484. Effect of alternative synchronisation treatments on the vaginal microbiota of ewes and their impact on pregnancy rate

E.L. Reinoso-Peláez[1*], O. González-Recio[1], C. González[1], M. Saura[1], A. Fernández[1], A. López-García[1], A. Saborío-Montero[1], J.H. Calvo[2], M. Ramón[3] and M. Serrano[1]

[1]Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria (INIA-CSIC), Ctra. de La Coruña, km 7.5, 28040 Madrid, Spain; [2]Centro de Investigación y Tecnología Agroalimentaria de Aragón (CITA-ARAID-IA2), Av. Montañana 930, 50059 Zaragoza, Spain; [3]Instituto Regional de Investigación y Desarrollo Agroalimentario y Forestal de Castilla-La Mancha (IRIAF), Av. del Vino s/n, 13300 Valdepeñas; edgar.reinoso@inia.es

Abstract

The low pregnancy rate by artificial insemination in sheep represents a fundamental challenge for breeding programmes. In this species, estrous synchronization is done by manipulating hormonal regimens through the insertion of progestogens in intravaginal sponges, which may alter the vaginal microbiota and the success of artificial insemination. In this study, we analysed the microbiome of 94 vaginal swabs from 47 ewes with alternative treatments applied to the progesterone-releasing intravaginal devices, in two sample periods (before and after removing the devices) using nanopore-based metagenome sequencing. A lower abundance of the genera *Neisseria* (Proteobacteria) and *Oenococcus* (Firmicutes) was observed in pregnant compared to non-pregnant ewes. Although differences between treatments were not detected, the progestogen of the synchronization devices seemed to increase the alpha-diversity and decrease the abundance of harmful microorganisms belonging to Gammaproteobacteria and Fusobacteriia classes, suggesting that progestogens may have a beneficial effect on vaginal microbiota favouring the artificial insemination success.

Introduction

Ovine production has a great economic value in Spain, which is the second producer in Europe and the fifth worldwide. Artificial insemination (AI) is the key technology in dairy ruminants breeding programmes for progeny testing, connecting herds and disseminating genetic improvement. However, the efficiency of AI in sheep is low as fertility rates range from 30 to 60%. This low efficiency is due to the particular morphology of the reproductive tract of the ewe, the requirement of using fresh semen and the difficulty to determine the exact stage of the ovulatory cycle at the time of AI. A typical practice for oestrus synchronization is the use of progesterone-releasing intravaginal devices (PRID) consisting on a stainless spiral coil coated with an inert silicone rubber matrix impregnated in flugestone acetate (20 mg), allowing the alteration of ewes hormonal regime. However, our understanding of other factors that may affect the success of AI in sheep, such as the genital tract microbiota, is still low.

In recent years, the study of the composition and abundance of microbial communities (microbiota) has been facilitated by the advances of high-throughput sequencing technologies. In particular, metagenomics has emerged as the reference technique to directly analyse the genomes contained in an environmental sample (microbiome). While there is strong evidence of the role of the reproductive tract microbiota on fertility and sexual disease in humans (e.g. Kindinger *et al.* 2017), its study in livestock species is more limited (Quadros *et al.* 2020; Pascottini *et al.* 2021), and very few in sheep (Serrano *et al.* 2020; Koester *et al.* 2021).

The aim of this study was to elucidate the role of the vaginal microbiota in fertility of Assaf sheep by sequencing the microbial metagenome from pregnant and non-pregnant ewes. In addition, we evaluated the effect of PRIDs supplemented with different additives on the microbiota composition, and whether potential changes in that composition were related with the pregnancy rate. Additionally, the advantages of third-generation

R.F. Veerkamp and Y. de Haas (eds)

DOI: 10.3920/978-90-8686-940-4_484, © E.L. Reinoso-Peláez *et al.* 2022

nanopore sequencing technology (Oxford Nanopore Technologies, ONT) were exploited to study microbial genes and functional pathways (KEGGs, Kyoto Encyclopaedia of Genes and Genomes) involved in fertility.

Materials & methods

Experimental design, DNA extraction and sequencing. A total of 94 vaginal exudate (swabs) samples from 47 ewes from an Assaf flock were obtained in two sampling times. A first vaginal swab was collected from each female, immediately prior to insertion of PRIDs (S1 group). Intravaginal devices were removed 14 days later, and AI was conducted 55 hours after PRID removal. Just before insemination, a second sample of vaginal exudate was taken from each ewe (S2 group) in the same way as the first. At the time of PRID administration the ewes were divided in four batches, depending on alternative treatments added to the PRID (200 mg lyophilized): (1) *probiotic* (n=13), containing *Lactobacillus rhamnosus* and maltodextrine (excipient); (2) *maltodextrine*, the probiotic excipient (n=10); (3) *antibiotic*, containing Framitecin (n=13); and (4) *control*, no additive added in the PRID (n=11). Microbial DNA was extracted using the QIAamp ©DNA Microbiome extraction kit (Qiagen Inc., Valencia, CA, USA). Metagenome sequencing was performed in a MinION device (Oxford Nanopore, Oxford, UK) using the ligation sequencing kit (SQK-LSK109, Oxford Nanopore, Oxford, UK). Pregnancy status was assessed using ultrasonography pregnancy testing 42 days after AI. Notice that all samples (n=94) were used for comparison between sampling times, while only samples from the S2 group (n=47) were used for the analysis of pregnancy outcome.

Bioinformatics and statistical analyses. Base calling was performed with the Guppy 4.2.2 software (ONT). A first filtering of the host genome was performed by mapping the reads against *Ovis aries* reference genome (Oar_rambouillet_v1.0, GCA_002742125.1) using the BBMap software. The retained sequences were analysed using the *sqm*_longreads.pl SqueezeMeta 1.1.0 pipeline software (Tamames and Puente-Sánchez, 2019), which runs a Blastx search of the translated nucleotide reads against GenBank nr, and Kyoto Encyclopedia of Genes and Genomes (KEGG) databases obtaining taxa (phylum, class, and genus) and KEGG. Subsequently, quality filters were applied to remove unclassified taxa and those at a relative abundance threshold <0.1%. Imputation of zero values was performed using a Bayesian multiplicative replacement procedure. Normalization was implemented by applying the Centred Log-Ratio (CLR) transformation. Microbial abundance was evaluated using SQMtools and alpha-diversity by the Phyloseq software. The effect of microbiota on pregnancy rate, as well as the effect of PRID were assessed with PCA, PERMANOVA, and a differential abundance analysis, LIMMA R package. Quality filters and analyses were carried out using R-project (https://www.r-project.org).

Results

Of the 47 inseminated ewes, 26 were pregnant (four in the *probiotic*, seven in the *antibiotic*, six in the *maltodextrine*, and nine in the *control* groups) and 21 were not pregnant (nine in the *probiotic*, six in the *antibiotic*, four in the *maltodextrine* and two in the *control* groups).

No significant differences were observed in microbial alpha-diversity when comparing pregnant and non-pregnant groups, although it was higher in pregnant (56±11.6) than in non-pregnant (47.1±17.2) ewes. Differential abundance analysis revealed that *Oenococcus* (Bacilli) and *Neisseria* (Betaproteobacteria) genera were significantly more abundant in non-pregnant ewes. The comparison of microorganism abundance did not reveal significant differences between probiotic, maltodextrine, antibiotic and control groups, both in the analysis of pregnancy outcome and when comparing sampling times S1 and S2.

Regarding sampling times, alpha-diversity was significant higher in S2 (54.1±14) compared to S1 (39±13.2). Also, significant differences between both groups were found in terms of abundance as revealed by PCA, PERMANOVA and differential abundance analysis for genus, phylum and KEGGs (Figure 1).

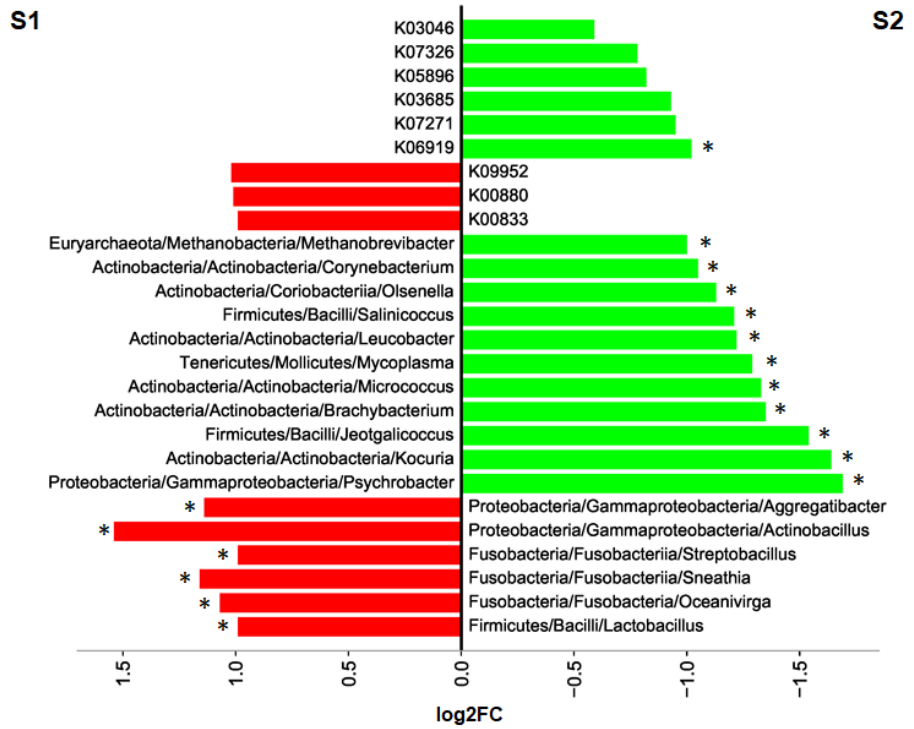

Figure 1. Differential abundance analysis for samples collected before PRIDs placement (S1) and after PRIDs removal (S2), corrected by PRID treatments, at phylum, class, genus and KEGG levels. Asterisks represent adjusted *P*-values at the 5% level. Bars without asterisk correspond to adjusted *P*-values at the 10% level. The x-axis represents the fold-change (FC).

So, Proteobacteria was the most abundant phylum in the S1 group, whereas Firmicutes was the most abundant phylum in S2. At the genus level, *Actinobacillus, Sneathia, Streptobacillus* and *Aggregatibacter* were significantly the most abundant in S1, whereas *Jeotgalicococcus, Kocuria* and *Psychrobacter* were significantly the most abundant in S2 (Figure 1). Concerning functional analysis (genes and genomes), K00800 (L-xylulokinase) and K09952 (CRISPR-associated endonuclease Csn1) KEGGs were significantly more abundant in the S1 group, while K06919 (DNA primase, phage- or plasmid-associated) was significantly more abundant in the S2 group.

Discussion

In this work we have investigated the role of vaginal microbiota in sheep AI pregnancy rate and the effect of different treatments added to PRIDs in its composition and abundance. Our results revealed a significantly higher abundance of the genus *Neisseria* in non-pregnant ewes, and this may be related with the AI failure. Species of this genus (*Neisseria gonorrhoeae*) have been found to trigger ectopic pregnancy and infertility in women (Smolarczyk *et al.* 2021).

In addition, we observed that the progestogen included in the synchronization sponges increased the alpha-diversity of the vaginal microbiota, suggesting a beneficial effect on pregnancy outcome. In line with this, a recent study by Koester *et al.* (2021) evaluating the microbial composition at a pre-breeding time point and after pregnancy testing found increased alpha-diversity associated with pregnancy rate.

Regarding the effect of the PRIDs (and irrespective of the treatment added) on the microbiota composition and abundance, our results showed that the genera *Actinobacillus* (Gammaproteobacteria) and *Sneathia* (Fusobacteriia) decreased in samples collected after the PRID removal (S2). Pathogenic species from these genera (e.g. *Actinobacillus seminis, Sneathia vaginalis* and *Streptobacillus notomytis*) have been associated to infertility in a recent studio by Serrano *et al.* (2020), which may suggest a beneficial effect of the synchronisation treatment for reproductive success. The authors also found that *Mageebacillus* and *Histophilus* were less abundant in the pregnant group, although this discrepancy with our results might be related with the different sequencing methods used (sequencing of the hypervariable regions V3-V4 of the 16S rRNA gene vs metagenome Nanopore sequencing).

From a functional perspective, we detected a higher abundance of sequences corresponding to L-xylulokinase (K00800) in the S2 sampling time. This KEGG is indirectly involved in the process of phage colonization of host bacteria, representing an essential enzyme for phage DNA replication (Wang *et al.* 2020). Its increase after the removal of the synchronization devices could be responsible for the decrease of harmful classes of bacteria such as Gammaproteobacteria and Fusobacteriia. Interestingly, these bacterial classes have more than 10 genes related to helicase and primase activities (K06919), involved in prokaryotic defence against foreign genetic elements, which were found in lower abundance in samples collected after the removal of the sponges (S2).

In conclusion, this study provides new insights about the effect of the progesterone-releasing intravaginal devices used to synchronize estrous on the vaginal microbial composition and abundance. Although preliminary, our results suggest a beneficial effect of the synchronization treatment, contributing to reduce the abundance of some genera associated with reproductive failure. This study also evidences the potential of nanopore sequencing not only to characterise the microbial populations present in a community but also to provide information about genes and pathways involved.

Acknowledgements

This work was funded by Ministerio de Ciencia e Innovación (RTI-2018-096487-R-C33) and the Convenio INIA-GENOVIS CON19-043. The authors gratefully acknowledge the computing time granted by the Centro de Supercomputación de Galicia (CESGA).

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