RBMO

ARTICLE

Effect of probiotics on vaginal *Ureaplasma parvum* in women suffering from unexplained infertility

BIOGRAPHY

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KEY MESSAGE

Probiotics did not change vaginal alpha diversity but contained relative abundances of *Ureaplasma parvum* compared with the control group. It is tempting to speculate that probiotics have the effect of protecting the vaginal microbiota by limiting non-beneficial bacteria.

ABSTRACT

Research question: Does oral probiotic supplementation influence the relative abundance of different vaginal microbiota in women experiencing infertility?

Design: A prospective, monocentric randomized controlled trial. To study the influence of probiotics on infertility, 80 patients with primary or secondary infertility were included. Patients were assigned to either a probiotic treatment or a control group. Participants in the treatment group (n = 40) took one sachet (2 g) a day of a defined probiotic supplement limiting *Lactobacillus* strains. Patients in the control group did not receive any additional probiotic supplements. Vaginal samples were taken on day 20 of the menstrual cycle and 4 weeks later, on day 20, of the consecutive cycle. Subsequently, 16s rRNA gene analysis of the vaginal samples was conducted.

Results: After the intervention phase, no effects on alpha diversity resulting from treatment could be observed. The between sample diversity of different women (beta diversity) at baseline had no effects of age, treatment group or body mass index. Primary or secondary sterility, however, had a significant effect on community. Three clusters (*Lactobacillus crispatus*, *Lactobacillus iners* and *Lactobacillus gasseri*) were identified as the leading representatives. Furthermore, patients treated with probiotics showed limited growth of *Ureaplasma parvum* compared with the control group (P = 0.021).

Conclusions: This study points to a possible protective effect of probiotic supplements on the vaginal microbiota. It is tempting to speculate that this effect assists in containing the growth of non-beneficial bacteria and helps to prevent or cure a dysbiotic vaginal flora.

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KEYWORDS

Infertility
Lactobacillus
Probiotics
Ureaplasma parvum

INTRODUCTION

Infertility is a global phenomenon affecting around 186 million people worldwide (*Inhorn and Patrizio, 2015*). Many circumstances have been identified to cause infertile conditions, with age being one of the most limiting factors of fertility. Besides acute, chronic or infectious diseases, environmental and occupational exposures, general lifestyle, genetic conditions and specific reproductive disorders can also affect either the man or woman attempting to conceive (*Cunningham, 2017*). In recent years, however, the microbiome has gained importance in the treatment of infertility and is considered an important player contributing to improved success rate of reproductive medicine treatments, such as IVF (*Sirota et al., 2014*).

Within the female reproductive tract, the dominance of *Lactobacilli* is associated with a healthy vaginal microbial community in healthy women of reproductive age (*Moreno et al., 2016*; *Miles et al., 2017*). In detail, certain community state types (CST), mainly CST I (*Lactobacilli crispatus*), CST II (*Lactobacilli gasseri*), CST III (*Lactobacilli iners*) and CST V (*Lactobacilli jensenii*) were found in non-pregnant, fertile and asymptomatic women from four ethnic groups (*Barrientos-Durán et al., 2020*). *Lactobacillus* strains produce lactic acid, causing an acidic environment of pH less than 4.5. This specific condition provides a highly protective environment against pathogens and is part of multiple defense mechanisms in the lower female genital tract (*Linhares et al., 2011*). Furthermore, *Lactobacillus* species have been shown to produce antimicrobial bacteriocins to inhibit growth of undesirable species, such as *Klebsiella species*, *Staphylococcus aureus*, *Escherichia coli* or *Enterococcus faecalis* (*Stoyancheva et al., 2014*).

A higher abundance of *Lactobacilli* is associated with better reproductive outcomes; however, the presence of *Enterococci*, *Enterobacteriaceae*, *Streptococci* or gram-negative bacteria is related to lower implantation rates, decreased numbers of preterm birth and increased numbers of miscarriages (*Egbase et al., 1996*; *Salim et al., 2002*; *Selman et al., 2007*). In addition, a decreased relative abundance of *Lactobacilli* has been associated with dysbiosis and vaginal inflammation (*Moreno and Franasiak, 2017*; *Kyono et al., 2018*). The most common dysbiotic state is bacterial vaginosis, described as an anaerobic polybacterial state in the lower female genital tract. Because of the overgrowth of anaerobes, infections and obstetrical complications are triggered by noxious substances, such as polyamines and small polycationic molecules, with a wide array of biological functions that were recently shown to be involved in bacterial pathogenesis (*Tofalo et al., 2019*). Enhanced production of proteolytic enzymes that act on vaginal peptides to release polyamines cause a shift in the balance of cytokine expression towards pro-inflammatory cytokines, such as interleukin (IL)-1 β and IL-8 (*Mastromarino et al., 2014*). Furthermore, bacterial lipoproteins adhere to and invade host cells of the genitourinary tract, trigger inflammation processes and lead to apoptosis and cell death (*You et al., 2008*). These changes in the vaginal environment increase the risk of acquiring sexually transmitted infections and affect women's reproductive health negatively. During pregnancy, bacterial vaginosis can lead to chorioamnionitis, preterm premature rupture of the membranes and preterm birth (*Redelinghuys et al., 2020*).

One of the most important contributors to bacterial vaginosis is *Ureaplasma parvum* (*Haggerty et al., 2009*). As part of the mycoplasma family, *Ureaplasma* belong to the smallest self-replicating microorganisms and human parasites, and are the most potentially pathogenic bacteria in the human urogenital tract (*Cassell et al., 1993*). They independently reproduce aerobic to facultatively anaerobic bacteria, and have a pleomorphic, variable, vesicular shape (*Viscardi, 2010*; *Rumyantseva et al., 2019*). *Ureaplasma species* do not possess a cell wall, and are surrounded only by a plasma membrane, contacting host cell surface via lipid-associated membrane proteins. Their pathogenicity is characterized by the production of proinflammatory cytokines as well as their ability to induce apoptosis in monocytes and macrophages (*Melgaço et al., 2018*). *Ureaplasma* colonization in the vagina is associated with infertility, stillbirth, histologic chorioamnionitis and neonatal morbidities, including congenital pneumonia, bronchopulmonary dysplasia, meningitis and perinatal death. Antibiotic resistance to macrolides, fluoroquinolones and tetracyclines has been reported (*Sprong et al., 2020*). Hence, it has been suggested that *Ureaplasma* colonization should be diagnosed and treated early to prevent long-term infection and subsequent complications (*Siles-Guerrero et al., 2020*).

Despite the importance the microbiome has gained over recent years, its influence in IVF treatments is still controversial. Some studies suggest a negative influence of vaginal dysbiosis on pregnancy rates (*Haahr et al., 2016*); however, a meta-analysis showed no association between abnormal vaginal flora and conception rates after IVF treatment (*van Oostrum et al., 2013*). The prevalence of bacterial vaginosis is significantly higher in infertile women compared with fertile women (*Salah et al., 2013*). Furthermore, bacterial vaginosis was associated with a significantly elevated risk of preclinical pregnancy loss (*van Oostrum et al., 2013*). Therefore, it is tempting to speculate that restoring a healthy vaginal microbiota is important in the treatment of women experiencing infertility.

One therapeutic approach to bacterial vaginosis is the administration of beneficial microorganisms (probiotics), mainly *Lactobacillus species*. The supplementation of exogenous *Lactobacilli* strains has been suggested as a cure for dysbiotic vaginal flora, reestablishing healthy conditions and improving female fertility health (*Mastromarino et al., 2014*), a practice dating back to the 1900s (*Sieber and Dietz, 1998*). A large number of clinical studies with oral supplementation have been conducted since then, for which a substantial number of literature reviews are available (*Strus et al., 2012*; *Homayouni et al., 2014*; *Huang et al., 2014*). The main mechanism studied of how orally administered *Lactobacilli* settle in the vagina is their migration from the intestine to the anus and finally to the vagina. This mechanism seems to combine mucosa-adhesive features of *Lactobacilli* and chemotactic factors that guide their migration towards the vaginal mucosa (*Reid, 2008*; *Borges et al., 2014*). Although oral versus vaginal probiotic applications have numerous advantages and disadvantages, the biggest benefit of oral supplementation is its convenience for the patient and the concomitant compliance.

A recent meta-analysis has shown short- and long-term effects of probiotics in the

treatment of bacterial vaginosis (*Wang et al., 2019*); however, the influence of probiotics on the abundance of unfavourable pathogens, such as *U. parvum*, has not been evaluated so far. Therefore, the aim of the present study was to investigate the effect of four probiotic strains (*L. crispatus* LBV88, *Lactobacillus rhamnosus* LBV96, *L. gasseri* LBV150N and *L. jensenii* LBV116) on the relative abundance of vaginal microbiota species in women experiencing infertility. We hypothesized that oral intake of probiotics may alter the vaginal microbiota, including the relative abundance of potentially pathogenic species, such as *U. parvum*.

MATERIALS AND METHODS

Sample collection

The study included 80 Austrian women experiencing unexplained primary or secondary infertility or male factor infertility during their first reproductive medicine treatment. All patients were aged between 18 and 40 years, with a body mass index (BMI) ranging from 19 to 29.9 kg/m^2. Ethnicity was self-reported as white with European ancestry. Patients who met the following criteria were excluded: severe disease; acute disease, chronic disease, or both, e.g. acute vaginal infection; obesity; hirsutism; antibiotic intake (within the last 30 days before intervention); endometriosis; polycystic ovary syndrome; and use of other pre- or symbiotics. Patients were assigned to either treatment or control group, using block randomization to avoid confounding through treatment modalities. Participants in the treatment group (n = 40) took one sachet (2 g) of the probiotic OMNi-BiOTiC® FLORA plus+ (Institut Allergosan Pharm, Produkte Forschungs- u. Vertriebs GmbH, Graz, Austria) dissolved in 125 ml of water a day for a period of 4 weeks starting on day 20 of the menstrual cycle. The supplement is composed of four bacterial strains: *L. crispatus* LBV88, *L. rhamnosus* LBV96, *L. gasseri* LBV150N and *L. jensenii* LBV116. Patients in the control group did not receive any additional probiotic supplements. Vaginal samples were taken with sterile swabs (jeSwabTM) (Copan Diagnostics Inc., Murrieta, CA, USA) on day 20 of the menstrual cycle. Luteal phase was confirmed by transvaginal ultrasonography and by urinary LH. A second vaginal sample of every patient was obtained 4 weeks later, on day 20 of the consecutive cycle. Samples were stored at –72°C and 16s rRNA gene analysis was conducted (Biovis Diagnostik MVZ GmbH, Limburg an der Lahn, Germany).

During the trial, 19 patients were prematurely discontinued owing to antibiotic intake (n = 16), cancellation of fertility treatment (n = 2) or withdrawal from consent (n = 1). All samples and clinical records were collected at the fertility clinic Das Kinderwunsch Institut Schenk Gmb, Dobl, Austria, between 2018 and 2019. All participants consented to use of their medical records in research. The study was approved by the Ethics Committee of the Medical University of Graz, Austria (approval number: 30–182 ex 17/18; date of approval: 25 July 2018).16s

rRNA analysis and data processing

Data were generated from DNA extracted from the supplied vaginal swabs using the QIAamp UCP Pathogen mini kit automated on the QIAcube. The swab was transferred to a Pathogen Lysis Tube S filled with 0.65 ml ATL buffer (including Reagent DX) and incubated for 10 min at 56°C with continuous shaking at 600 revolutions per min. Subsequently, bead beating was carried out using a SpeedMill PLUS for 45 s at 60 Hz. Samples were then heated to 95°C for 5 min and centrifuged afterwards for 1 min at 10,000 revolutions per min. A total of 400 µl of the resulting supernatant was transferred to a 1.5 ml microcentrifuge tube, which was placed in the QIAcube for follow-up automated DNA isolation according to the manufacturer's protocol. Elution volume was 100 µl.

Amplification of the 16S rRNA gene, targeting the variable regions V1-V2 (27F-338R), was carried out. Sequencing was executed using the Illumina MiSeq (Illumina, Netherlands) using the Illumina V3 chemistry kit (https://www.illumina.com/products/by-type/sequencing-kits/cluster-gen-sequencing-reagents/miseq-reagent-kit-v3.html) to obtain 2 × 300 bp reads. Subsequent downstream processing of sequencing data was carried out using the DADA2 package (https://benjjneb.github.io/dada2/index.html, v1.10) for R (v3.6) using the following parameters in the filterAndTrim() function: truncLen=c(230,180), trimLeft=c(5, 5), maxN=0, maxEE=c(2,2), truncQ=5, rm.phix=TRUE. Amplicon sequence variants were taxonomically annotated using the Genome Taxonomy Database (GTDB; release r86; https://gtdb.ecogenomic.org/) using a Bayesian classifier. Good-quality data could be obtained for 29 samples from the probiotic treatment group and 30 samples from the control group. Two samples were discarded owing to low sequence counts, not recovered by sequencing twice, most likely because of low DNA yield from the original sample.

Statistical analysis

Unless otherwise stated, statistical tests were carried out in R (v3.6) using the R base package (R Foundation for Statistical Computing, Vienna, Austria; https://www.R-project.org/) default functions. Shannon species equivalent, a sound index sensitive to species richness, was used as measure for alpha (within-sample/community) diversity (*DeJong, 1975*). Shannon diversity was calculated using the diversity() function of the *vegan* package for R (v.2.5-5; https://CRAN.R-project.org/package=vegan), followed by transformation using the exp() function. Baseline differences caused by primary and secondary sterility, and differences of treatment groups before treatment, were assessed using Wilcoxon Rank-Sum Test. Spearman's rank correlation test was used to assess the effects of BMI and age on alpha diversity. To assess differences in alpha diversity before and after intervention, paired Wilcoxon Rank-Sum test was used on data sets in the different treatment groups. Effects of BMI, age and cause of sterility, as well as differences between treatment groups on between-sample diversity of different women at baseline (beta diversity), were assessed using permutational multivariate analysis of variance using Bray–Curtis dissimilarity as implemented in the adonis() function of the *vegan* package for R. Unsupervised partitioning around medoids (PAM) clustering algorithm, using the pam() function of the *cluster* package for R (v.2.10; https://cran.r-project.org/web/packages/cluster/index.html), were used to assess potential clustering options. The main clustering candidates were selected based on Gini index. Community (in-) stabilities based on treatment were investigated by Fisher's exact test.

RESULTS

Per sample community composition of cohort

Forty-four out of 59 samples showed community composition patterns that were characterized by dominance of

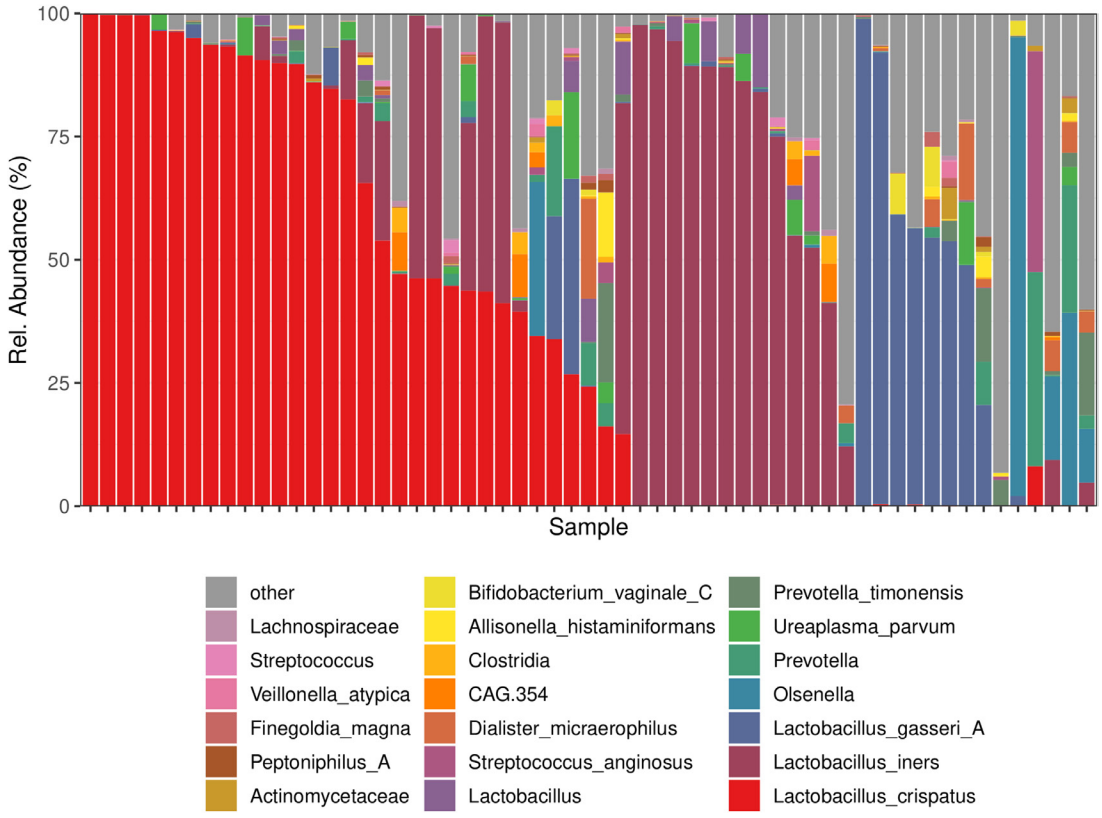

FIGURE 1 Overview of per sample community composition of the cohort before the intervention. Different colours represent different classes, genera and species.

Lactobacillus species (>50%) (FIGURE 1). The most abundant *Lactobacillus* species were *L. crispatus* (36% total, found in 41 out of 59 samples), *L. iners* (23% total, found in 16 out of 59 samples) and *L. gasseri* (10% total, found in 22 out of 59 samples). Six per cent of all *Lactobacillus* reads on genus level could not be allocated to subordinate species. Irrespective of *Lactobacillus* dominance, the most abundant bacteria not belonging to the *Lactobacillus* genus were *Olsenella*, which accounted for 3.38% of total community composition and were found in 18 out of 59 samples. Fifteen out of 59 samples were not dominated by members of the *Lactobacillus* genus but were also not dominated by any other genus except one (sample 55). Among these 15 samples, the most abundant bacteria belonged to the genus *Olsenella* and *Prevotella*. Three samples yielded reads that mostly could not be allocated to specific genera or species.

Within-sample analysis before treatment

Shannon species equivalent was used as measure for alpha (within-sample/community) (FIGURE 2A) diversity. Baseline differences caused by primary and secondary sterility, and differences of groups before treatment, yielded no significant differences (FIGURE 2B). Neither BMI (FIGURE 2C) nor age (FIGURE 2D) showed significant effects on alpha diversity. No significant changes in alpha diversity before and after intervention could be identified, independent of treatment (FIGURE 3).

Between-sample analysis before treatment

Effects of BMI, age and cause of sterility, as well as differences between groups on between-sample diversity of different women at baseline (beta diversity), were assessed. Sterility group (primary or secondary) seemed to affect community composition significantly (R^2 = 0.05026; P = 0.017) (FIGURE 4). No effects could be identified for BMI (explained variance R^2 = 0.015; P = 0.712), age (R^2 = 0.0096; P = 0.760) (data not shown) and treatment group (R^2 = 0.0071; P = 0.893) (FIGURE 5).

Cluster analysis

In advance of the between-sample analyses after treatment, potential clustering options were assessed to screen for possible clustering dynamics that would occur under treatment, i.e. significant change of cluster membership. The PAM clustering algorithm showed best results (Calinsky–Harabasz index) for a clustering into three clusters (FIGURE 6A). The most important features for cluster discrimination (based on Gini index) (FIGURE 6B) were the three *Lactobacillus* species: *L. crispatus*, *L. iners* and *L. gasseri*. A leave-one-out approach was used for Random Forest: 51 out of 59 (86%) individuals could be assigned to one of the clusters with high confidence (high confidence [>0.5], filled shapes; low confidence [<0.5], empty shapes) (FIGURE 6B). Intra-individual comparisons in Bray–Curtis dissimilarity before and after the intervention period in connection with intervention and *Lactobacillus* cluster membership before intervention are presented in FIGURE 6C and FIGURE 6D, respectively.

Clustering dynamics

Most of the samples had a stable cluster membership (FIGURE 7). Six samples in total transferred to a different cluster

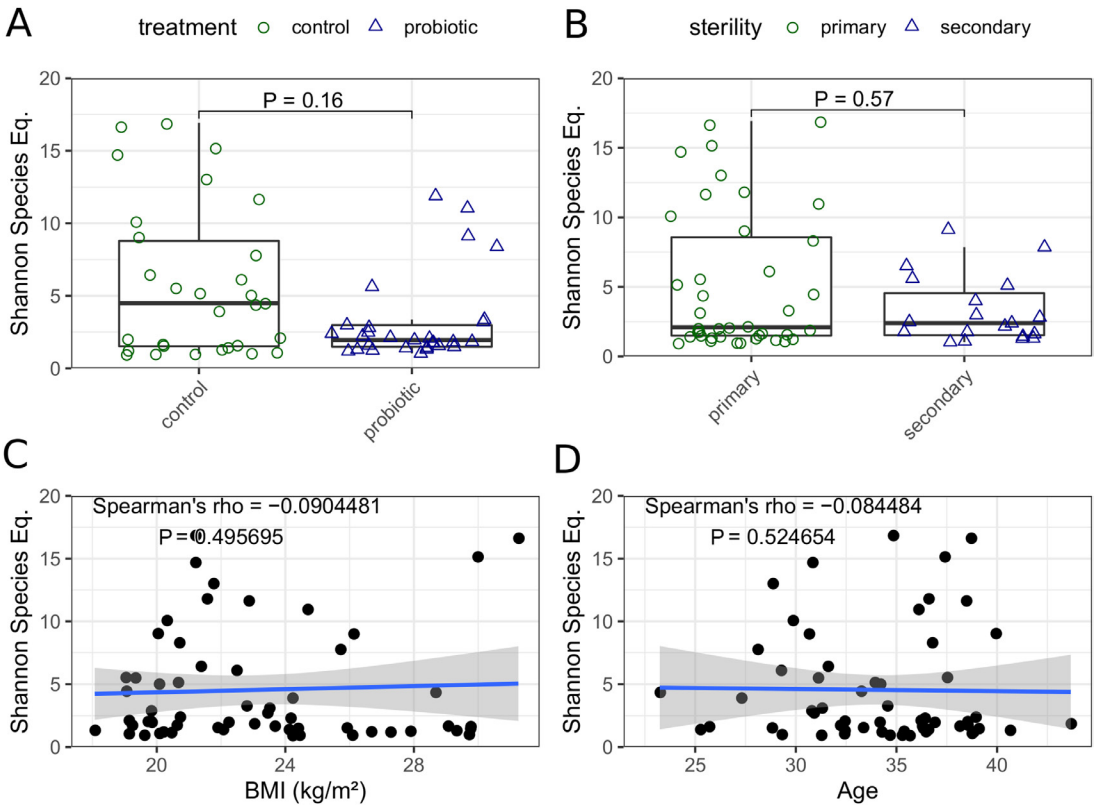

FIGURE 2 Alpha (within-sample/community) diversity measured by Shannon species equivalent before the intervention in connection with (A) intervention arm; (B) reason for sterility; (C) body mass index (BMI); and (D) age.

assignment after the intervention phase, four of which belonged to the probiotic treatment group (two from cluster 1 to cluster 3 and *vice versa*) and two from the control group (one from cluster 2 to cluster 3 and one from cluster 3 to cluster 1). No statistical effects on community (in-)stability could be identified based on treatment ($P = 0.4$) (Fisher's exact test).

Feature changes in time point and intervention

In accordance with the findings of the clustering dynamics analysis, direct comparison of relative abundances of individual species (where possible) or genera revealed that most representatives retained stable relative amounts irrespective of the comparison between time point or intervention (FIGURE 8). Exceptions with nominal statistical significance were the decrease of unclassified members of the genus *Clostridium* over time (mean abundance T1 = 0.815%; T2 = 0.048%, $P = 0.011$) in the control group, whereas it remained unchanged in the probiotics group. Obtained sequencing data did not yield sufficient information to provide higher-level resolution within the class as well as group difference in the relative abundance of the species *U. parvum* between control (3.52%) and intervention group (0.77%, $P = 0.021$) after the intervention period (FIGURE 9).

A pairwise comparison between timepoints within groups did not reveal a significant change in any of the groups ($P = 0.22$ [control], $P = 0.75$ [probiotics]).

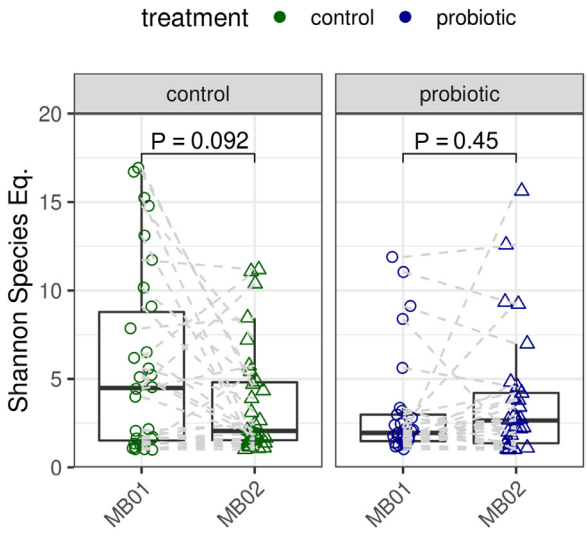

FIGURE 3 Paired Wilcoxon Rank-Sum test comparison of alpha diversity between timepoints separated by treatment. MB01, timepoint before intervention; MB02, timepoint after intervention.

DISCUSSION

In the present study, we showed that oral probiotics did not influence alpha or beta diversity of the vaginal microbiota

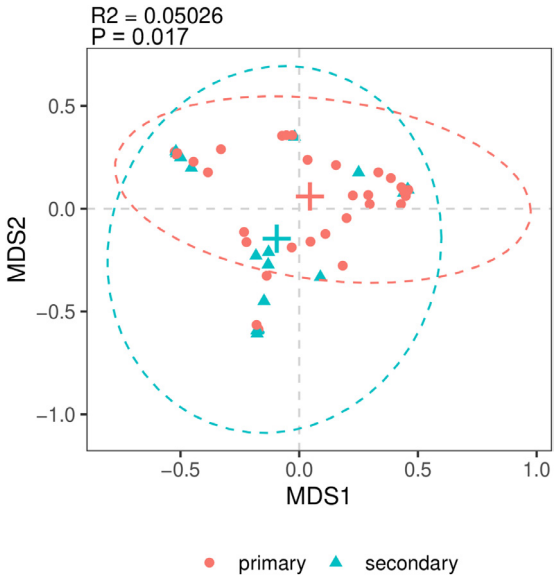

FIGURE 4 Multidimensional scaling (MDS) plot of Bray–Curtis dissimilarities. Each blue circle and red triangle represent one individual with primary and secondary infertility, respectively. Group centroids are marked with crosses of the groups' respective colours. Ellipses represent 95% confidence intervals of the groups' multivariate t-distribution.

in women experiencing unexplained infertility. Patients treated with probiotics, however, showed a contained relative abundance of *U. parvum* after the intervention period compared with the control group, indicating a possible protective effect of the natural microbiota caused by probiotics. To the best of our knowledge, this is the first study showing a possible direct influence of probiotics on the relative abundance of pathogenic bacterial strains.

The microbial composition of the female reproductive system has been intensively reviewed (*Koedooder et al. 2019*). The upper female reproductive system was considered a sterile niche for some time. Recent evidence, however, suggests the presence of microorganisms in the fallopian tubes, the uterus and ovaries (*Moreno and Simon, 2019*). The question of how microbiota maintain vaginal health and influence immune function is currently under investigation. During fertilization, cervical bacteria enter the uterus by spermatozoa and interact with the oocyte in the beginning of embryo development. This step could be critical for fertility, as gestational infections reduce fertility and increase the possibility of preterm birth and miscarriage (*Dixon et al., 2018*).

In the present study, 44 out of 59 samples showed community composition patterns that were characterized by dominance of *Lactobacillus* species and thus resembled composition patterns previously described (*DiGiulio et al., 2015*). The most abundant *Lactobacillus* species were *L. crispatus* (CST I), *L. gasseri* (CST II) and *L. iners* (CST III), reflecting the bacterial CST introduced by *Ravel et al. (2011)*. In this study, five CST were identified, depending on ethnic and biographical background. Four of the CST, however, are dominated by *Lactobacillus* species (*L. crispatus*, *L. gasseri*, *L. iners, L. jensenii*) and are associated with healthy women. The fifth CST is dominated by various anaerobic bacteria, associated with increased risk of sexually transmitted infections and preterm birth (*Romero et al., 2014*; *van de Wijgert et al., 2014*).

Besides the presence of *Lactobacillus* species, age, BMI, and primary and secondary infertility have been thought to influence bacterial composition in the vaginal microbiota. In the present study, neither BMI nor age showed significant effects on alpha or beta diversity before and after intervention. Only primary versus secondary infertility seemed to affect community composition before probiotic treatment significantly, which, to the best of our knowledge, has not yet been reported. Only a few studies have focused on the postpartum maternal microbiome, revealing the importance of biological and environmental influences, such as sleep deprivation or dietary changes on the commensal bacteria within the body (*Mutic et al., 2017*). Furthermore, a dramatic change in the vaginal microbiome after delivery was reported, displaying a less dominant *Lactobacillus* species dominant flora with a higher alpha diversity independent of ethnicity (*MacIntyre et al., 2015*). It is tempting to speculate that these changes may hamper the chance of a consecutive pregnancy in women with secondary infertility, and certainly warrants further research in this patient group.

In general, the vaginal microbiota is remarkably stable in its composition. Variations in aerobic and anaerobic communities during the menstrual cycle have been observed (*Chaban et al., 2014*). Deviations from the microbial stability correlate with time in the menstrual cycle, and may be influenced by bacterial community composition and sexual activity (*Gajer et al., 2012*). During menses in particular, a temporary instability of the vaginal flora has been described, associated with a decreased relative abundance of *Lactobacillus* species and a higher concentration of non-*Lactobacillus* species (*Eschenbach et al., 2000*). Our data suggest a stable cluster membership on day 20 of the menstrual cycle in women experiencing unexplained infertility, independent of probiotic treatment. Three clusters were discriminated owing to the relative abundance of the three *Lactobacillus* species (*L. crispatus, L. iners and L. gasseri*). Six samples in total transferred to a different cluster assignment after the intervention phase. No statistical effects on community (in-)stability could be identified based on treatment. Interestingly, we observed slight community variations in the *Clostridium* genus. We identified a decrease of unclassified members of *Clostridium* over time in the control group, which may be a result of the described given variability.

As previously mentioned, *Lactobacillus species* are thought to play a critical role; however, studies have shown that a healthy vaginal microbiome is not necessarily *Lactobacillus* dominated. Factors, such as ethnicities, contraceptive use and sexual behaviour, also contribute to the overall vaginal environment. A study of South African women found that only a minority (37%) had a *Lactobacillus* dominant vaginal microbiota (*Anahtar et al., 2015*). Findings like this challenge the traditional understanding of a

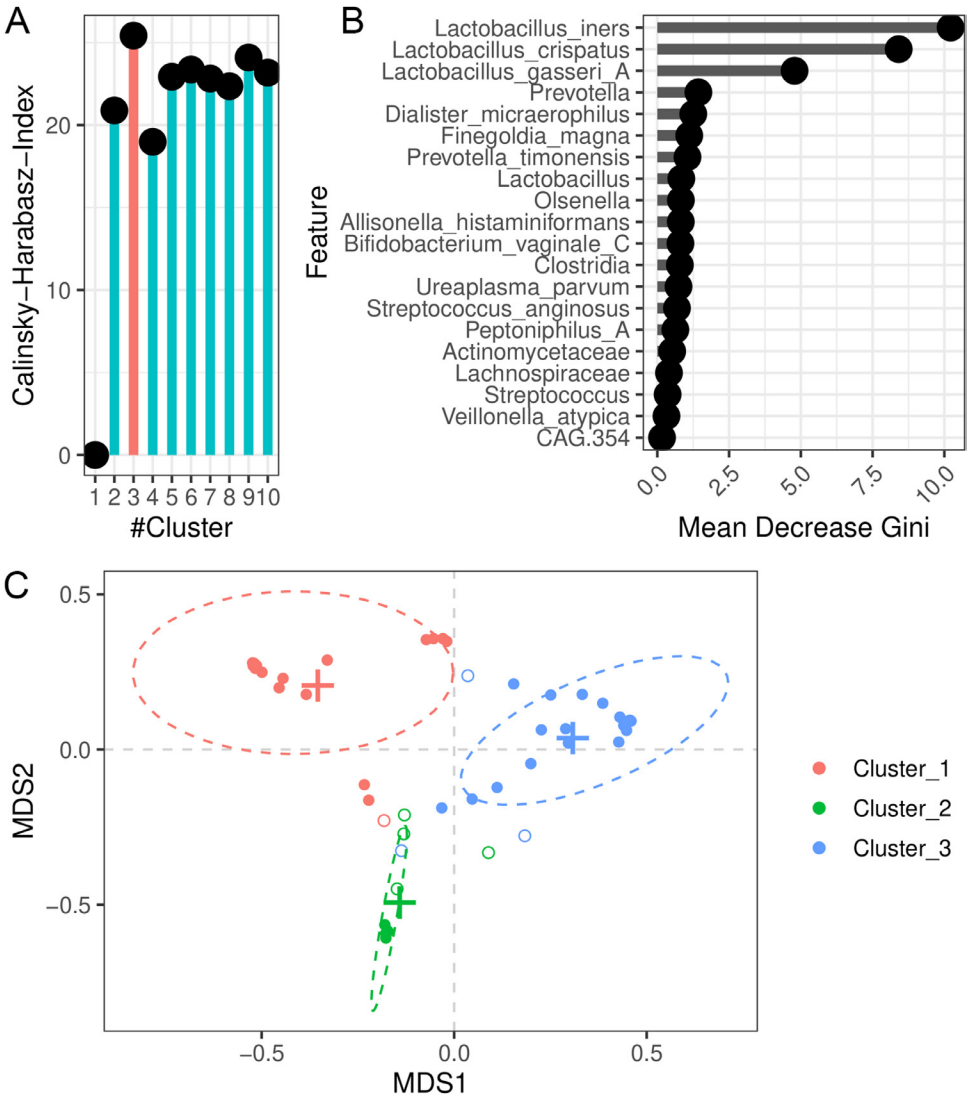

FIGURE 5 Cluster analysis. (A) Calinsky–Harabasz index of unsupervised partitioning around medoids. Orange bar = best result; (B) Gini index of bacterial species with largest influence on cluster membership; (C) multidimensional scaling (MDS) plot of Bray–Curtis dissimilarities. Samples are coloured by cluster membership. High confidence (>0.5), filled shapes; low confidence (<0.5), empty shapes.

healthy vaginal microbiome and require further investigation. Despite these novel findings, much evidence in reproductive medicine shows that a non-*Lactobacillus* dominant microbial flora is associated with lower implantation rates, pregnancy rates and live birth rates (*Moreno et al., 2016*). Hence, it is important to maintain a healthy vaginal microbiota to avoid dysbiosis states, such as bacterial vaginosis. Bacterial vaginosis directly affects fertility and reproductive health, as an ascending dissemination of the involved pathogenic species leads to tubal infertility and increases the risk of acquiring sexually transmitted diseases (*Mastromarino et al., 2014*). Interestingly, between 62 and 97% of all bacterial vaginosis cases are related to *Ureaplasma*, but its role as pathogenic is unclear (*Taylor-Robinson, 1996*). Yet, it has been related to implantation problems and miscarriages, and has been detected in the vaginal flora of women diagnosed with infertility (*Wee et al., 2018*). Furthermore, the stimulatory effect of *Ureaplasma species* on cytokine release (tumour necrosis factor-α, IL-8, IL-6), has been confirmed *in vitro* (*Namba et al., 2010*). Increased abundance of *U. parvum* are strongly correlated with a higher risk for spontaneous preterm birth, low birth weight and bronchopulmonary disease in the preterm neonate (*Viscardi, 2010*; *Donders et al., 2017*; *Rittenschober-Böhm et al., 2018*). Studies have shown that timing and duration of fetal exposure to *Ureaplasma*, and the fetal and maternal inflammatory response, play a key role for neonatal outcomes. Interestingly, the rate of *Ureaplasma* respiratory tract colonization increases with duration of rupture of membranes, indicating that *Ureaplasma* is vertically transmitted from mother to the child as a result of an ascending infection from the lower genital tract (*Grattard et al., 1995*; *Kafetzis et al., 2004*; *Viscardi, 2010*).

In the present study, we recognize a significant difference in the abundance of *U. parvum* between the probiotic group and the control group. In the probiotic group, the relative abundance of *U. parvum* remains low, whereas, in the control group, we report an increase of *U. parvum* abundance. Direct comparison of *U. parvum* abundance shows that the significant difference

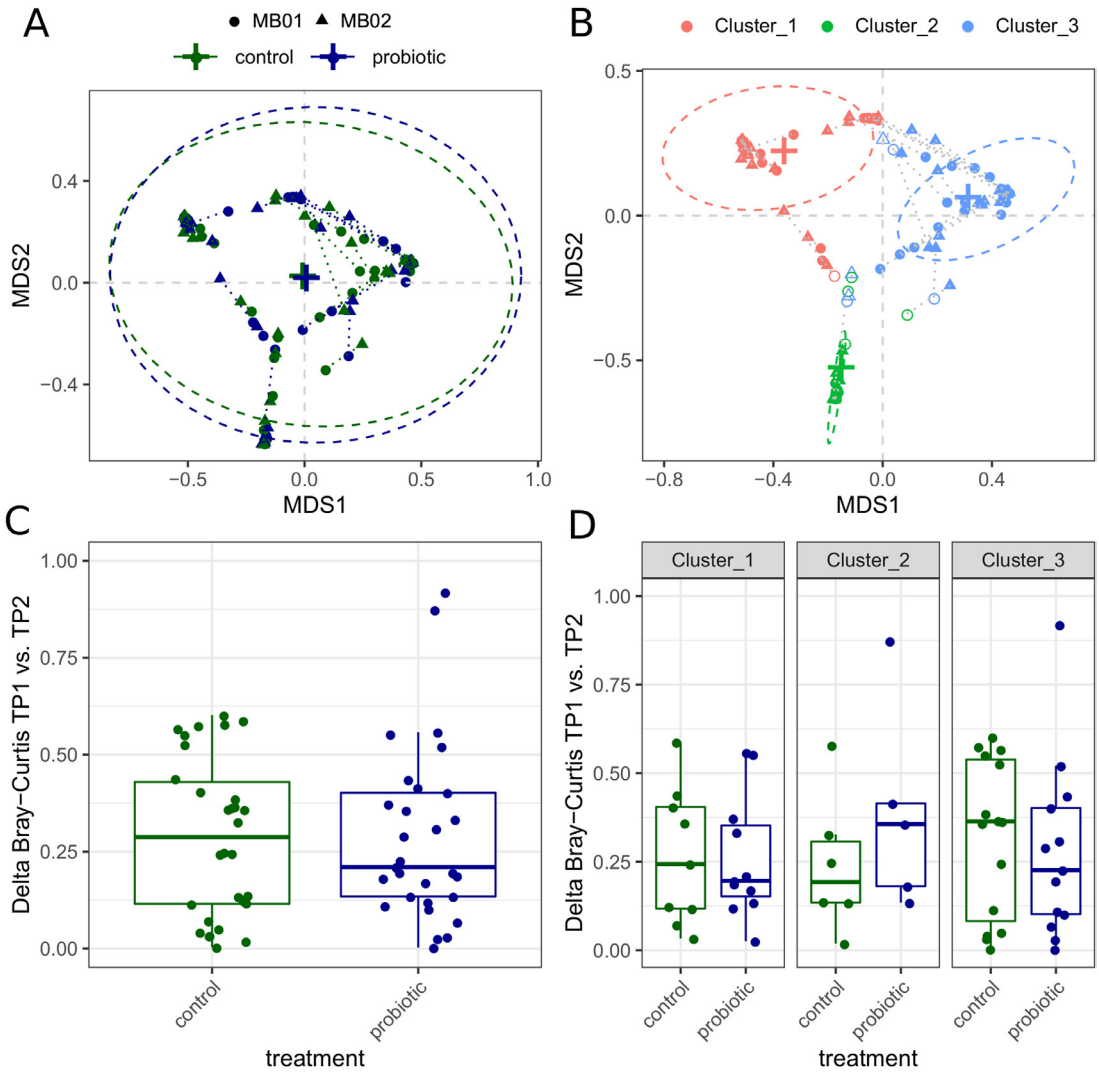

FIGURE 6 (A) Changes in beta (inter-sample) diversity as measured by Bray–Curtis dissimilarity before (circles) and after (triangles) the intervention period in the intervention and control groups; and (B) stability of Lactobacillus cluster membership. Filled shapes represent sample communities that could be assigned to one of the cluster with high confidence (>50%), samples from the same individual are connected by dashed lines. Intra-individual comparisons in Bray–Curtis dissimilarity before and after the intervention period in connection with (C) intervention arm and (D) Lactobacillus cluster membership before intervention. MB01, timepoint before intervention; MB02, timepoint after intervention. TP1, time period 1; TP2, time period 2.

between groups is caused by an increase of relative abundance in the non-treated control individuals, rather than a reduction in the probiotic treatment group. It is tempting to speculate that probiotics have a protective effect by temporarily hindering pathogenic strains to disseminate. The interaction of *Lactobacilli* and *Ureaplasma* has been studied recently by *Melgaço et al. (2018)*. They were able to demonstrate that *Lactobacilli* inhibited the death of vaginal epithelial cells after incubation with *U. parvum* and suggested their potential for maintaining a healthy vaginal environment, in line with our findings. One possible protective mechanism of *Lactobacilli* in the study by *Melgaço et al. (2018)* was competitive adhesion to host epithelial cells. The adhesion capacity and hydrophobicity of the probiotic strains suggested antagonistic effects on the adhesion of lipid-associated membrane proteins to the plasma membrane of the vaginal epithelium. This high hydrophobicity, coupled with the self-aggregation ability, may cause the lipid-associated membrane proteins to bind to the cell wall of these microorganisms rather than to the epithelial cell membrane (*Melgaço et al., 2018*). In addition, many antagonist effects of probiotic microorganism were described, such as competitive adhesion to the mucosa and epithelium; strengthening of the epithelial barrier; secretion of antibiotics substances, such as bacteriocins and organic acids; and modulation of the immune system (*Bermudez-Brito et al., 2012*).

The use of probiotics as a treatment for patients with dysbisis has been under debate for some time. As antibiotic treatments do not completely eradicate vaginal biofilms and negatively influence the existence of healthy vaginal microbiota (*Machado et al., 2016*; *Chetwin et al., 2019*), probiotics seem to be a good alternative or additional treatment option for patients suffering

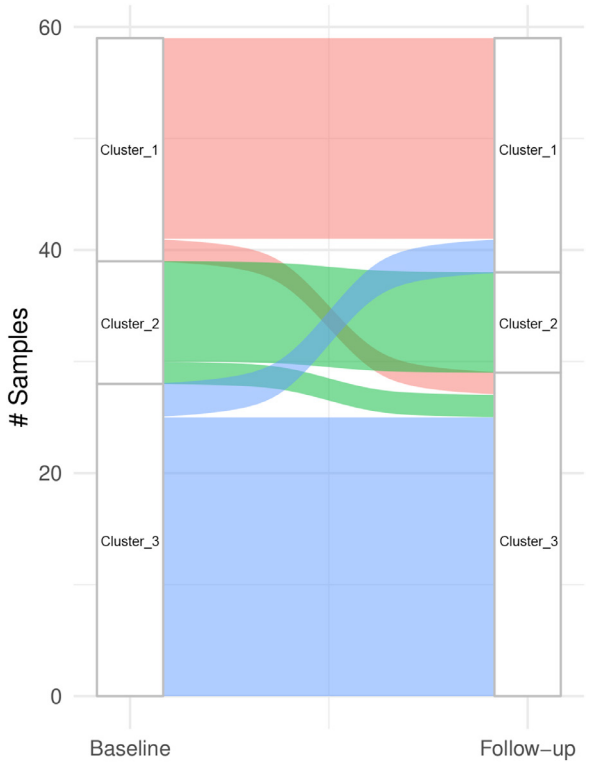

FIGURE 7 Between timepoint analysis of community stability of the whole cohort. MB01, timepoint before intervention; MB02, timepoint after intervention

from vaginal dysbiosis. Numerous meta-analyses, however, have concluded that probiotics only slightly enhance the temporary cure rate of dysbiotic states caused by bacterial vaginosis and vulvovaginal candidiasis and not changing alpha or beta diversity (*Xie et al., 2017*; *Buggio et al., 2019*), which is in line with our data.

The small sample size may be considered a study limitation; however, sound statistical analysis could be conducted for all analysed parameters. Furthermore, microbiota analysis was based on the amplification of variable regions of the 16S rRNA gene. These regions have been a mainstay of sequence-based bacterial analysis. Although high-throughput sequencing of the full genome would reveal additional information, the method used in the present study is considered convenient and powerful to infer likely taxonomy from the generated data (*Johnson et al., 2019*). In addition, the containment of *U. parvum* abundance is of nominal significance; therefore, a larger dataset in future studies should be envisaged to confirm this finding. The cause of infertility of the patients

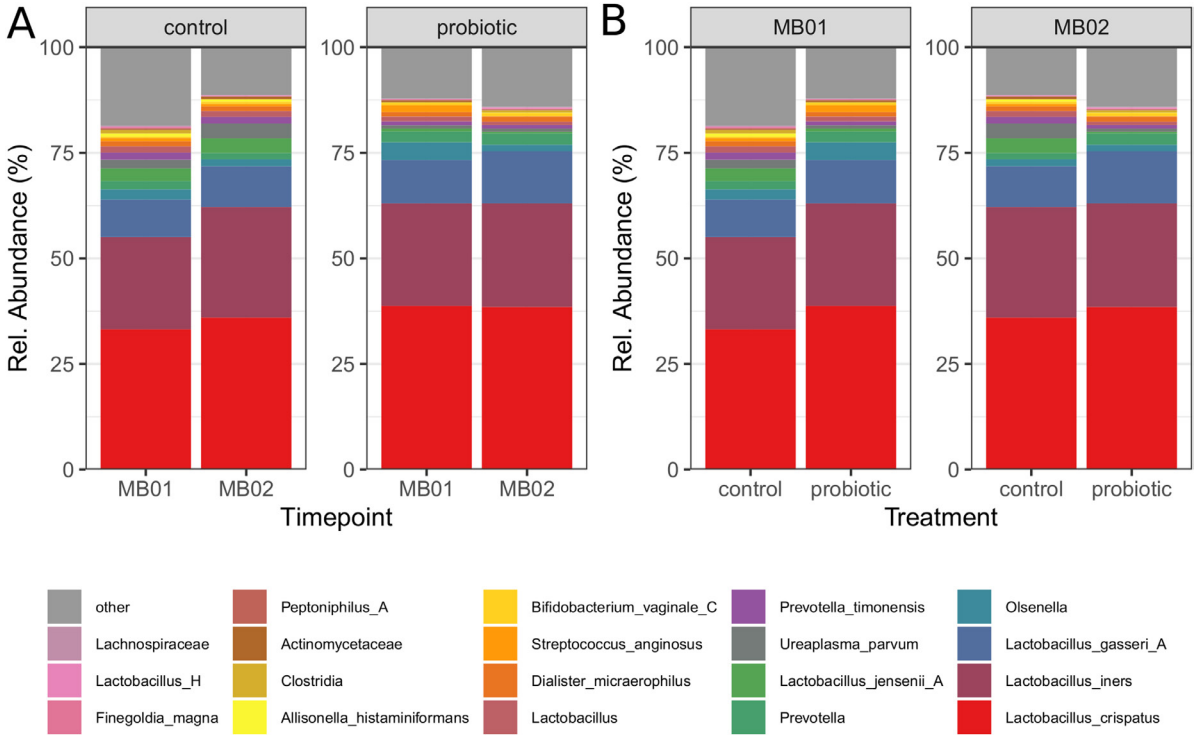

FIGURE 8 Overview of feature changes between (A) timepoints and (B) treatments. MB01, timepoint before intervention; MB02, timepoint after intervention

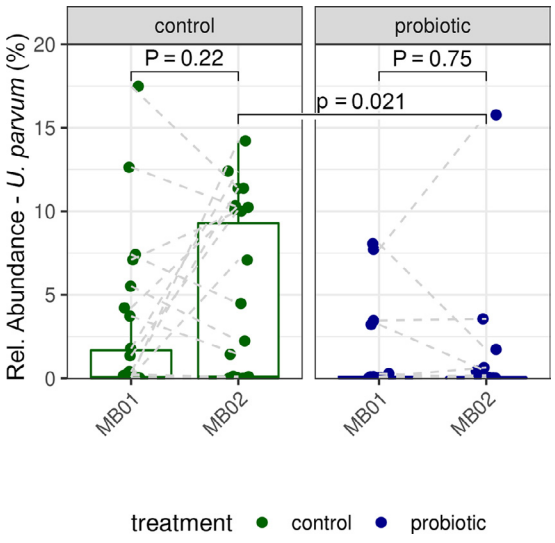

FIGURE 9 Between timepoint comparison of relative abundances of Ureaplasma parvum, separated by placebo or treatment. MB01, timepoint before intervention; MB02, timepoint after intervention

has not been considered, which may be seen as drawback of the study. Patients with polycystic ovary syndrome or endometriosis, both known to affect the microbiome in the reproductive tract, however, were excluded (*Salah et al., 2013*; *Chen et al., 2017*). Therefore, we created a female study population not affected by any obvious fertility issue and diagnosed with unexplained infertility or male factor infertility. The heterogeneity in study designs, especially in the composition of probiotic compounds, makes it difficult to achieve a consensus for probiotic administration. Therefore, future studies should attempt to adjust study designs to make results more comprehensible and comparable.

In conclusion, our results clearly show that probiotics do not influence alpha or beta diversity of the vaginal microbiome. Patients treated with probiotics showed contained growth of *U. parvum* compared with the control group. These results may provide novel insights into the interaction of *Lactobacillus* species and *U. parvum*. It is tempting to speculate that probiotics, especially *Lactobacillus* species, could produce a temporary protective effect of the vaginal microbiota by containing or suppressing non-beneficial bacteria, such as *U. parvum*. Further research is warranted, and professional medical associations should issue well-defined recommendations on the use of probiotics in gynaecological disorders, which may lead to novel approaches in infertility therapy.

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