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Development of an innovative intravaginal model of probiotic inoculation in dairy ovine flocks: positive effects on the vaginal microbiota, vaginitis and fertility

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ABSTRACT

In vitro studies have reported the antimicrobial effect of an inoculum (**L2**) made from commercial probiotic strains of *Lactobacillus* spp. (*Lactobacillus (L.) crispatus*, *L. brevis* and *L. gasseri*) against some ruminants' pathogens. However, their possible beneficial effects on controlling the vaginosis caused by the use of intravaginal sponges for oestrus synchronisation have not been evaluated in *in vivo* models. Therefore, the purpose of this study was to perform an intravaginal double inoculation of this same non-lyophilised (non-freeze-dried) L2 inoculum, in 120 ewes from four dairy commercial ovine flocks using straws similar to those used in artificial insemination. Globally, in the entire study population, the fertility percentage (65%) of the inoculated groups increased by 10% compared to the control groups (55%). Specifically, increases in the percentages of pregnant sheep in flocks 1 and 4 (10.7 and 5.7% respectively; $P > 0.05$) and flock 3 (27.3%; $P < 0.05$) were observed in inoculated ewes. Overall, the administration of L2 on the day of intravaginal sponge insertion decreased the number of ewes with vaginitis by 11.13% at the time of sponge removal (**T1**). In the first flock, the administration of the probiotic positively modulated the microbiota with an increased richness and presence of Lactobacillales accompanied by a reduction in *Mycoplasma* spp. Genera such as *Fusobacterium, Streptobacillus, Campylobacter or Trueperella* were linked to the presence of vaginitis at T1. Genera belonging to Lactobacillales, including some genera previously reported to be beneficial for pregnancy, significantly increased at the time of pregnancy diagnosis but significantly decreased at T1. The results of this study suggest that the use of probiotics via intravaginal inoculation as a strategy to positively modulate the microbiota and vaginal inflammation, derived from the use of intravaginal sponges, is also associated with improved fertility. This novel approach could improve animal welfare in oestrus-synchronisation protocols and represents an alternative to the prophylactic use of antibiotics.

Implications

The routine use of progestogens-impregnated intravaginal sponges to synchronise oestrus in sheep causes vaginosis that impairs fertility and motivates a preventive use of antibiotics, which raises the need of finding alternative therapies. This study reports a novel intravaginal probiotic inoculation system that had beneficial effects on dairy ewes by reducing the frequency of vaginitis, favourably modulating the vaginal microbiota and increasing fertility. This provides a new practical alternative strategy to antibiotic prophylaxis that could improve animal well-being and the low fertility of ovine artificial insemination, improving the economy of flocks and the success of genetic selection schemes.

Introduction

Vaginitis is a common consequence of synchronisation protocols using progestogen-releasing intravaginal treatments in small ruminants (Manes et al., 2013; Vasconcelos et al., 2016). Due to

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the presence of a foreign body in the vagina, induced physiological changes can favour the growth of pathogenic and opportunistic bacteria (Suárez et al., 2006; Manes et al., 2010, 2013; Oliveira et al., 2013). When removing the intravaginal devices, vaginal discharges are usually observed altogether with erythema, gas, foul odour, and the presence of inflammatory cells (Penna et al., 2013; Vasconcelos et al., 2016; Quereda et al., 2020a) and an increase in the vaginal pH (Martinez-Ros et al., 2018). Intravaginal sponges (**IS**) used for synchronisation protocols are more prone than progestogen-loaded silicon-based devices to generate adhesions, abnormal discharges and vaginosis (Suárez et al., 2006; Martinez-Ros et al., 2018). In addition, the vaginitis caused by IS has a negative effect on the fertility rate in sheep (Manes et al., 2014). In this sense, alterations in the dynamics of the sheep's vaginal ecosystem linked to the use of IS have been reported. This could increase the microbiota instability during fertilisation process (Quereda et al., 2020a). The need to obtain appropriate intravaginal probiotics to improve fertility rates linked to artificial insemination of ovine animals has been previously suggested (Reinoso-Peláez et al., 2023). Indeed, although more than 500 000 sheep are inseminated each year in Australia, 300 000 in France or 60 000 in Spain (Hernández Ballesteros et al., 2015), artificial insemination in sheep is not widespread worldwide due to problems related to the different insemination and semen conservation techniques (Alvarez et al., 2019) which generate unsteady artificial insemination outcomes with a fertility rate ranging between 30 and 70% (Anel et al., 2005; Faigl et al., 2012; Fernandez et al., 2019; Gómez-Brunet et al., 2007; Macías et al., 2020; Masoudi et al., 2017; Neila-Montero et al., 2023; Salamon and Maxwell, 2000).

The improper use of IS can also have public health consequences. Antibiotics are commonly combined with IS to control the vaginal inflammation produced by those (Berruga et al., 2008; Serrano et al., 2020). The administration of antibiotics combined with the use of IS has been reported to influence the ovine vaginal microbiota and can negatively affect the abundance of certain bacterial genera (Serrano et al., 2020). Unfortunately, this practice bears dangerous consequences such as the presence of antibiotic residues in milk (Berruga et al., 2008). The problems surrounding the use of long-term progestogen treatments motivated the need to explore alternative strategies to the use of antibiotics such as the administration of intravaginal probiotics (Quereda et al., 2020a). In recent years, the use of intravaginal probiotics in sheep around the use of IS has been evaluated in experimental meat flocks under natural mating conditions (Quereda et al., 2020a), inseminated ewes from commercial dairy flocks (Reinoso-Peláez et al., 2023) and Merino ewes (Güner et al., 2022; Güner et al., 2024), in order to evaluate its influence on the vaginitis, microbiota, fertility or health status of the animals. Some of these studies suggested that intravaginal administration of probiotics in sheep using *Lactobacillus* spp. may positively influence vaginal health when probiotics are administered in conjunction with synthetic progestogens-impregnated IS.

Lactobacillus spp. are lactic acid bacteria, a group of gram-positive bacteria found in several anatomical locations including the reproductive tract of ruminants (Otero et al., 2006; Wang et al., 2013; Swartz et al., 2014). Their use as probiotic bacteria can be considered as an antibiotic alternative given its ability to inhibit pathogens through different mechanisms (Vieco-Saiz et al., 2019; Toquet et al., 2023). Although *Lactobacillus* spp. are not abundant in the vaginal microbiota of sheep (Swartz et al., 2014), their vaginal use as antibacterial agents has been suggested by some authors in ovine species (Serrano et al., 2020; Güner et al., 2024). Intravaginal administration of a freeze-dried *Lactobacillus* (*L.*) *rhamnosus* inoculum, a bacterial species reported to have positive effects on human fertility, to ewes before and after the use of IS significantly reduced the abundance of *Campylobacter* spp. compared to that in the other experimental groups (Reinoso-Peláez et al., 2023) while the administration of a cell-free supernatant of *L. plantarum* in IS significantly reduced the presence of *Enterobacteriaceae* at IS removal (Güner et al., 2022). Other authors (Quereda et al., 2020a) have also shown the positive effects of the administration of a single dose of a commercial probiotic, intended for the control and prevention of vaginosis in women, just at the time of applying the IS in a study population of 21 yearling ewes. In this pilot experiment, the inoculum, made from the aforementioned commercial probiotic, contained freeze-dried *L. crispatus*, *L. brevis* and *L. gasseri* strains that were cultured in a broth to reach their maximal concentration before being transported in this same liquid medium and inoculated into the animals. Adverse effects on the health of the animals were ruled out, and improvements in the vaginosis control and fertility were observed. Subsequently, three *in vitro* studies have confirmed the bactericidal effect of this combination of *Lactobacillus* spp. against *Mycoplasma bovis* (García-Galán et al., 2020a; García-Galán et al., 2020b) and *Mycoplasma agalactiae* (Toquet et al., 2023), which are important pathogens of cattle and small ruminants, respectively. These results were obtained using a one hundred times more concentrated inoculum (**L2**) than the inoculum previously used for intravaginal inoculation in ewes (Quereda et al., 2020a). Nevertheless, to date, this more concentrated L2 inoculum has not been tested *in vivo*, so its effects on the vaginal ecosystem of small ruminants and its safety have not been tested.

Considering the need to address the negative effects of the use of IS in sheep, to try to improve the fertility indices of artificial insemination and look for alternatives to antibiotic therapy, we decided to conduct an *in vivo* study with the L2 dose of the probiotic in a larger number of animals from different commercial dairy sheep flocks. The present study is based on the hypothesis that the deep administration of L2 (non-lyophilised inoculum of *Lactobacillus* spp.) beyond the vaginal vestibule just before and after applying the IS positively influences ovine vaginal health and even fertility. This hypothesis is justified by the fact that: lower doses of this composition of *Lactobacillus* spp. in a single dose and administered at the entrance of the vagina did not have an impact on the cultivable bacterial vaginal microbiota of ewes and their overall health status but did show positive effects on vaginal neutrophilia and fertility when vaginal sponges were used (Quereda et al., 2020a); the inoculum L2 has shown antimicrobial effects against pathogens of small ruminants (Toquet et al., 2023, 2024); vaginal decrease in Lactobacillales has recently been linked to failure in sheep pregnancy (Barba et al., 2024).

Therefore, the aim of this study was to evaluate the safety and possible influence of the infusion of two L2 doses of a commercial probiotic (*Lactobacillus* spp.) on the fertility of dairy sheep from commercial flocks subjected to oestrus synchronisation protocols with intravaginal sponges and artificial insemination. Additionally, we aimed to evaluate the possible influence of this inoculation on the pH and vaginal inflammatory response as well as on the microbiota present in this anatomical location.

Material and methods

Study design

This experimental study involved a total number of 240 multiparous ewes from 3 years old to 6 years old, from four different farms. The flocks were located in four different autonomous regions of Spain: flock 1 was located in the Basque Country (Latxa sheep, blond-faced variety, one birth per year), flock 2 was located in Navarre (Latxa sheep, dark-faced variety, one birth per year), flock 3 was located in Castilla-La Mancha (Manchega sheep, one birth per year) and flock 4 was located in Castile and León (Assaf

sheep, three births every two years). This last flock was in an intensive permanent housing system and used artificial rearing while the other three farms allowed the sheep out to graze in a semi-extensive system and used natural rearing. None of the animals studied showed symptoms of disease or previous reproductive disorders and were officially free of any notifiable disease in Spain (Real Decreto 779/2023 of the Agriculture, Fishing and Alimentation Ministry of Spain). Moreover, all the animals selected an adequate body condition score, between 3 and 4 (Russel, 1984).

On each farm, the animals were randomly divided into two groups: a group of animals inoculated with the L2 probiotic (**P**) group and a control (**C**) group with non-inoculated animals. In flocks 1 and 2, 30 ewes were included in each experimental group and the inoculations were carried out between August and September, while in flocks 3 and 4, 25 and 35 ewes were included per experimental group and the inoculations were carried out in March and April, respectively. Later on, five animals were eliminated from the study due to a low body condition score (< 3) (Russel, 1984) or a loss of the IS, lowering the total number of animals involved in this study to 235.

Both groups from each flock underwent an oestrus synchronisation protocol with synthetic progestogens (flugestone acetate 20 mg) administered by IS (Chronogest. MSD Animal Health®, Kenilworth, NJ, USA) for 14 days. Just before inserting the IS (**T0**), the P group received a first dose of L2 whereas the C group did not receive this dose. On the day of IS removal (**T1**), the P group received a second L2 dose. At T1, 480 UI of PMSG (Foligón® 6000 UI, MSD Animal Health España) was administered to all animals in both groups. Two days after this time, the veterinarians of each farm routinely cervically inseminated all the animals included in the study with a refrigerated (maximum 2 h) semen dose of 300–400 millions of spermatozoids elaborated from ejaculates with a concentration > 2 500 millions of spermatozoids per mL and basal motility $\geq$ 4. The pregnancy diagnosis was established by ultrasound at 50–60 days after T1 (**T2**).

The health state of the animals around the two inoculations was evaluated, as well as the percentages of fecundity resulting from artificial insemination, for all animals involved in the study. In addition, in flocks 1 and 2, a vaginal cytology was performed, and the vaginal pH was measured in all the animals studied at T0, T1, just before inoculating the probiotics, and T2. At each time point, in these two flocks, the vaginal microbiota was also evaluated in 24 animals (P group = 12, C group = 12) using barcoding metagenomics.

Initially, the innocuousness of the probiotic was confirmed in the first phase in flocks 1 and 2 via the measurement of rectal temperature and vaginal pH and a robust study was carried out evaluating vaginitis and the vaginal microbiota. In the second phase, in flocks 3 and 4, only the fertility and general condition of the animals were evaluated because the study of all the parameters included in the first two flocks required having the animals immobilised for too long, which was incompatible with the management routine of these flocks.

Preparation and inoculation of the Lactobacillus inoculum

The lyophilised commercial probiotic Femibiotic® (NS Femibiotic, cinfa, Pamplona, Spain) based on a mixture of *Lactobacillus* spp. (60% *L. crispatus*, 20% *L. brevis* and 20% *L. gasseri*) was reconstituted in PH medium (Gómez-Martín et al., 2012) without antibiotics, and the L2 dose (3.24×10^8 CFU/mL) was prepared following a previously described methodology (García-Galán et al., 2020a; García-Galán et al., 2020b; Toquet et al., 2023). A volume of 400 µL was inserted in artificial insemination straws (0.5 mL Cassou straw, IMV Technologies®, L'Aigle, France) and sealed with beads. The whole process was performed in a class II biosafety cabinet.

Given the distance between the farms and the laboratory, where the inocula were prepared, ranged between 330 and 600 km and the need to adapt to the routine of the flocks, the conservation time of the straws was variable. The probiotic doses in the artificial insemination straws were kept refrigerated (4–6 °C) for 5 h before being intravaginally inoculated in flock 3, 24 h before inoculation in flocks 1 and 4, and 48 h before being inoculated in flock 2. To inoculate L2 into the vagina, a small ruminant insemination catheter (Minitüb®, Tiefenbach, Germany) and a vaginal speculum (20.3 cm sheep speculum, Humeco®, Huesca, Spain) were used. In this way, the dose of probiotic was deposited deep in the vagina, near the cervix.

Prior to the *in vivo* experiment, three straws containing the inoculum were prepared, sealed, and kept in the laboratory at 4–6 °C for 48 h in order to evaluate the impact of the transport time on its viability and optic density. Lactic acid bacteria colonies were counted on Man, Rogosa and Sharpe agar (MRS agar, Scharlau, Scharlab, Madrid) as previously described (Toquet et al., 2023), and the optic density was measured with a spectrophotometer at a wavelength of 600 nm.

Health status monitoring: physical examination and fertility rate

The general health status of every animal involved in the experiment was evaluated by external examination (corporal condition, presence of abnormal discharges in the vagina) and rectal temperature at T0, 24 h post-T0, T1 and 24 h post-T1 with digital thermometers. Rectal temperatures greater than 39.9 °C were considered to indicate fever. The conception rate (or fertility) was calculated based on the outcome of the gestation diagnosis. Ewes that were diagnosed as pregnant at T2 by ultrasound (Sonosite M−turbo C60xi 5–2 MHz abdominal probe, FUJIFILM SonoSite®, USA) were considered fertile.

pH measurement, vaginal cytology and metagenomics sampling (flocks 1 and 2)

Before inoculation, the vaginal pH was measured at T0 and T1 by rubbing a vaginal swab (Deltalab, Eurotubo®, Barcelona, Spain) on a pH-indicator strip (Merck KGaA, Damstadt, Germany). Vaginal cytology and metagenomic analysis were also performed at T0, T1 and T2. The vulvar area of each ewe was cleaned with 2% chlorhexidine, and a sterile microbiological swab (Deltalab, Eurotubo®, Barcelona, Spain) was used to collect the sample for the vaginal cytology in all 120 animals from flock 1 and flock 2. Vaginal swabs were taken from six animals of each experimental group in both flocks (n = 12 per flock) with DNA-free sterile swab (Deltalab, Eurotubo®, Barcelona, Spain) for metagenomic analysis. For the vaginal cytology, a previously described methodology was used (Quereda et al., 2020a), and the following scale was used to determine the presence of vaginitis: 0–3.9 neutrophils was considered a score of 0 (no vaginitis), while values > 3.9 were considered a score of 1 (presence of vaginitis).

Metabarcoding analysis (flocks 1–2)

The composition and structure of the sampled microbial communities were assessed through the amplification and sequencing the V3-V4 variable regions of the 16S rRNA gene following a methodology previously described (Barba et al., 2024). The Illumina Miseq sequencing 300×2 approach was used. Amplification was performed after 25 PCR cycles. Two negative controls, DNA extraction and amplification blank, were included as well as a positive mock community control during library preparation to ensure quality control. Raw demultiplexed forward and reverse reads were processed using QIIME2 version 2019.4 (Bolyen et al., 2019)

while DADA2 was used for quality filtering, denoising, pair-end merging and operational taxonomic units (**OTUs**) assignation (Callahan et al., 2016). To ensure appropriate quality, reads were trimmed in the position where Phred quality dropped under 20 (25th percentile) (300 nt for forward and 245 nt for reverse reads). The number of reads obtained for the negative and positive controls was as expected. The mock control profile obtained was consistent to the theoretical expected, validating the processes of library preparation. Negative control samples were used to detect environmentally derived contaminants. Taxonomic affiliation results revealed that contaminant amplicons were either absent in samples or its mean abundances were at least two orders of magnitude less abundant than in the negative control.

Operational taxonomic units were aligned via the MAFFT method (Katoh and Standley, 2013), and this was used to create a tree and to calculate phylogenetic relations between OTUs fasttree 1.0 (Price et al., 2009). OTUs data were used to calculate the following alpha diversity metrics: observed OTUs (i.e. richness) and Pielou's evenness index while OTUs and phylogenetic data were used to calculate the following beta diversity metrics: Unweighted UniFrac, Weighted UniFrac, Jaccard and Bray-Curtis dissimilarities. Taxonomic assignment of OTUs was performed using a Bayesian Classifier trained with the Silva database (v132) (99% OTUs full-length sequences) (Wang et al., 2007).

Statistical analysis

Statistical analyses for the evaluation of health status monitoring were carried out using the MIXED procedure implemented in the program Statistical Analysis System (SAS Institute, version 8). According to the model, rectal temperature, and vaginal pH were used as the dependent variables; moreover, the mixed model included the random effect of ewe nested within flock. The model used, which considered repeated measures along time, was: $Y_{ijkl} = \mu + \alpha_i + \beta_j + \gamma_k + (\alpha\gamma)_{ik} + u_{(i)l} + \epsilon_{ijkl}$.

where: Y_{ijkl} = temperature and vaginal pH (dependent variables); μ = constant; α_i = the fixed effect of flock; β_j = the fixed effect of the experimental group (P or C); γ_k = the fixed effect of the time (T0, T1 and T2); $(\alpha\gamma)_{ik}$ = the fixed effect of the interaction between flock and time; $u_{(i)l}$ = the random effect of ewe nested within flock; ϵ_{ijkl} = the random residual. To fit a time series type covariance structure, in which the correlation changed as a function of time, the autoregressive first−order covariance AR(1) from MIXED procedure was used. Estimating and comparing means were obtained from the least squares means statement of SAS with multiple comparisons adjusted using the Tukey method. The level of significance considered was $P < 0.05$.

The conception rate and vaginitis frequencies were studied per flock and experimental group using the EpiInfo software (available at https://www.cdc.gov/epiinfo/index.html) using chi-square correction (Yates). The neutrophils' mean value per time, experimental group and flock were compared in the EpiInfo Software using ANOVA or Mann–Whitney/Wilcoxon two-sample test (Kruskal–Wallis test for two groups) according to the inequality of population variances and with the significance level set at 0.05.

Alpha diversity comparisons were performed using the Kruskal-Wallis non-parametric test. Beta diversity distance matrices were used to calculate principal coordinates analysis and to construct ordination plots using the R software package version 3.6.0 (https://www.R-project.org). The significance of groups in community structure was tested using Permanova. The PermDisp test was used to identify location vs dispersion effects (Anderson and Walsh, 2013). The significance threshold was set at 0.05. BiodiversityR version 2.11–1, PMCMR version 4.3, RVAideMemoire version 0.9–7 and vegan version 2.5–5 packages were used. The differential abundance of taxa was tested using the Kruskal Wallis non-parametric test. After the Kruskal Wallis test, Conover's test with false discovery rate Benjamini-Hochberg correction was added for pairwise comparisons.

Results

Inocula concentration

The concentration of the L2 inoculum fluctuated between 1.1 and 7.26×10^8 CFU/mL in 4 mL of medium. Once the supernatant was discarded and the pellet was resuspended in 400 µL of medium and sealed in the straws, the final concentration of lactobacilli inside the straws was ten times greater (between 1.1 and 7.26×10^9 CFU/mL). The evolution of the average concentration of three refrigerated (4 °C) straws over time, measured before the *in vivo* experiments, is shown in Fig. 1.

Health status monitoring: physical examination and fertility rate

Throughout the experiment, we did not observe any adverse effects on the animals' health status except for one ewe in the P group from flock 1 who suffered clinical vaginitis and was treated with antibiotics and anti-inflammatories. The double inoculation of the probiotics did not significantly change the rectal temperature, as the experimental group (P or C) had no effect on this parameter. The only animals that exceeded a rectal temperature of 39.9 °C were three sheep from group C (flock 1), whose temperatures were 40.2, 40.3 and 40.5 °C. Overall, at T2 (38.6 °C ± 0.02), the temperature was significantly lower than at T0 (39.1 °C ± 0.02) and T1 (39.0 °C ± 0.02) ($P < 0.001$). The total number of animals per experimental group in each flock can be seen together with the conception rate in Table 1. Increases in the conception rate were observed in flocks 1 (10.7%; $P > 0.05$), 3 (27.3%; $P < 0.05$) and 4 (5.7%; $P > 0.05$) in the P group compared to the C group, while it remained the same in the P group as in the C group in flock 2.

Vaginal pH and cytology (flocks 1–2)

A significant increase ($P < 0.001$) in the pH was observed at T1 (pH 8.1) and T2 (pH 8.0) compared to T0 (pH 6.5). The experimental group (P or C) and the flock (1 or 2) did not have any effect on the pH observed. A significantly lower number of animals had vaginitis at T2 than at T1 in both experimental groups. There was also a significantly lower number of neutrophils per animal at T2 in the P group ($P < 0.001$) and C group ($P < 0.01$) in flock 2 and only in the P group ($P < 0.05$) in flock 1. Overall, in the P groups, the number of neutrophils in animals with vaginitis significantly decreased between T1 and T2, which was not the case in the C groups. The percentages of animals with vaginitis and the mean counts of neutrophils can be found in Table 2.

Vaginal microbiota (flocks 1–2)

Alpha and beta diversity

In flock 1, the richness (Supplementary Table S1; observed OTUs) of the P group at T2 was significantly greater than both experimental groups (P and C) at T1 ($P < 0.01$, Supplementary Table S2) and that of the C group at T2 ($P < 0.05$, Supplementary Table S2). No differences in richness were detected between the sampling points and groups in flock 2 (Supplementary Table S2). The bacterial richness of flock 1 for each time point and experimental group is represented in Fig. 2. Globally, evenness (Supplementary Table S1) was significantly greater in flock 1 than in flock 2 ($P < 0.05$, Supplementary Table S2).

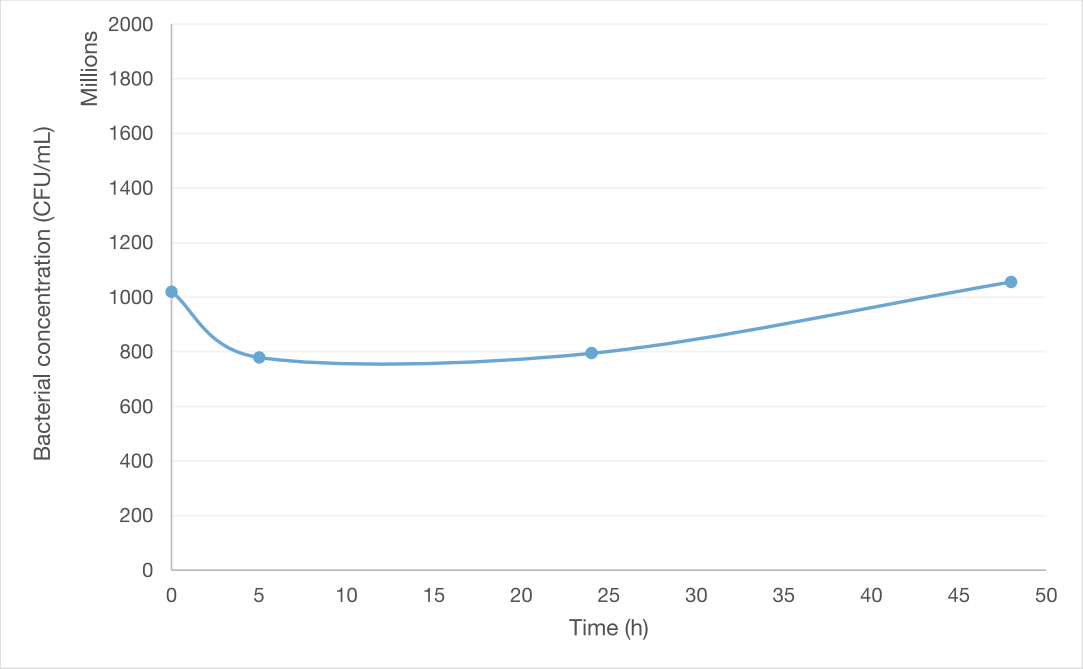

Fig. 1. Mean concentration of lactic acid bacteria in the refrigerated artificial insemination straws over time, measured before the *in vivo* ewe experiments.

Table 1
Number of ewes and conception rate per experimental group in each flock.

Flock	Experimental group	N total	Pregnant ewes	Non-pregnant ewes	Conception rate (%)
1	P	28	18	10	64.3[a]
1	C	28	15	13	53.6[a]
2	P	30	26	4	86.7[a]
2	C	30	26	4	86.7[a]
3	P	24	20	4	83.3[a]
3	C	25	14	11	56[b]
4	P	35	12	23	34.3[a]
4	C	35	10	25	28.6[a]

Abbreviations: P = probiotic group; C = control group; N = number of ewes.
Values with different superscripts between experimental groups within each flock differ significantly at $P < 0.05$.

Table 2
Vaginitis percentages and neutrophil mean values observed in ewes per time, treatment group and flock.

Time	Experimental group	EV (in %)	Nm per ewe flock 1	Nm per ewe flock 2	Nm per AV
T1	P	56.14[a]	1.8[a]	4.4[a]	5.7[a]
T1	C	67.27[a]	1.3[a]	4.1[a]	4.0[a]
T2	P	32.14[b]	0.3[b]	0.4[b]	1.0[b]
T2	C	29.63[b]	1.1[a]	0.1[b]	1.9[a]

Abbreviations: P = probiotic group; C = control group; Nm = neutrophil mean; EV = ewe with vaginitis.
0–3.9 neutrophils were considered a score of 0 (no vaginitis), while values >3.9 were considered a score of 1 (presence of vaginitis).
Values with different superscripts between time points within each experimental group differ significantly at $P < 0.05$.

There were significant differences in the bacterial community structure between T1 and the other two sampling points in both flock 1 and flock 2 for all four beta diversity distances, as represented by the blue dots in Fig. 3. Significant differences were also observed between the P and C groups in flock 1 at T2 ($P < 0.05$) for the Jaccard, Bray-Curtis, Weighted UniFrac indices (Supplementary Fig. S1, Supplementary Table S3).

Taxonomic analysis of bacterial phyla

At T0 in flock 1, there were 20 different bacterial phyla, and the most abundant phylum was Firmicutes (Bacillota) in the P group and Fusobacteria (Fusobacteriota) in the C group. The presence of Fusobacteria in the C group was significantly greater ($P < 0.05$). In flock 2, there were 16 different phyla, and Firmicutes was the most abundant phylum in the P group, while Proteobacteria (Pseudomonadota) was the most abundant in the C group. At T1, the number of different phyla decreased to seven in flock 1 and eight in flock 2. The most abundant phylum was Bacteroidetes (Bacteroidota), which was significantly more abundant than at T0 and T2 in both experimental groups of both flocks ($P < 0.01$). In flock 1, the P group had a significantly greater relative abundance (**RA**) of Fusobacteria ($P < 0.05$) at T1 than at T0 and T2. In flock 2, the abundance of Epsilonbacteraeota (Campylobacterota) was significantly greater at T1 than at T0 in both the C and P groups ($P < 0.05$). At

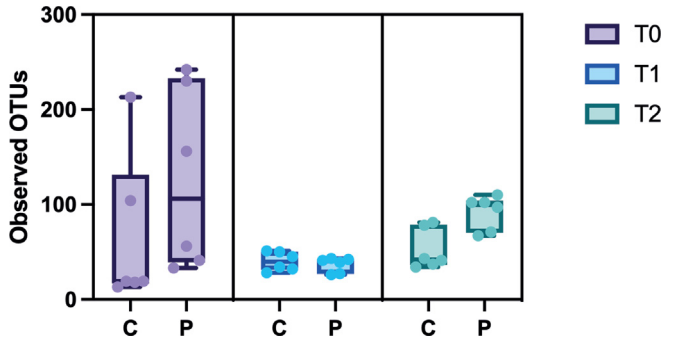

Fig. 2. Observed Operational Taxonomic Units (OTUs) in ewes from flock 1 for the probiotic group (P) and control group (C) the day of sponge insertion (T0), the day of sponge removal (T1) and the day of pregnancy diagnosis (T2).

T2, there were 14 phyla in flock 1, and the most abundant phylum was Firmicutes in the P group and Fusobacteria in the C group. In flock 2, there were 12 different phyla, and the most abundant phylum was Proteobacteria in the P group and Firmicutes in the C group. The abundance of Fusobacteria was significantly greater in the C group ($P < 0.05$), and the abundance of Verrucomicrobia (Verrucomicrobiota) was significantly greater in the P group ($P < 0.05$). The abundance of Tenericutes (Mycoplasmatota) was also significantly greater at T2 than at any other sampling point in the C group in the first flock ($P < 0.05$). In flock 2, the abundance of Epsilonbacteraeota significantly decreased at T2 in the C group ($P < 0.05$). The information regarding the RA of phyla is reflected in Fig. 4 and Supplementary Table S4 whereas all the significative differences can be found in Supplementary Table S5.

Taxonomic analysis of bacterial genera

In both flocks, the most abundant genus overall was *Porphyromonas* with a RA of 10.5% in flock 1 and 8% in flock 2. At T0 in flock 1, the five most abundant genera were *Ureaplasma, Porphyromonas, Oceanivirga, Actinobacillus,* and *Streptobacillus* while in flock 2, the five most abundant genera were *Escherichia-Shigella, Histophilus, Actinobacillus, Leptotrichiaceae,* and *Ureaplasma.* At the time of sponge removal (T1) in flock 1, the five most abundant genera were *Porphyromonas, Fusobacterium, Bacteroides, Streptobacillus,* and *Trueperella* while in flock 2, the five most abundant genera were *Porphyromonas, Bacteroides, Fusobacterium, Leptotrichiaceae* (unknown), and *Peptoniphilus.* On the day of the gestation diagnosis (T2) in flock 1, the most abundant genus in both experimental groups was *Ureaplasma.* In the C group, the other four most abundant genera (by order of decreasing RA) were *Oceanivirga, Actinobacillus, Mycoplasma* and *Fusobacterium,* whereas in the P group, *Acinetobacter*, *Porphyromonas, Aerococcus* and *Staphylococcus* were the other four abundant genera. At T2 in flock 2, the five most abundant genera in the C group were *Histophilus*, *Actinobacillus, Ureaplasma, Oceanivirga* and a genus of *Leptotrichiaceae*, while in the P group, the five most abundant genera were identical except that *Oceanivirga* was substituted by *Escherichia-Shigella.* The information regarding the RA of the most abundant genera is reflected in Fig. 5 and Supplementary Table S6 for flock 1 and Supplementary Table S7 for flock 2.

On the one hand, in flock 1, the presence of several abundant genera, including *Trueperella* ($P < 0.001$), *Peptoniphilus* ($P < 0.05$), *Tissierella* ($P < 0.001$), and *Porphyromonas* ($P < 0.05$), significantly increased at T1 and later decreased at T2 in both experimental groups. Some genera such as *Parvimonas* ($P < 0.05$) and *Streptobacillus* ($P < 0.05$) exhibited the same pattern but only in the P group from flock 1. On the other hand, the presence of *Corynebacterium* 1 ($P < 0.05$) significantly decreased at T1 and increased at T2 in both groups. Other abundant genera decreased at T1 only in the P group including *Staphylococcus* ($P < 0.001$), *Aerococcus* ($P < 0.05$), *Streptococcus* ($P < 0.05$), *Anaerococcus* ($P < 0.05$), and *Acinetobacter* ($P < 0.05$). *A posteriori*, at T2, *Staphylococcus* (P (C group) < 0.05; P (P group) < 0.001), *Anaerococcus* ($P < 0.05$) and *Fastidiopila* ($P < 0.01$) significantly increased in both groups, while some genera significantly increased only in the P group, such as *Aerococcus*

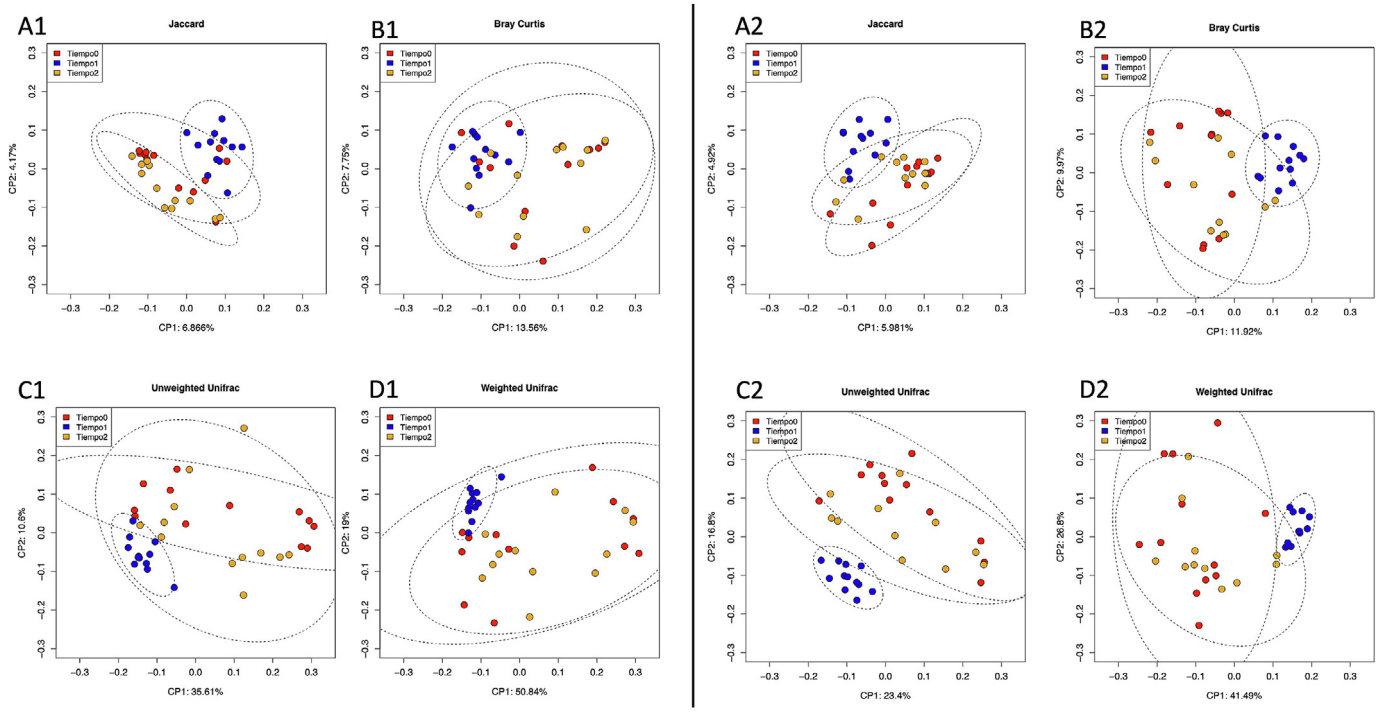

Fig. 3. Principle coordinates analysis (PCoA) of the Bray Curtis, Jaccard, Unweighted UniFrac and Weighted UniFrac distances for ewes of flock 1 (A1, B1, C1, D1) and flock 2 (A2, B2, C2, D2) the day of sponge insertion (Tiempo0), the day of sponge removal (Tiempo1) and the day of pregnancy diagnosis (Tiempo2).

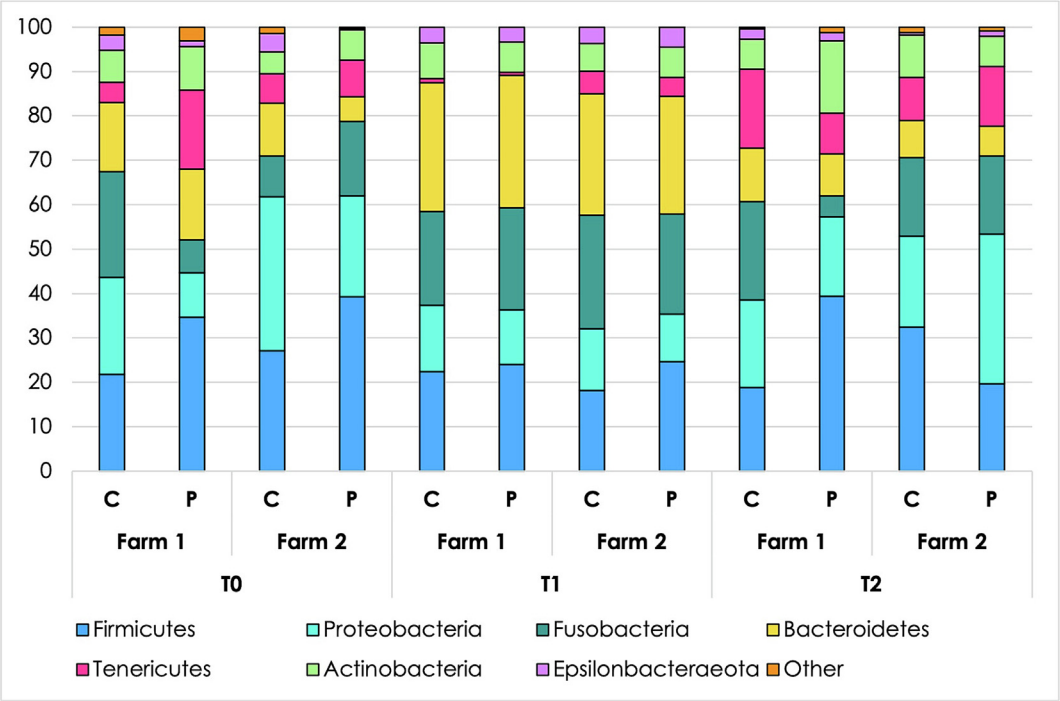

Fig. 4. Relative abundances of bacterial phyla in ewes from the probiotic group (P) and control group (C) the day of sponge insertion (T0), the day of sponge removal (T1) and the day of pregnancy diagnosis (T2). Only phyla with a relative abundance > 1% are represented.

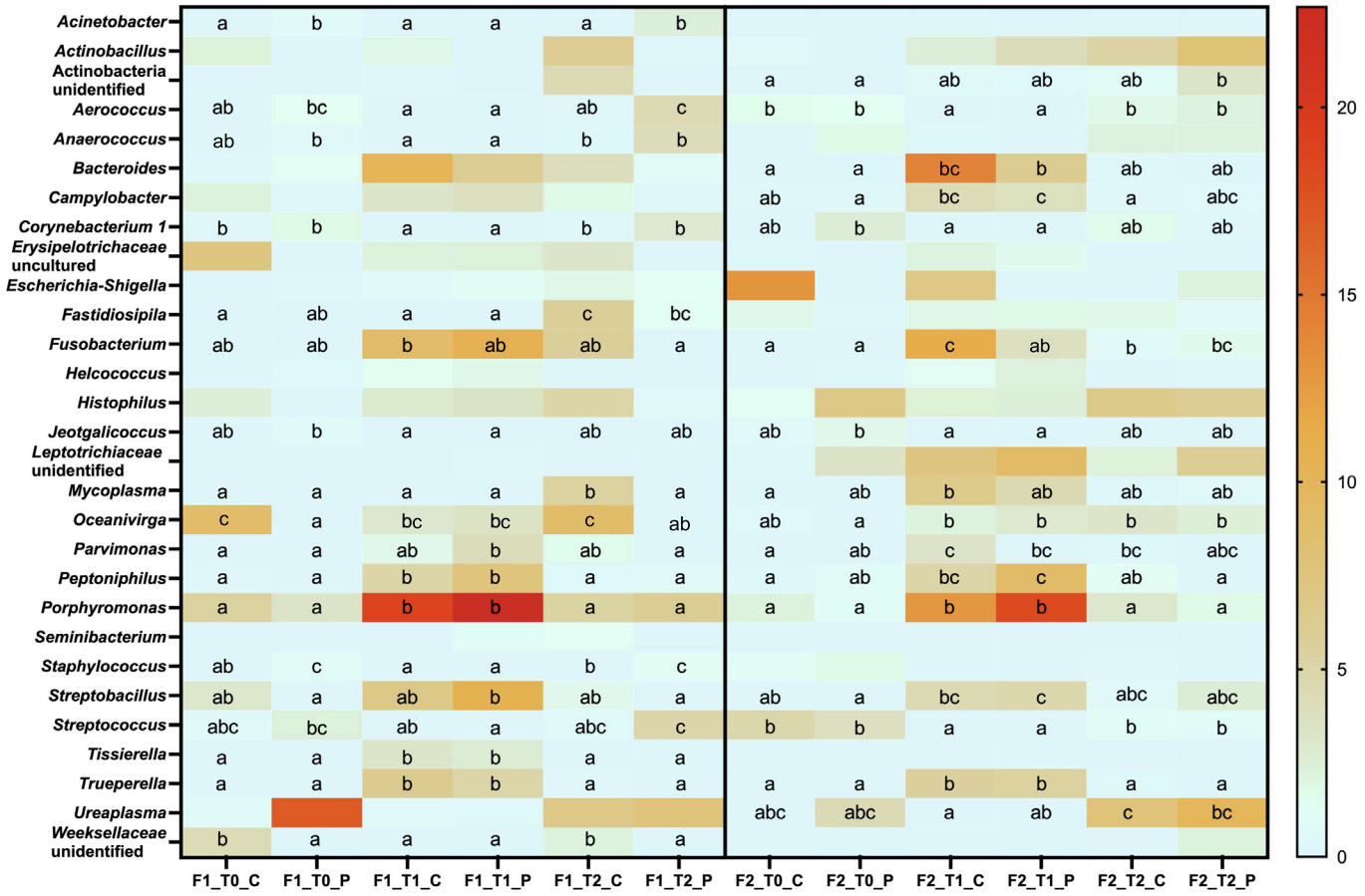

Fig. 5. Heatmap of the most abundant genera in ewes from the probiotic group (P) and control group (C) the day of sponge insertion (T0), the day of sponge removal (T1) and the day of pregnancy diagnosis (T2) in flock 1 (F1) and flock 2 (F2). The color scale represents the median value of each group at each time point. Only genera with a relative abundance > 1% are represented. Different letters (a, b, c) indicate a significant difference between sampling time points or experimental groups within one flock.

($P < 0.001$), *Streptococcus* ($P < 0.01$), and *Acinetobacter* ($P < 0.05$), and some significantly increased only in the C group such as, an unidentified *Weeksellaceae* genus ($P < 0.05$) and *Mycoplasma* ($P < 0.001$). In flock 1, several of the previously mentioned genera, *Acinetobacter* ($P < 0.05$), *Aerococcus* ($P < 0.05$), and *Staphylococcus* ($P < 0.05$), were significantly more abundant in the P group than in the C group at T2, while several genera, such as *Mycoplasma* ($P < 0.001$) and *Oceanivirga* ($P < 0.01$), were more abundant in the C group. The information about genera differential abundances in flock 1 is reflected in Fig. 5 and Supplementary Table S8.

On the one hand, in flock 2, there was a significant increase in the abundance of *Trueperella* and *Porphyromonas* ($P < 0.05$) at T1 and a decrease at T2 in both experimental groups ($P < 0.01$). Other abundant genera also increased at T1 in both groups, including *Bacteroides* ($P < 0.01$), *Peptoniphilus* ($P < 0.05$). In the P group only, there was a significant increase in abundance of *Campylobacter* ($P < 0.05$), and *Oceanivirga* ($P < 0.01$), while in the C group, there was a significant increase of *Parvimonas* ($P < 0.01$), *Fusobacterium* ($P < 0.001$) and *Mycoplasma* ($P < 0.05$). At T2 in the C group, there was a significant decrease in *Bacteroides* ($P < 0.05$), *Campylobacter* ($P < 0.05$) and *Fusobacterium* ($P < 0.05$). *Peptoniphilus* decreased significantly only in the P group ($P < 0.01$). On the other hand, at T1 in farm 2, there was a significant decrease of *Aerococcus* ($P < 0.01$) and *Streptococcus* ($P < 0.001$) in both experimental groups and a significant decrease only in the P group of *Corynebacterium* 1 ($P < 0.05$), *Jeotgalicoccus* ($P < 0.05$), and *Staphylococcus* ($P < 0.05$). At T2, there was a significant increase in the abundance of the Lactobacillales genera *Aerococcus* (P (C group) < 0.01; P (P group) < 0.05), *Alloiococcus* ($P < 0.01$) and *Streptococcus* (P (C group) < 0.001; P (P group) < 0.01) in both experimental groups. There was also a significant increase in the percentage of *Ureaplasma* in the C group ($P < 0.05$). No significative differences in abundant genera were observed between the two experimental groups at T2 in flock 2. The information about bacterial genera differential abundances in flock 2 is shown in Fig. 5 and Supplementary Table S9.

Lactic acid bacteria fluctuations

No *Lactobacillus* spp. was detected at T1 or T2. However, several genera from Lactobacillales such as *Aerococcus, Alloicococcus, Atopostipes, Facklamia* and *Streptococcus* were present and significantly fluctuated between sampling time points. Lactobacillales were significantly more abundant ($P < 0.05$) in the P group than in the C group at T2 in flock 1. In flock 1, their abundance also significantly decreased at T1 ($P < 0.01$) but increased during pregnancy diagnosis at T2 ($P < 0.001$). In flock 2, the amount of Lactobacillales significantly decreased at T1 ($P < 0.01$) and increased at T2 ($P < 0.01$) in both groups, but no significant differences were observed between the experimental groups. The fluctuations in the RA of the order Lactobacillales over the study period are shown in Fig. 6.

Discussion

To the best of the authors' knowledge, this is the first report on commercial dairy sheep of the possible beneficial effects on fertility, vaginitis and vaginal microbial composition of the administration of intravaginal probiotics. The combination of three species of *Lactobacillus* spp. with antimicrobial effects *in vitro* against reproductive pathogens of ruminants such as mycoplasmas, the inoculum preparation and the inoculation protocol used could have favoured the positive effects observed in this study. The need to seek solutions, such as the administration of intravaginal probiotics, to prevent vaginal complications derived from the use of IS has been suggested in previous studies (Quereda et al., 2020a; Serrano et al., 2020; Güner et al., 2024). The inoculum model preparation together with its transport and inoculation method developed here has several novel aspects: (1) the inoculated probiotic was based on a combination of *Lactobacillus* spp. (L2) with antibacterial effects previously reported *in vitro* (García-Galán et al., 2020a; García-Galán et al., 2020b; Toquet et al., 2023); (2) it uses a viable non-freeze-dried inoculum, one hundred times more concentrated than that used in a previous a pilot study (Quereda et al., 2020a), that ensures the viability of the inoculated bacteria; (3) we used sheep from commercial dairy flocks subjected to artificial insemination, while the previous studies used meat sheep from an experimental unit in a natural mating system (Quereda et al., 2020a) or merino ewes (Güner et al., 2024); (4) a double inoculation is used when only a single lyophilised (Reinoso-Peláez et al., 2023) or non-lyophilised (Quereda et al., 2020a) doses had been inoculated until now; (5) the probiotic inoculum is hygienically loaded into insemination straws and transported to the farms in refrigerated conditions; and (6) it is deposited at the entrance of the cervix through an insemination catheter. Previous studies did not use this transport system for the probiotic nor did they deposit the probiotic as deep (Quereda et al., 2020a; Reinoso-Peláez et al., 2023; Güner et al., 2024). The use of straws to transport and deposit the inoculum offers various advantages: preserving the probiotic concentration for up to 48 h, transporting it in the same way as seminal doses, preventing technicians from training to use this inoculation system, and allowing a deeper inoculation, which reduces the risk of reflux and favours its influence.

The use of probiotics such as lactic acid bacteria can confer several benefits (Vieco-Saiz et al., 2019). *L. crispatus*, which is the predominant *Lactobacillus* sp. in our L2 inoculum (Toquet et al., 2023), has been positively associated with improvements in sperm quality

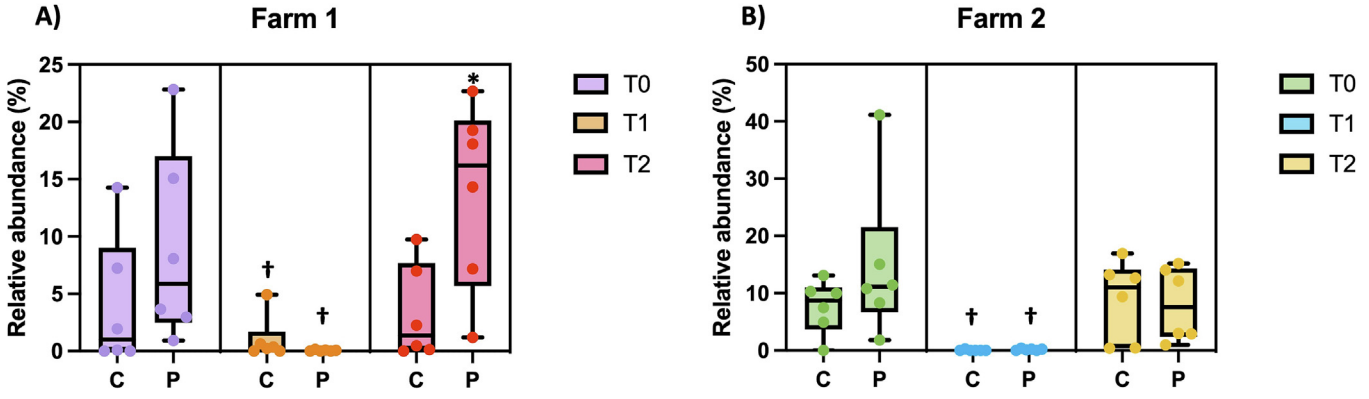

Fig. 6. Relative abundance of Lactobacillales in ewes from the probiotic group (P) and control group (C) the day of sponge insertion (T0), the day of sponge removal (T1) and the day of pregnancy diagnosis (T2) in flock 1 (A) and flock 2 (B). Significative differences between treatment groups at the same time sampling point are indicated by *. Significative differences between sampling points are indicated by †.

parameters or with the presence of leukocytes and IgA in semen (Weng et al., 2014). Moreover, it has been hypothesised that vaginal inoculation of *Lactobacillus* spp. or its presence in sheep semen could have antimicrobial effects against pathogenic bacteria and could reduce adverse effects on sperm quality (Serrano et al., 2020). In this sense, the presence of lactic acid bacteria has been described in buck and ram semen (Serrano et al., 2020; Mocé et al., 2022) and the antibacterial effect of some Lactobacillales strains against pathogens of small ruminants has been reported *in vitro* (Makete et al., 2017; Toquet et al., 2023). In a previous pilot experiment, a single administration of a less concentrated inoculum of *Lactobacillus* spp. did not cause adverse effects when administered intravaginally to 11 one−year-old sheep (Quereda et al., 2020a). Our results in 120 sheep support the absence of symptoms previously reported for this type of inoculum and suggest the *in vivo* safety of double inoculation with a L2 dose of *Lactobacillus* spp.

Far from observing negative effects, we were able to observe an increase in fertility in three of the four inoculated flocks. Indeed, the fertility rate in flocks 1, 3 and 4 increased by 10.7, 27.3 and 5.7% respectively. Interestingly, flock 3, where the L2 inoculum was refrigerated for less time, showed the greatest fertility improvement. The flock where L2 was preserved the longest before inoculation (flock 2) did not show any physiological or reproductive improvement although we did not observe any important loss of lactobacilli concentration up to 48 h in the refrigerated straws (Fig. 1). However, unlike the other flocks evaluated, the fertility of flock 2 was greater than 80%, and possibly, the positive effects of the administration of L2 were more pronounced in flocks with lower fertility. An increase in fertility was also observed with the previous lactobacilli inoculum (Quereda et al., 2020a). These results suggest the need to consider deep intravaginal probiotic inoculation models as a tool that could help improve the fertility percentages associated with artificial insemination in sheep where we believe there is room for improvement.

One factor that could influence sheep fertility is the vaginal physiological alterations derived from the placement of the IS. Under natural conditions, the vaginal pH of ewes is close to neutral (Swartz et al., 2014), and the pH observed at the time of placing the IS (6.5) could represent an adverse environment for reproductive pathogens sensitive to acidic pH, such as mycoplasmas (Gómez-Martín et al., 2015). The significant increase in the pH (8.1) at T1 in the two first flocks corroborates the presence of vaginosis observed in ewes after the oestrus-synchronisation protocol with IS, as observed in several other studies (Manes et al., 2015; Martinez-Ros et al., 2018; Ojeda-Hernández et al., 2019). Indeed, according to the beta diversity analysis, there was a clear difference in the community structure at T1 compared to that at T0 and T2 (Fig. 3) in both flocks indicating a shift in the bacterial composition of the microbiota after the use of IS. Vaginal dysbiosis was also corroborated by the significant decrease in the microbiota richness at T1 in flock 1 (Fig. 2). Changes in the vaginal microbiota induced by the use of oestrus synchronisation tools have been previously reported (Manes et al., 2010, 2013; Oliveira et al., 2013; Vasconcelos et al., 2016; Bragança et al., 2017; Quereda et al., 2020a). Indeed, this increase in pH at T1, two days before fecundation, could favour the colonisation of potentially pathogenic bacterial genera (Reinoso-Peláez et al., 2023). Although lactic acid bacteria are low in abundance in the ovine vaginal microbiota (Serrano et al., 2020), in our opinion, they play an important role in preventing excessive increases in the vaginal pH. In this sense, lactobacilli have been positively linked to fertility in women by improving vaginal dysbiosis (Lan and Chen, 2023). Supporting our hypothesis, in flock 1, the richness was greater at T2 in the group with inoculated ewes. In flock 1, the significant differences in the beta diversity metrics between the P and C groups at T2 (Supplementary Fig. S1, Supplementary Table S3) could also imply an effect of the probiotic on the structure of the bacterial communities present in the vagina. Moreover, although *Lactobacillus* spp. were unable to colonise the vagina, most likely due to their tendency to be host-specific (Toquet et al., 2021), the colonisation of certain microorganisms, such as the phylum Verrucomicrobia (Fig. 4, Supplementary Table S5), which has been positively linked to fertility (Koester et al., 2021), the order Lactobacillales (Fig. 6) and the Lactobacillales genus *Aerococcus* (Fig. 5), seemed to have been favoured at T2 in flock 1, while the colonisation of the genus *Mycoplasma,* represented by the species *Mycoplasma bovigenitalium*, which is linked to infertility in cattle (Parkinson, 2019), and another unidentified *Mycoplasma* sp., appeared inhibited in the P group at T2 in comparison to the C group (Fig. 5, Supplementary Table S8). These data support the previous theory that the viability of mycoplasmas in anatomical locations for which they have shown tropism could be negatively influenced by the presence of lactic acid bacteria (Toquet et al., 2023).

The use of IS in sheep is characterised by cytological changes such as neutrophilia (Manes et al., 2015) which is consistent with our results of increased neutrophils at T1. Nevertheless, the inoculation of the probiotic significantly reduced the numbers of neutrophils per ewe in flock 1 at T2. Globally, in flocks 1 and 2, the first administration of L2 reduced the number of ewes with vaginitis by 11.13% at T1 (Table 2), although this difference was non-significant. This finding supports previous results where the inoculation before IS insertion reduced the percentage of neutrophilia by 44% (Quereda et al., 2020a). Lactobacilli have been reported beneficial for vaginal inflammation in women (Witkin and Linhares, 2017) and cows (Ametaj et al., 2014).

The most abundant phyla in flocks 1 and 2, which accounted for more than 99% of the bacterial population, were Firmicutes, Proteobacteria, Fusobacteria, Bacteroidetes, Tenericutes, Actinobacteria, and Epsilonbacteraeota. Except for the latter, these phyla represented more than 95% of the bacterial population in another study (Greenwood et al., 2022). The significant increase in Bacteroidetes and Epsilonbacteraeota, which are Gram-negative bacteria usually present in the environment and faeces (Thomas et al., 2011; Van Der Stel and Wösten, 2019), at T1 in both groups of both flocks corroborates the presence of vaginosis. The increased presence of Gram-negative bacteria, after the use of IS, has previously been observed (Martins et al., 2009; Manes et al., 2010; Bragança et al., 2017; Ojeda-Hernández et al., 2019).

Overall, the most abundant genus was *Porphyromonas*, an important bacterial genus in the ewe vagina (Reinoso-Peláez et al., 2023). Additionally, the high RA of genera such as *Staphylococcus* and *Escherichia-Shigella* was in accordance with previous metagenomic studies (Koester et al., 2021; Reinoso-Peláez et al., 2023; Barba et al., 2024; Cassas et al., 2024). Another abundant genus observed in our study (Supplementary Table S6–7) that was also reported in other ewes' vaginal metagenomic studies was *Ureaplasma* (Koester et al., 2021; Greenwood et al., 2022; Reinoso-Peláez et al., 2023; Barba et al., 2024; Cassas et al., 2024). The need to clarify the reproductive implications of *Ureaplasma* spp. in ruminants has been suggested (Barba et al., 2024) since its presence has been reported not only in ruminants affected by reproductive disorders but also in healthy ones (Quereda et al., 2020b; Serrano et al., 2020; Koester et al., 2021; Mocé et al., 2022; Barba et al., 2024). In a previous study, *Ureaplasma* sp. USP128, which was the only *Ureaplasma* species observed in the present study, increased during pregnancy, and was significantly more abundant in ewes that were successfully fecundated after natural mating (Barba et al., 2024). Moreover, *Ureaplasma diversum* was more abundant in ovine herds with high pregnancy rates after the use of artificial insemination (Serrano et al., 2020). The *in vitro* ability of *Ureaplasma urealyticum* to influence sperm activity in rams, depending on parameters such as extracellular pH, has

been described (Reichart et al., 2001). We hypothesise that ovine and caprine ureaplasmas could alter spermatic metabolism and thus have an impact on fertility.

On the one hand, in both flock 1 and flock 2, the abundance of some microorganisms, such as *Porphyromonas*, *Bacteroides, Fusobacterium (Fusobacteriales), Streptobacillus (Fusobacteriales), Peptoniphilus, Campylobacter, Trueperella pyogenes, Parvimonas, Tissierella,* and *Anaerosalibacter*, significantly increased at T1 and decreased at T2 (Fig. 5). These bacteria could be linked to vaginitis and therefore be negatively associated to pregnancy such as *Fusobacterium* (Koester et al., 2021). It has been reported that IS produces a proliferation of *Enterobacteriaceae*, directly correlated with a loss of diversity, which could negatively affect Lactobacillales populations (Quereda et al., 2020a). On the other hand, *Streptococcus, Aerococcus, Alloicoccus, Facklamia* and *Atopostipes* (all belonging to the order Lactobacillales), as well as *Staphylococcus*, *Acinetobacter*, *Corynebacterium* 1, and *Anaerococcus,* exhibited the opposite pattern and significantly decreased at T1 and significantly increased at T2, indicating a possible beneficial role in the gestation process. In this sense, a significant decrease in the RA of *Alloicoccus* and *Atopostipes* has previously been observed in ewes that failed to get pregnant (Barba et al., 2024). One study has also reported significant increases in *Corynebacterium, Facklamia* and *Streptococcus* at the time of pregnancy diagnosis. In this same study, *Streptococcus* sp. and *Staphylococcus* spp. were also more abundant in pregnant ewes than in non-pregnant ewes (Koester et al., 2021). In a more recent study, *Streptococcus*, *Staphylococcus*, *Corynebacterium* and *Anaerococcus* showed an increased abundance at different times during gestation (Cassas et al., 2024).

Conclusions

Probiotic inoculation based on non-freeze-dried inoculum of *Lactobacillus* spp. was beneficial on the vaginal microbiota by restricting the proliferation of pathogenic or opportunistic bacteria after the use of intravaginal sponges. This novel approach to vaginal probiotic inoculation protocols represents a step forward in promoting alternatives to the prophylactic use of antibiotics and could help improve the well-being of animals when oestrus-synchronisation intravaginal devices are used.

Supplementary material

Supplementary Material for this article (https://doi.org/10.1016/j.animal.2024.101417) can be found at the foot of the online page, in the Appendix section.

Ethics approval

All animal experiments complied with the ARRIVE guidelines and were carried out in accordance with the EU Directive 2010/63/EU for animal experiments. The Animal Experimentation Ethics Committee (CEEA) of the CEU Cardenal Herrera University reviewed the methods employed in this study to manipulate the animals to obtain biological samples. Following the Spanish Royal Decree 53/2013, said committee considered that the method is exempt from ethical approval by the authorised body since it does not include any activity considered a procedure on animals (CEEA report 20/006).

Data and model availability statement

The data/models of the metagenomic analysis were deposited in the European Nucleotide Archive (ENA) (European Molecular Biology Laboratory, European Bioinformatics Institute (EMBL-EBI)): https://www.ebi.ac.uk/ena/browser/home, with the study ID: PRJEB83796. The rest of the datasets used and/or analysed during the current study are available from the corresponding author upon reasonable request.

Declaration of Generative AI and AI-assisted technologies in the writing process

During the preparation of this work the author(s) did not use any AI and AI-assisted technologies.

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Declaration of interest

The authors declare that they have no competing interests.

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