started after administration of levofloxacin 500 mg/d per os for 7 days. The reason why levofloxacin was selected was the same as in Case 2. Twenty days after the initiation of prebiotics (60 days from her first endometrial microbial analysis), a second endometrial microbial analysis was performed, still presenting NLDM, with changes in dominant microbiota (Figure 2A, D-1B). The patient then underwent oocyte pickup (OPU) with GnRH antagonist protocol. Twelve oocytes were retrieved, seven were fertilized by ICSI, and four blastocysts were vitrified. In our clinic, we make it a rule to administer doxycycline to patients prior to OPU and cefdinir after OPU for the purpose of preventing infection, so she was administered 100 mg/d doxycycline for 7 days per os prior to OPU and 300 mg/d cefdinir for 3 days per os after OPU. Just after her OPU cycle, she used probiotic tampons during her menstruation. Sixty-four days after the initiation

FIGURE 7 Course of treatment in Case 3 (identical to Case D in Figure 2). CFDN, cefdinir; DOXY, doxycycline; FBT, frozen-thawed blastocyst transfer; LVFX, levofloxacin; OPU, oocyte pickup
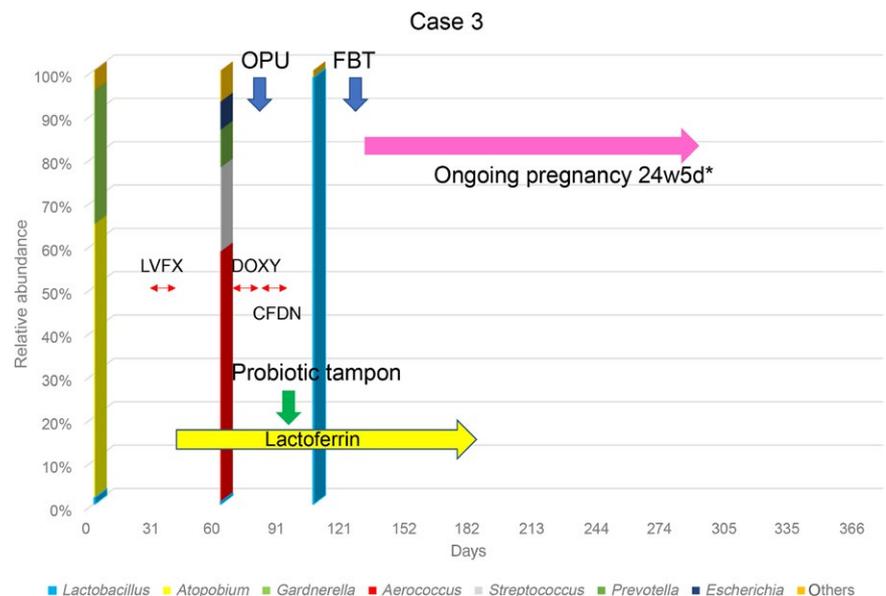
of Lf and 16 days from the usage of probiotic tampons (104 days from her first endometrial microbial analysis), a third endometrial microbial analysis was performed, finally resulting in LDM (Figure 2B, D-2). She had a successful ongoing pregnancy in her next FBT (Figure 7). We recommended that she continue LF through her pregnancy.

4 | DISCUSSION

Analysis of endometrial microbiota by next-generation sequencing has become commercially available recently, but to what extent and how the human uterine microbiomes are involved in implantation is still not clear; also, the treatment strategy for dysbiotic endometrium is still not established. We have endeavored to establish a way of recovering *Lactobacilli*-dominant endometrium in this pilot study, and to the best of our knowledge, this is the first study reporting about treatment strategies for dysbiotic endometrium in infertile women in Japan.

Compared to the LDM group, the NLDM group was significantly older, had a higher percentage of multipara patients, shorter duration of infertility, and fewer previous ET cycles (Table 2). The reason for this may be simply due to patient recruitment, or there may have been more patients hoping for a second child in the NLDM group, suggesting that childbirth, either by vaginal delivery or cesarean section, may interfere with maternal endometrial microbiota. Other factors that may interfere with endometrial microbiota may be derived from IVF procedures or backgrounds of IVF patients such as infertility period, seminal factor, frequent exposure to gynecological examinations/interventions such as transvaginal examination, uterine catheterization such as IUI or hysterosalpingography, oocyte retrieval, embryo transfer, frequent administration of antibiotics, hormonal fluctuation due to controlled ovarian stimulation, etc.[6] Also, lifestyle habits including sexual activities and sanitary conditions may have an impact on endometrial microbiome, but these were not analyzed in this study.

In our preliminary study, *Lactobacillus* dominancy was favorable in terms of pregnancy outcome (Table 2, Table S2), but the result was not as significant as in the previous report.[5] The reasons for this may be due to the limited study numbers, short follow-up period, ethnic differences, or specific reasons in current Japanese reproductive medicine; no permission to use preimplantation genetic testing for aneuploidy or oocyte donations, and enforcement of single embryo transfer. Moreno et al[5] reported that pregnancy outcome could be predicted by the relative abundance of *Lactobacilli* in EF, and <90% *Lactobacilli* in the endometrium had an adverse effect on pregnancy. In our study, patients classified as NLDM but having more than 80% *Lactobacilli* in the endometrium showed good pregnancy outcomes (Figure 3, Table S2); thus, the percentage of endometrial *Lactobacilli* >80% might be enough for embryo implantation. Also, even if classified as NLDM, *Bifidobacterium*-dominant endometrium might also be an acceptable environment for implantation (Figure 3).

As there were differences between the background of LDM and NLDM groups as described above, the outcomes between NLDM→LDM patients (nine cases; Figure 2B) and no-treatment NLDM patients (19 cases; Figure 3) should have been compared, in order to assess the actual effect of microbial status on implantation. The prognosis of the no-treatment NLDM group was good (pregnancy rates of 63.2% per patient and 57.1% per FBT, and miscarriage rate of 16.7%) compared to that of NLDM→LDM patients (pregnancy rates of 55.6% per patient and 33.3% per FBT, and miscarriage rate of 40%), although the differences were not statistically significant. The good outcome of this no-treatment NLDM group was considered to be due to the pregnancies achieved by five patients presenting >80% endometrial *Lactobacilli* and two patients with dominant *Bifidobacterium* (Figure 3); when those seven patients were excluded, the pregnancy rates of the no-treatment NLDM group were 41.7% per patient and 35.7% per FBT, and the miscarriage rate was 40%, respectively, still comparable to the outcome of NLDM→LDM patients. As the number of studied cases was limited, we cannot conclude that intervention for NLDM is not necessary.

Moreover, there were a few cases who achieved pregnancy in spite of the non-*Lactobacillus*-dominated endometrial status (Figures 3 and 4), but the fact should not be interpreted literally, because there is a possibility that the endometrial microbial status when embryo implantation occurred was different from the time of analysis. Implantation may occur sometimes at low *Lactobacillus* endometrium but may eventually turn into miscarriage. In this study, a patient was analyzed for her endometrial microbiota at the time of testing catheter just before her FBT, and the result was NLDM with 4.5% *Lactobacillus*; but she now has an ongoing pregnancy. Pathogenicity may differ with the types of bacteria dominant in the endometrium, but we could not find a significant trend in microbiomes between pregnant and nonpregnant cases in this preliminary study. Moreno et al[5] reported that the adverse effect of NLDM on pregnancy was more evident in participants presenting dominant *Gardnerella* and *Streptococcus* genera. Bacteria which is reported to be responsible for chronic endometritis (CE), such as *Enterococcus*, *Enterobacteriaceae*, *Streptococcus*, *Staphylococcus*, *Gardnerella*, *Mycoplasma*, *Ureaplasma*, *Chlamydia*, and *Neisseria*,[9] may have adverse effects on implantation. But the mechanism of how the pathogenic bacteria affect the embryo implantation is still not clear.

Currently, CE is diagnosed by hysteroscopy, histology, CD-138 immunostaining, and microbial culture, alone or in a combination of those methods. Recently, a report demonstrated that a molecular microbiology method using next-generation sequencing may be a faster and better diagnostic tool for the determination of CE compared to three diagnostic methods, with a high degree of concordance with these three classical methods altogether.[9] However, the diagnostic criteria of CE have not yet been established.[10] We did not focus on the association of microbiomes and CE in this study, because there had been substantial interobserver variability in the diagnosis of CE between the pathologists and between the gynecologists who performed hysteroscopy; thus, we thought it was inappropriate to analyze endometrial microbiomes in terms of CE. For the treatment of CE, several kinds of antibiotics are reported

to be effective in many cases.[11] Meanwhile, we have experienced several patients presenting *Lactobacillus*-dominant endometrium who were also presenting CD138-positive cells in the endometrium (data not shown). There is a risk of disturbing uterine/vaginal normal bacterial flora with the blind, cumulative usage of antibiotics. Whether CE is truly caused by specific bacteria, and non-*Lactobacillus*-dominated endometrium is truly a dysbiosis or not, remains to be elucidated.

Administration of antibiotics only may not be helpful in creating an LDM because the *Lactobacilli* may also be the target of some antibiotics. Concurrent administration of prebiotic and/or probiotic drugs containing *Lactobacilli* spp was expected to be one treatment option, as several studies using probiotics for treating bacterial vaginosis have been conducted.[12,13] Appropriate administration of antibiotics for the target microbiome is critical, and broad-spectrum antibiotics may not be always effective. There is always a problem of time lag, which was about 4 weeks in our study, between the date of microbial analysis and the date of starting antibiotics; and there are possibilities that microbiomes change over time. How fast NLDM turns into LDM may depend upon the types and percentages of the non-*Lactobacillus* microbiomes and their susceptibility to antibiotics. There is a possibility that it is not necessary to eradicate every single non-*Lactobacillus* microbe from the endometrium, because they may be simply residents, not pathogens of the endometrial cavity.[14]

Antibiotics followed by prebiotics and/or probiotics were effective in the restoration of LDM in the uterine cavity of NLDM patients (Table S1a, Figure 2B). Lf was helpful in recovering LDM in the endometrium, as reported by Otsuki et al,[7,8] although in our study there were several cases who discontinued Lf due to diarrhea. In their study, *Lactobacilli* gradually became dominant after the administration of LF for one month.[7,8] In our study, *Lactobacilli* seemed to have become dominant in the endometrium 1-3 months from the start of Lf and about a month from the start of probiotics (Figures 5-7) in combination with the antibiotics, but there were variations between the patients, and such variation was suspected to derive from the patient's basal *Lactobacilli* percentage in the endometrium. When and where to stop prebiotics/probiotics is a difficult issue. In the previous study of Otsuki et al,[8] *Lactobacilli* remained dominant throughout pregnancy by continuing LF until delivery, with no adverse effect on either mothers or babies.

There are limitations of this present study: short follow-up period, limited study numbers, and not analyzing other aspects of gynecological histories such as sexual contact, past oral contraceptive usage, past antibiotic usage, miscarriage, endometriosis, and bacterial vaginosis. In terms of sample collection, EF specimens may have contained some endocervical fluid, which was distinguished in a previous study,[15] but the endometrial microbiota was not suspected to be carried over from the vaginal microbiota.[6]

The results of this pilot study could not necessarily prove the clear benefit of establishing *Lactobacillus*-dominated endometrium in terms of pregnancy outcome, but we believe that there is significance in searching for endometrial microbial status of infertile patients and recovering *Lactobacillus*-dominated endometrium may benefit embryo implantation. The mechanism of how the pathogenic bacteria affect the embryo implantation remains to be elucidated. Antibiotics followed by prebiotics and/or probiotics were effective in the restoration of LDM in the uterine cavity of NLDM patients, and the selection of antibiotics is critical and needs expert knowledge. Further studies are needed to establish regimens for the treatment of endometrial NLDM and remain an issue for the future. By transferring euploid embryos in a personal window of implantation[16] under the *Lactobacillus*-dominated endometrium, much better pregnancy rates are expected.

ACKNOWLEDGEMENTS

DISCLOSURES

Conflict of interest: Yoshiyuki Sakuraba and Yoko Nagai are employed by Varinos, Inc. *Human rights statements and informed consent*: All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1964 and its later amendments. Informed consent was obtained from all patients for being included in the study. *Animal rights*: This article does not contain any studies with animal participants performed by any of the authors.

ETHICAL APPROVAL

This study was approved by the Institutional Review Board of Kyono ART Clinic and Kyono ART Clinic Takanawa.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.