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ORIGINAL ARTICLE

Ligilactobacillus salivarius PS11610 exerts an effect on the microbial and immunological profile of couples suffering unknown infertility

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Abstract

Problem: Unknown or idiopathic infertility has been associated with urogenital tract dysbiosis, reducing pregnancy and delivery ratios during assisted reproductive treatments (ART). The *Ligilactobacillus salivarius* PS11610 strain has shown extraordinary antimicrobial activity in vitro against urogenital pathogens as well as other probiotic characteristics. Therefore, an intervention study was performed to evaluate the effect of *L. salivarius* PS11610 on the microbial composition of urogenital tract in infertile couples with bacterial dysbiosis.

Method of study: Seventeen couples undergoing ART diagnosed with unknown infertility were selected. After confirming urogenital dysbiosis, they started a 6-month treatment with *L. salivarius* PS11610 (1 dose/12 h for female and 1 dose/24 h for male). Vaginal, seminal, glans, uterine and plasma samples were collected for determination of the microbiome and immune profile at the beginning and the end of the treatment.

Results: Supplementation with *L. salivarius* PS11610 significantly modified the urogenital microbiome composition in male and female samples, solving dysbiosis of 67% of the couples. Pathogens disappeared from the vaginal samples whereas Lactobacilli percentage increased after 3 and 6 months of treatment. Moreover, *L. salivarius* PS11610 changed the uterine microbiome that could be associated with a change of the uterine immune profile. Additionally, the probiotic intake could be associated with the observed change in the systemic immunological profile of couples. Finally, the pregnant and delivery ratio were improved.

Conclusions: Probiotic supplementation with *L. salivarius* PS11610 improved the male and female urogenital tract microbiome, modulating the immune system and increasing pregnancy success in couples undergoing ART.

KEYWORDS
dysbiosis, genital tract microbiota, immune profile, *Lactobacillus*, *Ligilactobacillus*, probiotics, unknown infertility

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1 | INTRODUCTION

Human infertility is defined by the World Health Organization (WHO) as the failure to achieve a clinical pregnancy after 12 months of regular unprotected sexual intercourse.[1] Infertility causes are associated to male, female, or combined failure. However, approximately 15%–30% of infertility problems have unknown causes and are called idiopathic.[2]

In the last decades, numerous publications have broken the old paradigm that considered the urogenital tracts as sterile, demonstrating that microorganisms present in the urogenital tract represent the 9% of the whole human microbiome.[3–6] Healthy urogenital microbiome improves implantation rate and pregnancy outcomes, whereas 40% of dysbiosis prevalence is observed in women under assisted reproductive treatment (ART).[5,7–9]

In females, urogenital microbiota is characterized by low bacterial diversity and *Lactobacillus* genus predominance (>90%).[8,10,11] *Lactobacilli* protect against pathogens by lactic acid production, which decreases the vaginal pH. Moreover, *Lactobacilli* inhibit the pathogen's colonization by blocking the adhesion molecules in epithelial cells or producing bacteriocins and/or H_2O_2.[12–14] Moreno and collaborators showed better implantation rates in women with endometrial microbiota dominated by *Lactobacillus* during In Vitro Fertilization (IVF).[8]

Bacterial vaginosis (BV) is the most common urogenital dysbiosis in women. BV is a polymicrobial disease characterized by the replacement of *Lactobacillus* by a plethora of pro-inflammatory microorganisms such as *Gardnerella vaginalis*, *Atopobium vaginae*, *Prevotella spp*, *Veillonella spp*.[14–16] Although women may be asymptomatic, BV induces several symptoms such as vaginal itching, pain, or vaginal secretions, as well as increases the probability of new infections, infertility and preterm birth or miscarriage.[17–19]

In males, the urogenital microbiome plays an important role in spermatogenesis. The absence of *Lactobacilli* and the increase of *Prevotella* spp. has been associated with alterations in the semen quality and fertility.[4,20–23]

It has been shown that oral or vaginal probiotic treatment, mainly with *Lactobacillus*, recovers a healthy vaginal microbiota without safety concerns.[24–29] However, the direct effect of probiotic supplementation in fertility and endometrial microbiome have not been deeply characterized.[30] In males, probiotic treatment improves sperm quality markers such as volume, concentration, velocity or oxidative stress in asthenozoospermic donors.[31–33]

Despite the appearance of new probiotic-based treatments, diseases associated to male and female urogenital dysbiosis are mainly treated with antibiotic therapy.[27,34]

Ligilactobacillus salivarius (formerly named as *Lactobacillus salivarius*) PS11610 strain has shown extraordinary antimicrobial activity in vitro against pathogens associated with bacterial dysbiosis of the female and male genital tract. Therefore, the purpose of this study was to investigate the effect of the probiotic strain *L. salivarius* PS11610 in couples with idiopathic infertility and genitourinary dysbiosis. We hypothesized that couples with idiopathic infertility and genitourinary dysbiosis who are treated with probiotics would solve the dysbiosis and improve the pregnancy rate.

2 | MATERIALS AND METHODS

2.1 | Study design

An intervention study was performed between January 2019 and May 2020 in the Reproduction Section of the Hospital Universitario La Paz. Research Ethics Committee of the Hospital Universitario La Paz approval was obtained on the 13th of July 2018. The study was conducted according to Good Clinical Practice (GCP) defined by the International Council for Harmonization of Technical Requirements for Pharmaceuticals for Human Use (ICH E6 (R2), 2016), in compliance with the Declaration of Helsinki developed by the "World Medical Association Declaration of Helsinki" (64th WMA General Assembly, Fortaleza, Brazil, October 2013). This study was registered on clinicaltrials.gov under the number NCT03701893.

Couples with ages between 20 and 40 years old under an ART: Artificial Insemination (AI) or IVF were eligible to participate in this trial. In order to include couples with unknown infertility, a wide range of exclusion criteria were selected: previous history of anovulation, hyperprolactinemia, hypogonadotropic hypogonadism, hyperandrogenisms, polycystic ovary syndrome, endometriosis, pelvic adhesions, myomas, polyps and/or uterine synechia, diagnosis of tubal factor (hydrosalpinx, tubal obstructions), low ovarian reserve, azoospermia, sperm motility (A + B) < 25%, sperm morphology ≤2%, chronic diseases that cause intestinal malabsorption, congenital or acquired immunodeficiency or obesity (IMC ≥ 30). Written informed consent was obtained from each participant.

Participants attended four visits at the hospital: at the beginning of the study (visit 1 or preselection visit); after the diagnose of bacterial dysbiosis in the male and/or female genital tracts (visit 2); 3 months after the initiation of the probiotic intake (visit 3), and at the end of the 6-months treatment period (visit 4).

Because the main objective of this study was to determine the effect of a probiotic strain on the genitourinary dysbiosis, couples had to be diagnosed for that condition before the treatment intake. Therefore, after visit 1, women collected vaginal samples with swabs weekly for 1 month. At the end of this month, men collected a glans swab and a semen sample and together with the previous collected vaginal samples, that were kept in frozen conditions, were all sent to the laboratory of Probisearch SLU (Tres Cantos, Madrid) to determine the presence of bacterial dysbiosis.

Bacterial dysbiosis criteria were stated having in consideration previous published microbiological data.[35–38] The criteria were bacterial counts below 50 CFU in vaginal exudates, *Lactobacilli* counts during ovulation below 10^2 CFU; corynebacterial, enterococci and/or *Staphylococcus aureus* counts over 10^5 CFU in male and/or female samples and presence of *Actinomyces neuii*, *G. vaginalis* and/or *Enterobacteriaceae* in male and/or female samples.

After bacterial dysbiosis diagnose, female participants took two capsules of *L. salivarius* PS11610 (10^9 CFU) every day (1/12 h) whereas male participants took one capsule every day (1/24 h) for 6 months. In the case of pregnancy, only women took one capsule every day (1/24 h) during the first 12 weeks of gestation and men stopped the treatment. The probiotic product was manufactured by Zinereo Pharma SL, and storage at 4–8°C.

In the case of pregnancy, two additional visits were done, one upon knowing the pregnancy status and the other one 12 weeks later.

2.2 | Clinical and safety parameters

Relevant medical history and pre-existing conditions were recorded by the gynecologists at visit 1, including history of miscarriages and recurrent infections.

Adverse events (AEs) were coded according to the Medical Dictionary for Regulatory Activities (MedDRA version 17.1). System Organ Classes (SOC, the most general level), and the Preferred Term (a distinct descriptor for a symptom) were used to code the AE data into an international standardized medical terminology.

2.3 | Samples' collection and analysis

To evaluate dysbiosis status, vaginal, glans, and semen samples were collected the day before visits 3 and 4. Moreover, to describe the local and systemic immune profile, a uterine and a blood sample from both members of the couple were collected in visits 2 and 4. Blood samples were freshly processed to obtain plasma.

Uterine samples were collected with an *Endoflower* device that protects the endometrial sample in a cannula through the vaginal cavity avoiding the contamination with the vaginal microbiota. Samples were frozen at -20°C after collection and were kept at that temperature until analysis.

The primary outcome was the effect of the probiotic strain *L. salivarius* PS11610 on the bacterial composition of female and male genital tracts. Therefore, samples were analyzed with culture-dependent techniques. First of all, vaginal, glans and semen samples were diluted in peptone water and spread onto CNA (Columbia nalidixic acid, BioMérieux, Marcy l'Etoile, France), MCK (MacConkey culture media, BioMérieux, Marcy l'Etoile, France), GAR (Gardnerella agar, BioMérieux, Marcy l'Etoile, France) and MRScysBP (de Man, Rogosa, Sharp (Oxoid, Basingstoke, UK) + .25% cysteine (w/v) (Sigma-Aldrich, St. Louis, USA) + .05% bromophenol blue (w/v) (Sigma-Aldrich)) for selective isolation and quantification of the present bacteria. After incubation, identification of the isolates was determined by MALDI-TOF, mass spectrometry on a Vitek- MS™, BioMérieux, Marcy l'Etoile, France) or by partial sequencing of the 16S rRNA gene.

Additionally, the microbiome composition of uterine, vaginal, glans and semen samples was characterized by 16S rRNA sequencing with Illumina technology. Initially, DNA from uterine, vaginal, glans and semen samples were extracted using QIAAMP DNA MINI KIT (Qiagen, Hilden, Germany). Subsequently, the bacterial 16S rRNA gene was amplified using primers that flanked the variable regions V3 and V4. The PCR primer sequences were V3V4-CS1 (ACACTGACGACATGGTTCTACACCTACGGGNGGCWGCAG) and V3V4-CS2 (TACGGTAGCAGAGACTTGGTCTGACTACHVGGGTATCTAATCC). Resulting reads of quality controls were assembled and taxonomically classified by comparison with Ribosomal Database Project using a Bayesian classification method and a level of similarity of at least 97%.

2.4 | Immunological analysis

The concentration (pg/ml) of APRIL/TNFSF13, BAFF/TNFSF13B, sCD30/TNFRSF8, sCD163, Chitinase 3-like 1, gp 130/sIL-6Rβ, INF-α2, INF-β, INF-γ, IL-2, sIL-6Rα, IL-8, IL-10, IL-11, IL-12p40, IL-12p70, IL-19, IL-20, IL-22, IL-26, IL-27p28, IL-28A/INF-λ2, IL-29/INF-λ1, IL-32, IL-34, IL-35, LIGHT/TNFSF14, MMP-1, MMP-2, MMP-3, Osteocalcin, Osteopontin, Pentraxin-3, sTNF-R1, sTNF-R2, TSLP, TWEAK/TNFSF12, TGF-β1, TGF-β2, TGF-β3 was measured in uterus and plasma samples. Prior to analysis, the uterine samples were suspended in peptone water, 1:1 (w/v), homogenized and centrifuged for 15 min at 14 000 × g at 4°C; then supernatants were collected. The analysis was carried out in duplicate using Bio-Plex Pro™ Human Inflammation Panel 1 (Bio-Rad Laboratories Inc., Hercules, CA, USA) and Milliplex Map TGFß (Merk., Darmstadt, Germany) Assay kits on a Luminex 200 (Luminexcorp., The Netherlands) following the manufacturer's instructions.

2.5 | Statistical analysis

Clinical and safety outcomes were evaluated in the total population (couples who consent participation).

The efficacy analysis was performed with the modified intention to treat population (couples who finished the treatment). Normally distributed data were reported as means and standard deviations (SD), and non-normally distributed data by medians and quartile ranges (Q1–Q3). The qualitative variables were described using absolute and/or relative frequencies.

The Friedman test was used to evaluate the evolution of the number of dysbiosis criteria met by couples throughout the study. Cultivable bacteria were grouped as Pathogens (*G. vaginalis*, *Actinomyces neuii*, *Klebsiella pneumoniae*, *Escherichia coli*, *Corynebacterium amycolatum/xerosis*, *Corynebacterium glucuronolyticum*, *Corynebacterium aurimucosum*, *Corynebacterium simulans*, *Corynebacterium tuberculostearicum*, *Corynebacterium spp.*, *Staphylococcus aureus*, *Enterococcus faecalis* and *Enterococcus faecium*), Staphylococcus spp., Streptococcus spp. and Others in order to evaluate de bacterial dysbiosis of vagina, glans and semen samples. Other secondary qualitative results were analyzed by Chi-squared or Fishert's test. The secondary quantitative results were analyzed by ANOVA or Kruskal–Wallis test according to data

distribution. When needed, posthoc pairwise Tukey (HSD) test or Nemenyi test with Holm correction were used, respectively. Exploratory multifactorial principal component analysis (PCA) was performed to show the global impact of the supplementation with the probiotic strain on the male urogenital bacterial composition, uterine and vaginal microbiome and the immunological profiles of plasma and uterine samples.

Results were considered statistically significant with *p*-value lower than .05. Statistical analyses were conducted using R (3.5.1, R-project, http: www.R-project.org) and GraphPad Prism 8.0 (GraphPad Software, La Jolla, USA).

3 | RESULTS

3.1 | Study population

In this study, a total of 17 couples were contacted and recruited. Among them, 14 began the intervention period (intention to treat, ITT) and nine completed the 6-months intervention (modified intention to treat, mITT). Three couples withdrew before probiotic intake and two before the end of the trial. All participants were Caucasian with a median age of 36 years for males and 35 years for female.

3.2 | Safety data

In this study, five AEs were reported and classified according to MedDRA as nasal polypectomy, urinary infection, right iliac fossa pain, vaginal candidiasis and gluteal abscess. Moreover, a serious adverse event (SAE) was reported. One participant suffered a cornual ectopic pregnancy and required of one hospitalization day, methotrexate treatment and 44 days of medical monitoring.

The concomitant medication reported was in accordance with the fertility treatment and AEs reported during the study.

All AEs were classified as mild intensity and, including the SAE, unrelated with the probiotic treatment.

3.3 | Microbiological analysis

The bacterial composition of vaginal, glans and seminal samples from enrolled couples was analyzed at the beginning of the study and after 3 and 6 months of treatment. Dysbiosis was evaluated by culture-dependent techniques. All analyzed couples at the beginning of the study (14) presented bacterial dysbiosis, reporting 37 positive dysbiosis criteria (Table 1). Interestingly, positive dysbiosis criteria significantly decreased to 10 in 10 couples analyzed after 3 months of probiotic intake and to 6 in 9 couples after 6 months ($p = .030$) (Table 1). In addition, the median of dysbiosis criteria per couple decreased significantly after 6 months of probiotic treatment ($p = .034$) (Figure 1A).

The analysis of vaginal microbiota composition, after 3 and 6 months of probiotic intake, shows a significant reduction of not only total bacterial counts, but also *Staphylococcus* spp., *Streptococcus* spp., Pathogens and Others potentially harmful bacterial populations ($p = .014$, $p = .001$, $p = .003$, $p = .032$, respectively) (Table 2). The percentage of *Lactobacillus* in relation to the total bacterial counts increased in the vaginal microbiome (Figure 1B). The mean of *Lactobacillus* spp. percentage in vaginal samples increased from 10 (1% of total bacteria at the beginning of the study) to 38 (5% after 3 months of probiotic intake) and 61 (7% after 6 months of study) (Figure 1B). In males, although significant changes of the urogenital microbiota composition after probiotic treatment were not found, pathogens and *Staphylococcus* spp. slightly decreased (Table 2).

The bacterial composition change was evaluated using a PCA. Vaginal microbiota samples mainly changed its distribution in the PCA because of the increase of the *Lactobacillus* genus (Figure 2A). In male samples, a change in the axis of the ellipsoid was observed indicating

TABLE 1 Number of couples that fulfill dysbiosis criteria at the beginning of the study and after 3 and 6 months of probiotic intake

Dysbiosis criteria	Visit 2 ($N = 14$)	Visit 3 ($N = 10$)	Visit 4 ($N = 9$)
<50 CFU in vaginal samples	2	0	1
<100 CFU Lactobacilli in Luteus Phase III < > Ovulation	4	1	1
Male and/or female samples with counts $>10^5$ CFU of			
Corynebacterium	12	4	3
Enterococcus	4	2	1
Staphylococcus aureus	1	0	0
Detection in male and/or female samples of			
Actinomyces neuii	4	2	0
Gardnerella vaginalis	6	0	1
Enterobacteriaceae	4	1	0

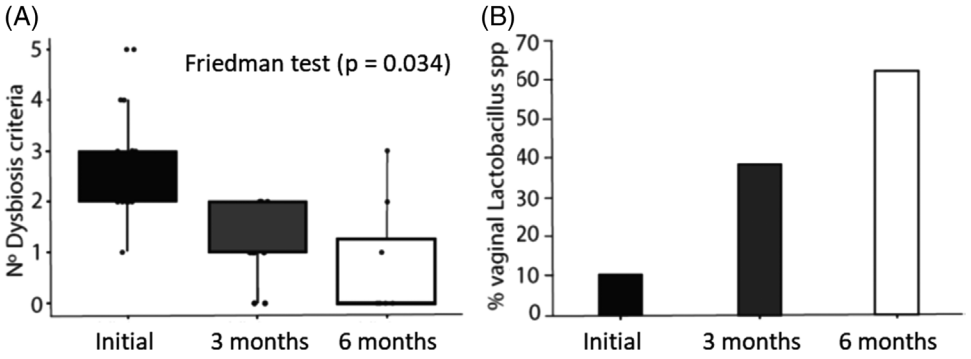

FIGURE 1 (A) Dysbiosis criteria quantification per couple at the beginning, after 3 and 6 months of treatment. Data were tested by Friedman test ($p = .034$). (B) Percentage mean of *Lactobacillus* spp. in vaginal samples microbiome at the beginning, after 3 and 6 months of treatment

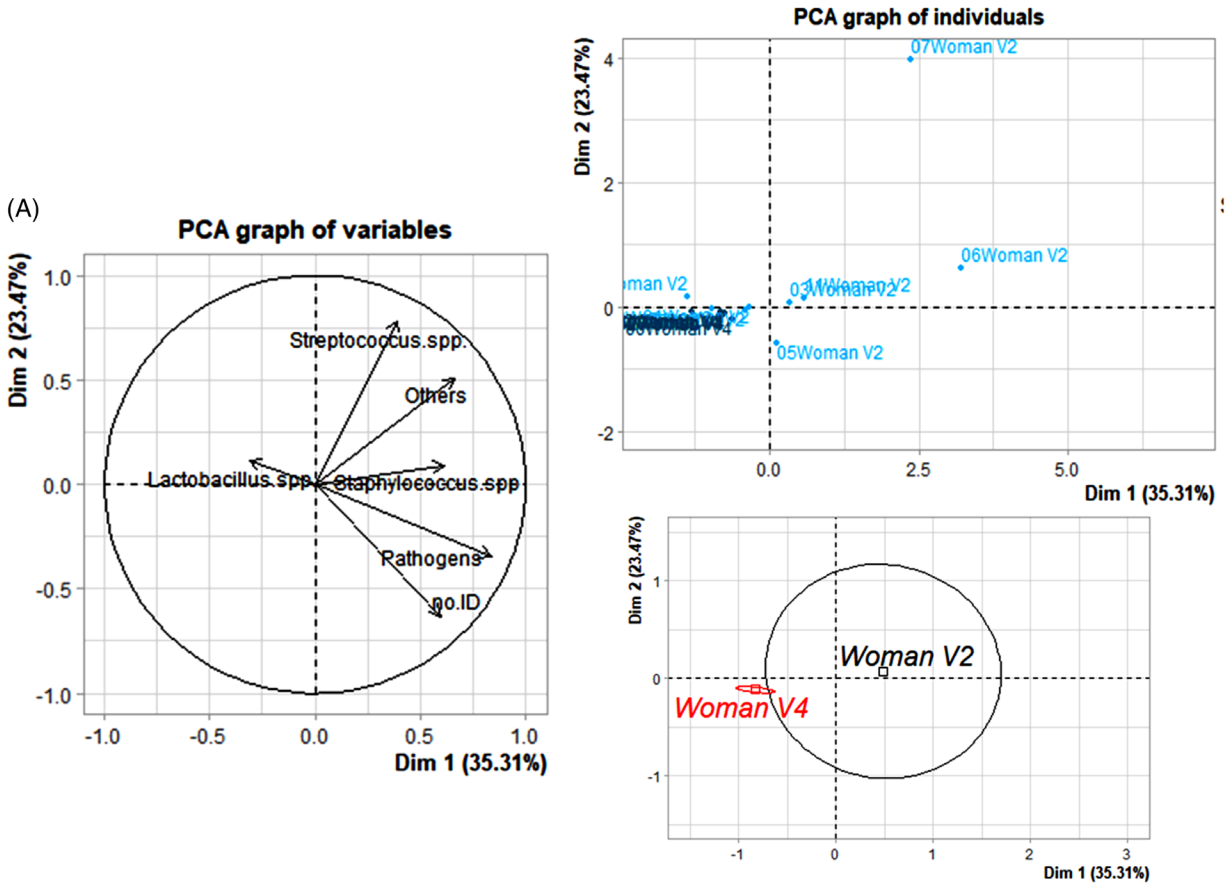

FIGURE 2 Principal component analysis (PCA) plot with the cultured bacterial composition of urogenital mucosa from female (A) and male samples (B) at the beginning (black) and at end of treatment period (red). The values on each axis label represent the percentage of the total variance explained by that axis. The ellipses represent the 95% confidence interval of the centroids for each group plotted

a change in the microbial composition although not significantly (Figure 2B).

To have a wider picture of the bacterial composition of the genital samples before and after the product intake the 16S rRNA gene sequencing technique was used (Supplemental Figure S1A–D). Regarding male samples, glans and semen, a mean of 103 029 (±15 378) and 95 384 (±9922) reads were obtained for every sample and an average of 93% and 92% of the reads were assigned at genus level, respectively. In relation to female samples, uterus and vagina, a mean of 70 664 (±31 418) and 82 430 (±12 603) reads were obtained for every sample and an average of 87% and 94% were assigned at genus level, respectively.

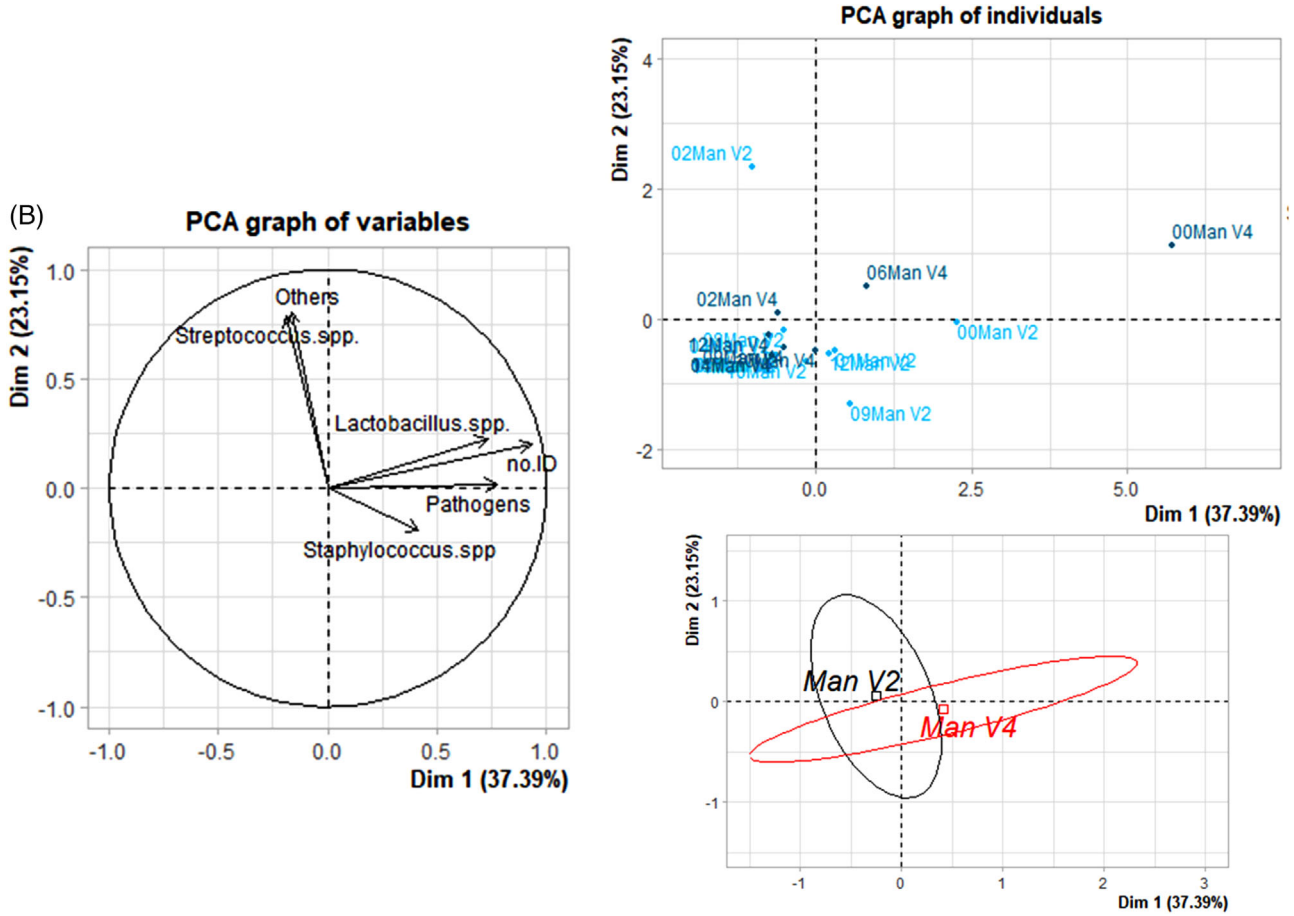

FIGURE 2 Continued

With this technique, male samples showed higher bacterial diversity at genus level than female samples (Supplemental Figure S1A–D). Shannon Diversity Index ranged from 1.004 to 1.151 in male samples during all intervention while in vagina it decreased from .228 (initial visit) to .190 (final visit) and in uterus from .431 (initial visit) to .325 (final visit).

No statistically significant changes were observed at phylum and genus level when comparing samples before and after the treatment although PCA plots revealed a change in the microbiome composition of uterus samples after 6 months of probiotic treatment (Figure 3). Initial (V2) and final (V4) uterus samples were located completely separated in the graph. Vaginal samples did not showed a change at genus level and clustered according to their time of collection (Figure 3).

3.4 | Immunological analysis

The concentration of 40 pro and anti-inflammatory markers was determined in male and female blood samples at the beginning and after 6 months of the intervention with *L. salivarius* PS11610 (Table 3).

There was a significant decrease of the proinflammatory markers IL-12p40, IL29/INFλ1, IL-34 in both members of the couples and IL28A/INFλ2 only in males. In addition, results showed lower levels of IL12-p70, pentraxin-3 and osteocalcin in females ($p < .1$). Moreover, lower levels of anti-inflammatory cytokines TGFβ1, TGFβ2, and IL-35 were observed in women after 6 months of probiotic intake (Table 3).

The immune response is a complex and interconnected system, in which different cocktails of cytokines play important roles in distinct effector cells. For this reason, we decided to perform relational analysis such as PCA which indicated a change from a proinflammatory to an anti-inflammatory profile of the couples at systemic level at the end of the study (Figure 4).

To address the immunological status at local level, we determined the concentrations of the same 40 cytokines in endometrial samples at the beginning and after 6 months of the treatment (Supplementary Table S1). Although only Chitinase 3-like 1 (CHI3L1) was increased ($p = .072$) after *L. salivarius* PS11610 treatment, PCA showed that the local proinflammatory profile was modified after the treatment (Supplementary Figure S2).

3.5 | Pregnancy outcomes

In this study, four women became pregnant, three during the first 3 months of the intervention period and the fourth at the end of the

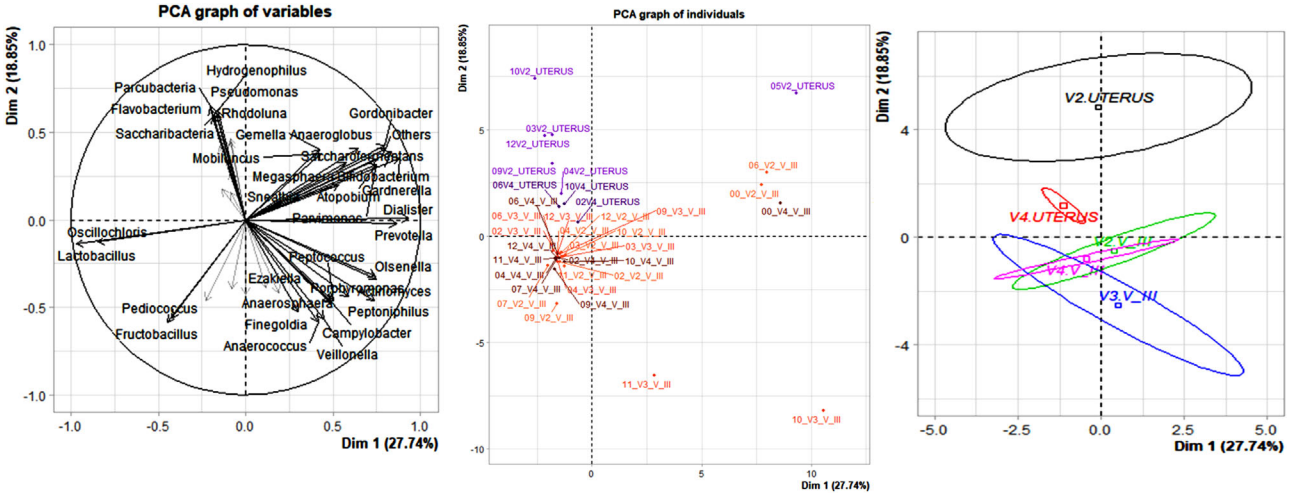

FIGURE 3 Principal component analysis (PCA) plot shows the uterine and vaginal microbiome composition at genus level. Samples from V2 were collected at the beginning of the treatment, V3 after 3 months of probiotic intake and V4 after 6 months. The values on each axis label represent the percentage of the total variance explained by that axis. The ellipses represent the 95% confidence interval of the centroids for each group plotted

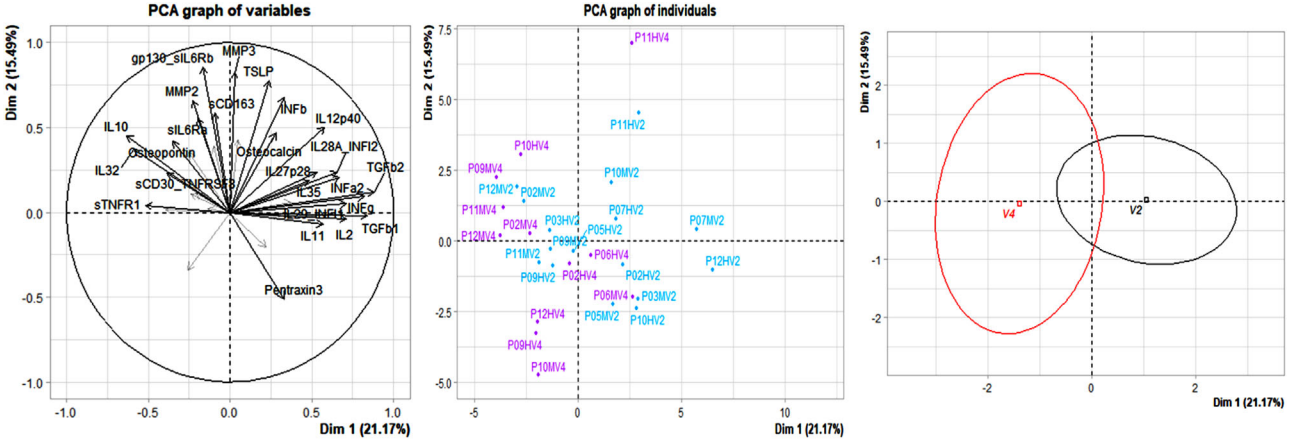

FIGURE 4 Principal component analysis (PCA) plot shows the systemic immunological profile of males and females analyzed by multiplex system at the beginning (black) and at the end of the treatment period (red). The values on each axis label represent the percentage of the total variance explained by that axis. The ellipses represent the 95% confidence interval of the centroids for each group plotted

second 3 months of the intervention period. More precisely, 1 after 2 months of treatment and an IA cycle that unfortunately suffered an ectopic pregnancy. One after three months of the probiotic intake and an IA cycle that delivered successfully. This woman suffered three previous IA failures before the study participation. One after three weeks of probiotic intake, spontaneously that gave birth successfully. One spontaneously after five and a half months of probiotic intake and gave birth successfully.

4 | DISCUSSION

This study investigated the effect of the probiotic strain *L. salivarius* PS11610 in the genital dysbiosis of couples with problems of fertility. All recruited couples (17) met at least one of the dysbiosis criteria established in this study, indicating a high prevalence of urogenital dysbiosis in couples with idiopathic infertility. The results obtained in this study by culture-dependent techniques and 16s rRNA sequencing showed that the treatment with *L. salivarius* PS11610 modified the microbiota composition of the vagina, uterus, semen, and glans samples, solving the dysbiosis in 88.9% of the couples. Additionally, a change in the immunological profile at local and systemic levels was detected. Consequentially, *L. salivarius* PS11610 intake slightly improved the pregnancy and delivery rates in idiopathic infertile couples during assisted reproductive processes.

Metagenomic analysis indicated a modification of the microbiota composition, eliminating pathogens and increasing *Lactobacillus* genus proportion. The change in the uterine microbiome, after the probiotic

TABLE 2 Analysis of the bacterial genera detected in male and female samples (Log10 CFU/ml) by culture dependent techniques at the beginning, after 3 and 6 months of probiotic treatment

	Male				Female			
	Initial	After 3 months of treatment	After 6 months of treatment	*p*-Value*	Initial	After 3 months of treatment	After 6 months of treatment	*p*-Value*
	Median (Q1–Q3)				Median (Q1–Q3)			
Total counts	7.14 (6.21–7.59)[a]	6.18 (5.02–6.92)[a]	6.89 (5.54–7.34)[a]	.543	6.72 (6.16–7.57)[a]	3.90 (3.70–5.85)[b]	5.46 (3.88–6.29)[a,b]	.014
Pathogens	6.02 (4.40–7.13)[a]	4.79 (3.87–5.85)[a]	3.97 (3.06–6.07)[a]	.273	6.10 (4.20–7.03)[a]	.05 (.05–2.70)[b]	.05 (.05–3.21)[b]	.003
Staphylococcus spp.	4.28 (3.20–4.98)[a]	3.83 (3.04–4.27)[a]	3.02 (2.06–5.37)[a]	.765	5.00 (3.60–5.44)[a]	.05 (.05–2.70)[b]	.05 (.05–.05)[b]	.001
Streptococcus spp.	2.65 (1.70–3.22)[a]	.05 (.05–.05)[a]	3.00 (2.04–4.16)[a]	.147	.05 (.05–4.02)[a]	.05 (.05–.05)[a]	.05 (.05–.05)[a]	.152
Others	4.63 (.05–6.88)[a]	6.18 (3.13–6.87)[a]	5.95 (4.51–6.96)[a]	.686	4.85 (3.18–6.02)[a]	.05 (.05–4.18)[a,b]	.05 (.05–.94)[b]	.032

Note: Different superscripts letters mean differences in the Nemenyi post hoc test using holm method for multiplicity correction.
Group "Pathogens" includes *Gardnerella vaginalis*, *Actinomyces neuii*, *Klebsiella pneumoniae*, *Escherichia coli*, *Corynebacterium amycolatum/xerosis*, *Corynebacterium glucuronolyticum*, *Corynebacterium aurimucosum*, *Corynebacterium simulans*, *Corynebacterium tuberculostearicum*, *Corynebacterium* spp., *Staphylococcus aureus*, *Enterococcus faecalis* and *Enterococcus faecium*.
Group Staphylococcus spp. only includes those species that are negative coagulase.
*Kruskal–Wallis test.

intake, is one of the main findings of this study. It has been shown that different mechanisms contribute to uterine microbiome formation and modification. Despite the connection between uterus and vagina through cervical canal, they only share 30% of the microbiota. Probiotics can reach the vagina and finally the uterus by rectal colonization but also internally from as the gut.[3,39,40]

Recent studies have proposed *Lactobacillus* spp. as a potential marker of vaginal health and fertility. The dominance by four species of *Lactobacillus* in the female urogenital microbiome, is associated with better rates of embryonic implantation and protection against pathogens.[8,11] Our obtained data using dependent culture techniques reported an increase of *Lactobacillus* rate in vaginal samples after 3 and 6 months of the probiotic intake. Similarly, metagenomic data showed an increasing trend of *Lactobacillus inners* level, which is one of the four dominant species of *Lactobacillus* in the urogenital tract and have been identified previously as a potential fertility marker in idiopathic cases.[41]

In male microbiome, levels of pathogens and staphylococci were slightly lower but significant differences were not observed after the probiotic treatment. One of the reasons of this unexpected result may be due to the lower probiotic dosage administrated to men in the study (1 capsule/24 h). Moreover, the different microbial colonizing mechanism of male genitourinary tract can contribute to the minor effect of probiotic treatment. Although most fertility treatments do not take men into account, we consider that their treatment is necessary, not only to improve their fertility markers but also to prevent the spread of pathogens during sexual intercourse.[42–44]

In the current trial, we have characterized the immunological profile of the couples in plasma. Initially, couples showed a proinflammatory status when analyzing their systemic immune profile. It has been described that obesity, a Western diet, psychological stress and a lack of exercise are associated with impaired intestinal mucosa barrier function ("leaky gut").[45] This condition may result in the passage of endotoxin-containing gut bacteria into the systemic circulation triggering inflammation and impaired testicular function.[46] Our results showed an association between the reduction of inflammatory cytokines and the probiotic treatment. IL-12p40 and IL12-p70 are implicated in the activation of proinflammatory lymphocytes Th1 and Th17 whereas IL-34 is involved in inflammatory macrophages activation. Surprisingly, lower levels of anti-inflammatory cytokines TGFβ1, TGFβ2, and IL-35 were observed in women after 6 months of probiotic intake. However, due to the pleiotropic effect of TGFβ1 and TGFβ2, their role in fertility remains partially unclear.[47]

Moreover, exploratory PCA showed a change from proinflammatory to an anti-inflammatory profile. Previous studies have reported a high INFγ/IL10 ratio in idiopathic infertile women and an association with miscarriage.[48,49] Thus, a healthy immune system may contribute to solve infertility in both genders and to improve implantation rates.

Endometrium has been proposed as a tertiary lymphatic organ like structure. The specific cytokines balance prevents infections, stimulates embryo implantation and pregnancy progression.[50,51] Our analysis of inflammatory markers showed an altered proportion of cytokines after probiotic treatment, but not significative differences

TABLE 3 Analysis of the immunologic systemic profile in plasma samples at the beginning and after 6 months of probiotic treatment

	Plasma					
	Male			Female		
	Initial	Final	*p*-Value*	Initial	Final	*p*-Value*
APRIL/TNFSF13	288.31 (207.49–343.84)	252.85 (217.01–330.74)	.711	324.58 (273.62–366.24)	289.81 (264.86–315.35)	.427
BAFF/TNFSF13B	12.96 (11.93–16.93)	13.64 (10.14–15.31)	.711	11.10 (10.74–15.84)	11.39 (10.50–12.20)	.791
sCD30/TNFRSF8	.78 (.67–1.19)	.81 (.64–1.07)	.958	.89 (.72–1.05)	.93 (.81–1.40)	.560
sCD163	161.74 (113.01–177.83)	120.77 (90.79–226.67)	.560	136.56 (81.13–188.25)	177.30 (127.12–192.38)	.427
Chitinase 3-like 1	14.32 (13.48–15.04)	8.87 (6.84–9.48)	.186	9.28 (6.75–13.82)	13.03 (11.00–14.88)	.224
gp 130/sIL-6Rβ	127.64 (114.23–137.84)	136.87 (103.84–169.47)	.958	104.58 (95.21–146.74)	111.55 (98.45–154.48)	.491
INF-α2	36.34 (31.56–50.52)	23.40 (17.59–34.50)	**.059**	36.27 (28.09–44.58)	32.77 (27.50–36.32)	.649
INF-β	49.87 (43.77–61.08)	47.84 (42.29–50.36)	.368	50.87 (44.84–57.00)	46.80 (43.27–49.88)	.186
INF-γ	16.26 (14.10–22.73)	15.18 (10.87–19.49)	.479	18.42 (14.10–20.57)	14.10 (9.26–16.25)	.202
IL-2	22.47 (20.68 –28.59)	24.67 (19.13–28.92)	.932	23.47 (18.68–26.24)	21.59 (17.85–25.33)	.737
sIL-6Rα	12.56 (11.22–13.73)	11.68 (11.39–14.25)	.791	14.21 (12.60–15.46)	12.01 (10.56–14.84)	.368
IL-8	.03 (.03–.04)	.03 (.02–.04)	.643	.02 (.02–.03)	.02 (.02–.02)	.221
IL-10	265.73 (230.16–284.87)	270.17 (245.13–307.61)	.427	262.92 (183.12–308.64)	270.09 (216.17–342.86)	.560
IL-11	7.03 (6.14–12.57)	6.95 (6.46–7.68)	.634	5.97 (2.80–8.07)	4.15 (3.28–5.08)	.427
IL-12p40	48.44 (48.36–53.55)	43.22 (32.97–48.36)	**.078**	53.59 (48.36–58.84)	38.03 (32.93–43.24)	**.039**
IL-12p70	6.55 (3.98–9.78)	3.98 (3.66–4.30)	.643	4.62 (4.62–5.91)	2.39 (2.22–2.55)	**.060**
IL-19	.14 (.12–.16)	.13 (.12–.14)	.560	.13 (.13–.15)	.11 (.09–.14)	.368
IL-20	37.56 (20.01–45.60)	33.65 (24.83–75.81)	.958	24.14 (18.15–66.20)	18.47 (18.15–32.95)	.637
IL-22	48.40 (20.33–89.64)	NA		53.06 (25.12–91.09)	NA	
IL-26	4.33 (4.12–4.75)	5.38 (4.64–5.80)	.427	4.75 (3.49–6.13)	4.75 (3.33–4.91)	.751
IL-27p28	.27 (.22–.36)	.16 (.16–.16)	.480	.23 (.12–.24)	NA	
IL-28A/INF-λ2	95.37 (86.24–154.53)	55.26 (42.40–91.07)	**.048**	91.39 (70.95–122.50)	72.96 (59.68–99.82)	.297
IL-29/INF-λ1	.19 (.14–.24)	.09 (.07–.13)	**.009**	.18 (.11–.29)	.10 (.08–.12)	**.080**
IL-32	.77 (.71–1.09)	.74 (.73–.87)	.791	.88 (.76–.93)	.88 (.76–1.12)	.791
IL-34	1.19 (.75–1.38)	.75 (.56–.86)	**.050**	.93 (.73–1.06)	.59 (.57–.65)	**.050**
IL-35	.21 (.19–.29)	.20 (.17–.27)	.491	.23 (.20–.26)	.18 (.17–.21)	**.044**
LIGHT/TNFSF14	87.98 (87.98–87.98)	NA		45.10 (30.12–60.07)	8.82 (8.82–8.82)	.221
MMP-1	1.15 (1.15–1.15)	NA		NA	NA	
MMP-2	40.44 (29.48–53.01)	61.09 (36.38–62.69)	.224	39.58 (28.41–43.68)	49.83 (31.49–60.85)	.224
MMP-3	33.30 (16.23–40.47)	23.53 (19.05–35.96)	.874	25.11 (16.42–31.93)	16.86 (15.81–38.97)	.711
Osteocalcin	2.24 (2.08–3.02)	2.86 (1.95–2.94)	.711	2.55 (2.32–3.02)	2.40 (1.73–2.55)	**.080**
Osteopontin	32.40 (31.00–35.10)	37.90 (30.83–39.84)	.186	41.72 (28.87–42.54)	36.55 (31.52–39.54)	.560
Pentraxin-3	23.80 (16.80–30.31)	25.84 (19.52–35.59)	.711	25.26 (21.10–26.23)	18.31 (16.18–20.58)	**.064**
sTNF-R1	.60 (.57–.68)	.57 (.53–.58)	.186	.53 (.51–.67)	.66 (.57–.76)	.186
sTNF-R2	.84 (.78–.94)	.74 (.73–.95)	.427	.87 (.76–.91)	.92 (.83–.98)	.315
TSLP	69.06 (54.68–77.29)	54.68 (46.33–66.45)	.152	64.90 (51.45–76.05)	53.09 (47.49–67.41)	.491
TWEAK/TNFSF12	3.66 (3.41–3.75)	3.57 (3.17–3.98)	.874	4.21 (3.78–4.91)	3.91 (3.45–4.35)	.368
TGF-β1	6.61 (3.45–10.66)	1.94 (1.35–3.09)	**.023**	5.13 (3.03–6.58)	1.83 (1.27–3.50)	**.050**
TGF-β2	.45 (.17–.47)	.08 (.07–.15)	.128	.23 (.12–.30)	.07 (.05–.16)	**.039**
TGF-β3	NA	NA		NA	NA	

Note: Results were expressed as median (Q1 – Q3) in pg/ml.
Abbreviation: NA: Not applicable.
Bold values mean with statistical significance at 90%.
*Kruskal–Wallis test.

were observed regarding the concentration of those markers, probably due to the low sample size. Only CHI3L1 showed higher levels after 6 months of probiotic intake. CHI3L1, also known as YKL-40, is a secreted glycoprotein associated with inflammatory processes. CHI3L1 has been found upregulated in cervicovaginal mucus during the oestrus phase of ovine reproductive cycle.[52] Additional studies are necessary for a complete understanding of the immunomodulator effect of oral *L. salivarius* PS11610 treatment.

The immunomodulatory function of the gut microbiome has been deeply characterized in several contexts. In the present study, we have found that *L. salivarius* PS11610 treatment might be associated with the change of the immune profile from proinflammatory to anti-inflammatory, revealing its potential beneficial effects in gastrointestinal mucosa or even other mucosas. Moreover, studies in males have reported a correlation between semen and gut microbiota after high fat diet.[53] For these reasons, we have considered the oral administration pathway the best approach to solve idiopathic infertility as multifactorial condition.

In this study, four women became pregnant (44.4%) and three of them had a live birth (33.3%). These results were compared with theoretical pregnancy and live birth probabilities, which were calculated using the data reported by the Spanish Fertility Society.[54] The theoretical average probability was 27.59% for pregnancy and 21.31% for live birth.

The main limitation of this pilot study is the low number of couples participants, which has hampered the analysis and the evaluation of the effects of probiotic supplementation in pregnancy success and live birth rates. Moreover, a control group treated with placebo would allow to obtain more robust conclusions. In conclusion, probiotic supplementation with *L. salivarius* PS11610 in couples with idiopathic infertility under assisted reproduction treatment improved the urogenital tract microbiome and might be associated with the modulation of the inflammatory profile, increasing the pregnancy rate. This study provides data supporting the use of probiotics as adjuvants during fertility treatments.

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CONFLICT OF INTEREST

Authors SE, SM, IEM, NC and EJ are employees of Probisearch, SLU Spain. All other authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

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SUPPORTING INFORMATION

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

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