Characterizing the vaginal microbiota of high and low producing Poll Merino and White Suffolk ewes

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ABSTRACT

There is a substantial, and growing, body of research focused on manipulating gastrointestinal microbes to affect health and production. However, the maternal vaginal microbiota and its effects on neonatal inoculation and lifetime production have received little attention. We aimed to characterize the vaginal microbes of domesticated sheep to determine whether they differ across sheep breeds with differing meat and wool growth potentials and to determine a link between vaginal microbes and high and low producing animals. A flock of White Suffolk (n = 136) and Poll Merino (n = 210) ewes were sorted by the Australian Sheep Breeding Values (ASBV), for yearling fleece weight in the Merino and by post-weaning weight in the Suffolk ewes. The top and bottom ASBV sheep were selected for sampling and the resulting treatment groups were; High Suffolk (n = 12), Low Suffolk (n = 12), High Merino (n = 12), and Low Merino (n = 12) ewes. A double guarded culture swab was used to sample from the surface of the vaginal epithelium. Diversity profiling analysis of vaginal bacterial communities was conducted using 16S rRNA amplicon sequencing. Breed and ASBV group differences in bacterial communities were tested. Within breed, there were no significant differences in ewe vaginal bacterial communities associated with ewe production parameters; however, there was a significant difference in ewe vaginal bacterial communities between breeds. We have been able to characterize the normal vaginal microbiota of nonpregnant ewes and demonstrate a rich microbial community.

INTRODUCTION

Gastrointestinal microbes and their manipulation to affect health and production have become a widely researched area, particularly in production animals. In sheep, studies so far have mainly investigated vaginal microbiota in relation to vaginal health and a way of understanding and reducing the incidence of vaginosis. Most of these studies focus on the use of progesterone controlled internal drug release devices widely used to synchronize estrus in ewes (Manes et al., 2010; Quereda et al., 2020). However, recently studies have begun to focus on the link between vaginal microbial populations and production targets. The neonatal microbiota is much more susceptible to change than the adult microbiota. Therefore, any changes in microbial inoculum source during this period (such as from the maternal birthing tract) may affect lifelong changes in the neonatal gut population and impact health and performance. For example, Serrano et al., (2020) reported that Mageeibacillus, Histophilus, Actinobacillus, and Sneathia were significantly more abundant in ewes found to be non-pregnant after insemination than in pregnant ewes. These bacterial genera were also more abundant in two farms with higher artificial insemination (AI) failure, indicating a potential link between reproductive failure and vaginal microbiota. Recent evidence from buffalo cows demonstrated distinct differences in the vaginal microbial communities during the various phases of the estrus cycle (Mahalingam, et al., 2019). Together, these studies lead to the conclusion that understanding and, ultimately, manipulating, the vaginal microbial composition could aid in improved reproductive management of production ruminants.

The aim of the current study is to: (1) characterize the vaginal microbes of domesticated sheep, 2) determine whether vaginal microbes differ between sheep breeds, and (3)
determine whether there is a link between vaginal microbial populations and high and low meat and wool producing animals within breed. We hypothesized that vaginal microbes would differ between sheep breeds with differing meat and wool growth potentials and high and low producing animals.

MATERIALS AND METHODS

Animals

The study was conducted in accordance with the guidelines set out in the ‘Code of Practice for the Care and Use of Animals for Scientific Purposes’ (NHMRC 2013) and with the approval of The University of Adelaide Animal Ethics Committee (Animal Ethics Committee Project Number: S-2020-004). All animal works were done at a White Suffolk and Poll Merino stud, in the south-east of South Australia, which volunteered the use of their ewes. All ewes sampled were born between 2016 and 2018, with sampling taking place in February 2020. The two breeds were run on the same property side by side all year round. The Merino ewes selected were either non-pregnant test negative or being held back to be surrogate mothers for an embryo transfer program (previous joining prior to this sampling would have been for 5 weeks, starting in early to mid-November 2018 to lambing in March/April in 2019). The White Suffolk ewes were due to be artificially inseminated in late February 2020 and had been brought into the yards for synchronization following our sampling. Although ewes which were selected for the study were all non-pregnant it is not known at what stage they were in their estrus cycle. Nutrition was provided to maintain a body condition score (BCS) of 3.0 – 4.0 and an average of above 3 at joining. Supplementary feed was provided by the farm from March to May as required (with hay-oats and clover-barley). Therefore, the ewes would have not received any supplementary nutrition, above what they receive from the pasture, since May of the previous year. As the animals sampled were not pregnant, there would have been no other relevant nutritional supplementation/alterations, such as feeding of twin bearing ewes.

Sheep were selected from a mob of White Suffolk (n = 136) and Poll Merino (n = 210) ewes. Individual Australian Sheep Breeding Value (ASBV) data were downloaded from the Sheep Genetics database (http://www.sheepgenetics.org.au/Home). Australian Sheep Breeding Values are a prediction of an animal’s genetic merit for a particular trait and are comparable across flocks due to genetic connectedness. A subset of 12 high and low genetic merit ewes based on ASBV for fleece weight for Merino and ASBV post-weaning live-weight for Suffolk were selected for the trial. The treatment groups were: High Suffolk (n = 12), Low Suffolk (n = 12), High Merino (n = 12), and Low Merino (n = 12) ewes. These traits were selected to split the ewes into high and low ‘production’ groups, as each trait is important to the efficiency and value of a ewe from that particular breed.

Vaginal sampling and bacterial profiling

All ewes were walked into the yard as a full flock and had their electronic tags scanned. If ewes had been selected for the study, then they were sampled in a straight raceway. A double guarded culture swab (Uterus culture swab, Minitube Australia Pty., Ltd.) was inserted into the vagina and moved to the posterior fornix, the inner swab was then pushed past the guard and onto the surface of the vaginal epithelium. The swab was moved about on the vaginal epithelium for 30 s per sheep and retracted back into the guard, before being removed from the sheep. Once the swab and guard was removed from the ewe, it was snapped from the extended swab, capped, labeled with the ewe identification number and breed and then immediately placed in a −20 °C portable freezer. Following sampling, ewes re-joined their original mob and were returned to their paddock by the farm staff. On the same day as sampling, the swabs were delivered to the laboratory and stored at −80 °C until analysis.

Total nucleic acid was extracted from the vaginal swabs of individual ewes using a modification of a South Australian Research and Development Institute (SARDI, Adelaide, Australia) proprietary method (Stirling et al. 2004; Torok et al. 2008, 2014). Swabs were added to 1.2 mL extraction buffer (1.3 M guanidine thiocyanate, 1.5 M NaCl, 30 mM Tris–HCl, 65 mM phosphate buffer, 3.4% (w/v) sarkosyl, and 1.7% (w/v) polyvinylpolypyrrolidone) and incubated for 30 min at 70 °C, prior to proceeding with the proprietary extraction method. Diversity profiling analysis of bacterial communities was done using 16S rRNA amplicon sequencing with the 341F (5ʹ-CCTAYGGGRBGCASCAG-3ʹ) and 806R (5ʹ-GGACTACNNGGATCTAAT-3ʹ) primers on the Illumina MiSeq platform using the 300 bp paired end protocol (Australian Genome Research Facility, Melbourne Node). Paired end reads were assembled and trimmed to remove primer sequences and then quality filtered and sorted by abundance using QIIME 1.8, USEARCH and UPARSE software. A Phred quality score of +33 was used and singletons and unique sequence reads were removed from the data set. Sequencing reads were mapped back to operational taxonomic units (OTU) with a minimum identity of 97%, and taxonomy assigned using the Greengenes database (version 13.8) in QIIME.

Statistics

The ewe vaginal 16S rRNA bacterial sequencing data were analyzed using multivariate statistical techniques (PRIMER7 version 7.0.20, PRIMER-E Ltd., Quest Research Limited). These analyses were used to examine differences in bacterial communities associated with breed and ASBV group within breed. For alpha diversity analysis species richness was measured by the total number of species (S), diversity was measured by the Shannon diversity index (H’), and evenness of the bacterial community was measured by Pielou’s evenness index (J’) using DIVERSE. For the beta diversity analysis, Bray–Curtis measures of similarity (Bray and Curtis 1957) were calculated to examine similarities between vaginal bacterial communities from the 16S rRNA profiling data matrices, following standardization by total and square-root transformation.

One-way analysis of similarity (ANOSIM) (Clarke 1993) on the Bray–Curtis similarity matrix data was used to test if there were significant breed and ASBV group differences among the vaginal bacterial communities. The R statistic value describes the extent of similarity among or between groups, with values close to unity (1) indicating that groups are entirely separate and a zero-value indicating that there is no difference among or between groups. To determine which individual bacterial taxa contributed most to the overall dissimilarity between statistically different groups, similarity percentages (SIMPER) (Clarke, 1999) analyses were conducted. Significant breed related taxa (average dissimilarity/standard deviation > 1)
contribute to the top 70% of the average dissimilarities were identified and displayed as box and whisker plots of taxa abundance. Non-metric multidimensional scaling (nMDS) (Kruskal 1964; Shepard 1962a, 1962b) on Bray-Curtis similarity data was done to graphically illustrate relationships with breed. Statistical analysis of both alpha bacterial diversity ($S$, $H'$, and $J$) and ASBV production data was done in IBM SPSS statistics 24, using an Unianova with type 3 sums of squares, with both ASBV group and breed as fixed effects for alpha diversity analysis or treatment (within breed) for the ASBV analysis.

**Results**

**Ewe production traits**

The top and bottom ewes sampled, based on their ASBV, were found to be significantly divergent ($P < 0.0001$) based on separation for the ASBV post-weaning weight of the lambs born to the Suffolk ewes (low Suffolk 12.33 ± 0.25 kg and high Suffolk 18.23 ± 0.21 kg) and ASBV yearling clean fleece weight for the Merino ewes (low Merino 6.97 ± 0.53 kg and high Merino 26.62 ± 0.60 kg). This simply shows that the ASBV groupings were statistically different before analysis of the bacterial communities.

**Vaginal bacterial communities**

The V3–V4 region of the 16S rRNA was sequenced from 48 ewe vaginal swab samples. Following quality control, there were on average 120,481 sequence reads per sample with a median of 125,139 sequence reads per sample. Sequence reads were assigned to 400 taxonomic OTU.

For the alpha diversity measures there were no significant differences in ewe vaginal bacterial communities associated with ASBV grouping by breed at the genus level; total number of species ($P = 0.376$), Shannon diversity index ($P = 0.497$) and Pielou’s evenness index ($P = 0.847$; Fig. 1). For beta diversity measures there were also no significant differences in vaginal bacterial communities associated with ASBV, within breed, at any taxonomic level investigated (phyla: Poll Merino $R = −0.019$, $P = 0.604$, White Suffolk $R = −0.006$, $P = 0.454$; family: Poll Merino $R = 0.001$, $P = 0.419$, White Suffolk $R = −0.055$, $P = 0.949$; genera: Poll Merino $R = −0.007$, $P = 0.491$, White Suffolk $R = −0.046$, $P = 0.914$; and species: Poll Merino $R = −0.007$, $P = 0.513$, White Suffolk $R = −0.044$, $P = 0.890$). However, significant beta diversity differences in vaginal bacterial communities associated with breeds, irrespective of ASBV, were detected at both the genus ($R = 0.004$, $P = 0.049$) and species levels ($R = 0.042$, $P = 0.043$), although not at the phyla ($R = −0.016$, $P = 0.572$) or family ($R = 0.007$, $P = 0.304$) levels. The observed breed-related vaginal microbiota difference is graphically demonstrated at the genus level in Fig. 2.

The dominant bacterial phyla in the vagina of ewes regardless of breed or ASBV (in decreasing order) were Proteobacteria, Fusobacteria, Firmicutes, Bacteroidetes, Tenericutes, and Actinobacteria; collectively accounting for over 95% of the bacterial population (Fig. 3). Dominant bacterial class contributing to the ewe vaginal microbiota (in decreasing order) were Fusobacteria, Gammaproteobacteria, Clostridia, Mollicutes, Bacteroidia, Actinobacteria, Bacilli, Betaproteobacteria, Erysipelotrichi, and Alphaproteobacteria, collectively accounting for over 90% of the bacterial population. While dominant bacterial order contributing to the ewe vaginal microbiota (in decreasing order) were Fusobacteriales, Pasteurellales, Clostridiales, Mycoplasmatales, Actinomycetales, Bacteroidales, Erysipelotrichales, Rickettsiales, Flavobacteriales, Gemellales, Campylobacterales, Burkholderiales, Neisseriales, collectively accounting for approximately 90% of the bacterial population.

Figure 4 shows the bacterial families contributing to at least 5% of the total vaginal population in individual ewes. In decreasing order of abundance, Pasteurellaceae, Leptotrichiaceae, Fusobacteriaceae, and Mycoplasmataceae collectively contributed to over 70% of the bacterial population. Bacterial genera contributing at least 5% to the vaginal bacterial population in individual ewes are shown in Fig. 5. Uncharacterized Leptotrichiaceae, uncharacterized Pasteurellaceae, Actinobacillus, Fusobacterium, and Mycoplasma collectively contributed to over 70% of the bacterial population. Genera identified by SIMPER analysis as significantly driving breed related vaginal microbiota differences are shown in Fig. 6. Coprococcus, Mycoplasma,
Fusobacterium, and uncharacterized Pasteurellaceae were more abundant in the Suffolk ewes, while Actinobacillus and uncharacterized Leptotrichiaceae were more abundant in Merino ewes. Where bacterial genera could be classified to species level, it was found that Actinobacillus seminis was significantly more abundant in the vagina of Merino ewes as compared with the Suffolk ewes.

Discussion

Two ASBV production traits (yearling fleece weight for Poll Merino and post-weaning progeny weight for White Suffolk ewes) were chosen to represent performance, as these traits best describe the production targets of the two breeds investigated. Despite identifying divergent ASBV “high” and “low” animals for each trait within the two breeds, there were no significant vaginal microbiota differences detectable in either alpha or beta diversity measures. However, there were significant differences in vaginal microbiota between the two breeds, regardless of the ASBV production trait. Most ovine reproductive microbiota studies to date have focused on vaginal health, the microbial changes which result from estrus synchronization programs and reproductive success. These studies have also used a variety of microbiological or molecular methodologies to identify bacteria, which have impaired the direct comparison of bacterial population structure amongst studies.

Two common bacterial genera identified in the normal vaginal microbiota of the sheep, namely Mycoplasma and Ureaplasma, have both been previously linked with increased incidence of pelvic inflammatory disease and premature birth in women (Larsen and Hwang, 2010). Ureaplasma has also been linked to abortions, granular vulvovaginitis, subclinical endometritis and decreased milk production in cattle, sheep, and goats (Díaz et al., 2019; Santos et al., 2021). Actinobacillus seminis was also identified in the normal vaginal microbiota of our sheep, and was more abundant in Merino ewes. This species of bacterium has also been associated with low fertility, and occasional abortions in sheep and...
goats (Foster et al., 1999; García et al., 2020; Van Tonder 1973).

Manes et al. (2010) analyzed the vaginal mucosal microbiota, using culture-based microbiology, in Texel ewes in Argentina around the point of estrus synchronization and found the predominant microbes were mostly (90%) gram positive bacteria (Bacillus spp., Staphylococcus spp., and Corynebacterium spp.). These bacterial genera were also identified in our study, although the contribution of these bacteria to the overall ewe vaginal microbiota were low with Bacillus spp. contributing 0.02–0.11% and Corynebacterium spp. contributing 0.06–0.34%. These discrepancies in percentage contribution of these bacterial taxa are most likely due the different methodologies employed. Swartz et al (2014) characterized the vaginal microbiota of Rambouillet sheep and crossbred beef cattle using 16S rRNA profiling and found that both ewes and cows were predominately colonized by members of the Proteobacteria (almost exclusively Gammaproteobacteria), Fusobacteria, and Bacteroidetes. This is consistent with findings that these three phyla are amongst the most abundant. Furthermore, Aggregatibacter and Streptobacillus are typically the most abundant genera in both ewes and cows (Swartz et al., 2014). Both these genera were identified in our ewes, but with a low percentage contribution to the overall vaginal microbiota.

Oliveira et al (2013) assessed the change in goat vaginal bacterial populations after estrus synchronization with progestogen sponges, using bacterial culture dependant techniques. They found the most prevalent bacteria identified belonged to the genus Staphylococcus, except at the time of sponge withdrawal, when the most prevalent bacterium was Escherichia coli. The results of that study demonstrated that the vaginal microbiota in goats subjected to a short-term protocol of estrus induction and synchronization was altered, with a rapid re-establishment of the normal microbiota following sponge removal. E. coli and Staphylococcus spp. were also identified in the normal vaginal microbiota of our sheep regardless of breed (data not shown), although the contribution of these two species to the overall bacterial population was low (E. coli 0.17–1.41% and Staphylococcus spp. 0.01–0.03%). Quereda et al. (2020) also recently investigated the effect of intravaginal sponges soaked in probiotics on ewe estrus synchronization. They used a probiotic mixture of Lactobacillus spp. (60% Lactobacillus crispatus, 20% Lactobacillus brevis, and 20% Lactobacillus gasseri) and found that it did not affect the general health status of the ewes and did not interfere or have negative effects on ovine fertility during natural mating. This is a promising result for the use of vaginal probiotics, as no ill health was induced with the treatments. The prevalence of Lactobacillus spp. was low within the normal ewe vaginal environment of our sheep. Hence, such a probiotic would most likely target maintenance of vaginal health, rather than affecting the neonate’s health and production.

Serrano et al (2020) and Deng et al (2019) investigated the genital microbiota of sheep and cattle, respectively, and the effect on AI outcomes, linking vagina microbiota with production. Differences in vaginal microbiota between pregnant and non-pregnant ewes, and between ewes carrying...
progesterone-releasing intravaginal devices with or without antibiotic were investigated, with *Magee*, *Histophilus*, *Actinobacillus*, and *Sneathia* significantly less abundant in pregnant ewes (Serrano et al., 2020). In addition, these genera were more abundant in ewes from two farms with higher AI failure. These genera were not present in the sperm samples of AI rams, but were found in the foreskin samples of rams belonging to a flock with a higher AI failure rate, suggesting the presence in the ewes’ vagina could be due to prior transmission via natural mating with rams reared in that flock. This is a promising finding, regarding inoculation of the ewe’s vagina with ‘designer’ microbes, as inoculation from rams upon previous mating was able to colonize the vaginal and affect subsequent production. The trial by Serrano et al. (2020) supports the idea that vaginal microbiota does play a part in production targets, in the case of pregnancy rates.

A focus of our research was the characterization of the normal vaginal microbiota in sheep and not the inoculation and production outcome of the young. The role of the vaginal microbiota in the inoculation of neonates and the establishment of their early gut microbiota may be an overlooked area of possible manipulation, which would be minimally invasive to both the dam and her offspring. Identifying the normal variations and those caused by breed and production differences in vaginal microbial populations is important. These differences may potentially represent the first opportunity to understand and create beneficial neonatal microbial populations. It is generally supported that in humans a dysbiosis of the vaginal microbiota increases the risk of bacterial vaginosis and contributes to an overall decrease in vaginal health (Barrientos-Durán et al., 2020). In pregnant women, there is an increase in vaginal bacterial diversity from week 24 of pregnancy and leading up to birth, showing a natural increase in the diversity of the vaginal microbiota before parturition (Rasmussen et al., 2020). It would be reasonable to assume that an increased diversity in vaginal microbiota would also be positive for ewe vaginal health. It would be interesting to investigate if there is a gestational increase in bacterial diversity in ewes throughout pregnancy, and if so, if the increase in diversity around parturition is a mechanism to assist in inoculation of the neonate or simply to ensure maintenance of vaginal health.

A ruminant animal is a very efficient converter of low quality feed to high quality protein in the form of meat, wool and milk. Microbial fermentation and rumen nutrient absorption are key steps in the energy metabolism of ruminants and the ruminant microbiota is highly associated with production of the host animal, such as feed conversion efficiency and growth (Zhou et al., 2009) and wool production (De Barbieri et al., 2015). Therefore, we believe that the production parameters used to separate the two breeds were relevant to production efficiencies of each breed. The two ASBV values chosen (yearling fleece weight for Merinos and post weaning

**Figure 5.** Ewe vaginal bacterial genera. Shade plots of bacterial genera contributing at least 5% to overall community structure. Depth of rectangle shading represents abundances from the standardised and square root transformed taxa-by-samples matrix, on a continuously linear grey-scale from absent (white) to the maximum value in that matrix (black). Black triangle = high Merino, inverted black triangle = low Merino, grey square = high Suffolk, and grey diamond = low Suffolk. * indicates uncharacterized bacterial genera.
It could be argued that our “high” and “low” ASBV divergent grouping were artificial and not sufficiently divergent, despite being analyzed as statistically different. For example, our selected “low” groups may not have accurately represented an industry relevant “low” production trait, given the high-quality stock from which our experiment animals were chosen because these single traits best describe the production targets of each individual breed. Even though the “high” and “low” ASBV grouping for each breed were significantly divergent, there were no significant vaginal microbiota differences observed in either the alpha or the beta diversity measures.
were selected. However, if we look at the Australian flock ASBVAs for 2020 (study sampling year) we believe that we did capture a reasonable representative spread of animals with our selected animals. With our White Suffolks, the low ASBV align with the 70–75% percentile band and the high ASBV align with the 4–5% band. For Merinos, the lows align with the 85–90% percentile band and the highs align with the 15–20% band (2020 Australian flock ASBV Data from LAMBPLAN and MERINOSELECT as supplied by direct communication with Sheep Genetics). Therefore, we believe that we did achieve meaningful genetic separation between high and low ASBV groups for both breeds. Alternatively, our sample size for the two different ASBV traits investigated (n = 12/treatment) may have been too low to detect significant microbial differences due to the animal-to-animal variability within group. We believe that there is merit in further investigating production related vaginal microbiota differences in more divergent ASBV groups with higher animal replication.

There could be an argument that post-weaning weight would have been a better measure of production for other breeds and potentially more comparable across breeds. Weight may be more likely to be a gut and microbial related factor than fleece production. There are a number of other ASBV factors, such as birth weight, fat depth, eye muscle depth, wool fiber diameter and intestinal worm egg count. Some of these may be more relevant ASBV parameters to investigate vaginal microbiota against, in particular birth weight, reproductive ASBV with the 85–90% percentile band and intestinal worm egg count, as these are more likely to be linked to gut and vaginal health. The above-mentioned research shows that there is a great deal of interest in the vaginal microbiota at the point of synchronization, but that production efficiency and its link to vaginal microbiota is overlooked, especially in ovine research.

Significant differences in ewe vaginal bacterial communities associated with breed were detected. However, we did not find any differences in the vaginal microbiota associated with the two ASBV production traits investigated in Poll Merino (yearling fleece weight) or White Suffolk (post weaning progeny weight) ewes. This may be due to our low sample size. However, we still believe that there is merit in further investigating linkages between ewe vaginal microbiota and these and other production traits as significant breed related differences were observed in vaginal microbiota between the Suffolk and Merino ewes. This area of research offers significant opportunity for manipulation of production parameters in livestock.

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CONFLICT OF INTEREST STATEMENT

None declared.

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