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Characterising the vaginal microbes of high and low producing Poll Merino and White Suffolk ewes

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Executive summary

In the neonatal gut, microbial diversity is relatively low at birth but increases rapidly as bacteria colonise the gut. The initial microbial community is acquired by sampling from the surrounding environment, including the vagina during delivery. The neonatal microbiota (early life microbial community) is more susceptible to change than the adult microbiota. Any changes during this period have the ability to effect lifelong change in the population and alter health and productivity. There is a substantial, and growing, body of research focussed on manipulating gastrointestinal microbes to affect health and production. However, the vaginal microbiota and its effects on neonatal inoculation and lifetime production have received little attention. Manipulation of the neonatal inoculation via the vagina would be minimally invasive to both the dam and offspring, and represents a significant opportunity to positively alter the microbiota of production animals. Identifying the normal variations caused by breed and production in vaginal microbes could represent the first opportunity to understand and manipulate neonatal microbial populations and production through this pathway.

Objectives:

1. To characterise the vaginal microbes of domesticated sheep.
2. To determine if vaginal microbes differ across sheep breeds with differing growth and wool growth potentials.
3. To determine a link between vaginal microbes and high and low producing animals.

A mob of White Suffolk (n=136) and Poll Merino (n=210) ewes were sorted by ASBV, for yearling fleece weight in the Merinos and by post-weaning weight in the Suffolks. The top and bottom ASBV sheep were selected for sampling and the resulting treatment groups were; High ASBV White Suffolk ewes (n=12), Low ASBV White Suffolk ewes (n=12), High ASBV Poll Merino ewes (n=12), Low ASBV Poll Merino ewes (n=12). A double guarded culture swab was used to sample from the surface of the vaginal epithelium, while avoiding contaminants at the entrance of the vagina. Total nucleic acid was extracted from the vaginal swabs from individual ewes. Diversity profiling analysis of vaginal bacterial communities was done using 16S rRNA amplicon sequencing. Bacterial community were analysed against breed and production group.

Despite the fact that the divergence between ASBV between our allocated treatment groups, within breed, was significantly different, there were no significant differences in ewe vaginal bacterial communities associated with ewe breed or ewe production parameters within breed. However, there was a trend towards the two breeds having different populations at the genus and species levels. Suffolk sheep tended to have a great number for taxa and Shannon's diversity, although not significantly different from Merino sheep.

We have been able to characterise the normal vaginal microbiota of non-pregnant ewes and demonstrate a rich microbial community. Perhaps, with greater than n=24/breed, we might have seen significant differences rather than just a trend between breeds. Also, the ASBV were significantly different between our high/low groups, but it can be questioned if the "low" group is really representative of an industry "low", given the high quality stock from which our experiment animals were selected. To further research this area, we would suggest several possibilities leading from the more simple to more complex studies; ASBV study using larger animal numbers with industry relevant divergence in ASBV in Suffolks only, investigate other ASBVs likely linked with gut health, utilise production records to analysed microbial data against rather than ASBV data, investigate pregnant ewes and their microbes' impact on production of offspring, vaginal probiotics or use of microbiological 'seeds' in order to alter the ewe vaginal microbial populations just before lambing and difference in breed vaginal microbes and cross inoculation with different breed microbial populations.

Table of contents

1 Background

1.1 Current knowledge base

In the neonatal gut, microbial diversity is relatively low at birth but increases rapidly as the gut matures and is colonised with microbes. The initial microbial community (made up of bacteria, fungi, protozoa and viruses) is acquired by sampling from the surrounding environment, including the vagina during birth (Curtis and Sloan, 2004; Rey et al., 2013). Once established, the adult gut microbiota is highly resilient to any changes (Benson et al., 2010). The neonatal microbiota is much more susceptible to change than the adult microbiota. Therefore, any changes in microbial inoculum source during this period may have the ability to effect lifelong changes in the neonatal gut population and impact health and performance.

1.2 Why was this research undertaken?

Gastrointestinal microbes and their manipulation to affect health and production has become a widely researched area, particularly in production animals. However, there appears to be no research into the vaginal microbiota of Australian domestic/production sheep. Also, research into ruminant vaginal microbial populations have mainly been focused on maintenance of vaginal health and not on the inoculation of neonates and the establishment of their early gut microbiota. This 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 population is important. These differences may potentially represent the first opportunity to understand and create beneficial neonatal microbial populations. This knowledge can then lead us in attempting to change lifetime population, through manipulation of their populations.

1.3 Significance for industry

There is no research to date that characterises the vaginal microbiota of Australian sheep commonly used in production, and very little overall in sheep. There is massive interest in manipulating the neonatal microbiota of production animals in order to increase production efficiency world-wide. However, the link between this 'first' gut microbiota and the dam vaginal population is not yet fully understood. One of the first inoculation sources is the vaginal microbiota and we believe that this will be the next population to be researched in the effort to learn how to successfully manipulate neonatal gut inoculation, with a special interest into how we can affect lifelong production of the inoculated neonate.

1.4 Overarching aims

There were three aims of the current trial;

1) Characterise the vaginal microbes of domesticated sheep,
2) Determine if vaginal microbes differ across sheep breeds with differing growth and wool potentials and
3) Determine if there is a link between vaginal microbial populations and high and low producing animals.

2 Project objectives

2.1 Objective 1

To characterise the vaginal microbes of domesticated sheep.

2.2 Objective 2

To determine if vaginal microbes differ across sheep breeds with differing growth and wool growth potentials.

2.3 Objective 3

To determine a link between vaginal microbes and high- and low-producing animals.

3 Methodology

3.1 Animals and treatments

The study was conducted in accordance with the guidelines set out in 'Code of Practice for the Care and Use of Animals for Scientific Purposes' (NHMRC 2004) and with the approval of The University of Adelaide Animal Ethics Committee (Animal Ethics Committee Project Number: S-2020-004). All animal work was done at a White Suffolk and Poll Merino stud, in the South-East of South Australia, which volunteered the use of their ewes. No ewes (either Poll Merino or White Suffolk) in this study were pregnant.

Sheep were selected from a mob of White Suffolk (n=136) and Poll Merino (n=210). Individual sheep ASBV data was downloaded from the web database 'sheep genetics' (http://www.sheepgenetics.org.au/Home). Sheep were then sorted by ASBV (within breed), for yearling fleece weight in the Merinos and by post-weaning weight in the Suffolks. The top and bottom ASBV (high- and low-producing) sheep were selected for sampling for each breed. Treatment groups were: High ASBV White Suffolk ewes or HS (n=12), Low ASBV White Suffolk ewes or LS (n=12), High ASBV Poll Merino Ewes or HM (n=12) and Low ASBV Poll Merino Ewes or LM (n=12). We accept that a limitation of this study may be the overall high quality of sheep produced by this particular stud, meaning that the 'tail-ends' of this flock may not represent industry 'tail-ends'. However, there was still a large difference between the ewes selected for sampling in the high- and low-producing groups for the trial and, therefore, we still believe that this cohort was adequate to answer our current aims. Table 1 shows the means for the treatment split and individual sheep ASBV numbers. Following analysis the ASBV treatment split per breed was found to be significant ($P<0.0001$; Table 1).

Table 1: Treatment mean and standard error (SEM) of ASBV for Merino and Suffolk ewes sampled and individual ewe values.

SUFFOLK		**MERINO**	
Treatment	Post weaning weight, kg	Treatment	Yearling clean fleece weight, kg
mean Low	12.333	mean Low	6.967
SEM Low	0.247	SEM Low	0.527
mean High	18.233	mean High	26.617
SEM High	0.211	SEM High	0.598
P value	< 0.0001	P value	< 0.0001
Low	10.5	Low	3.6
Low	11.2	Low	4
Low	11.6	Low	5.2
Low	11.9	Low	5.9
Low	12.4	Low	7.6
Low	12.5	Low	7.6
Low	12.9	Low	7.7
Low	12.9	Low	8
Low	12.9	Low	8.1
Low	12.9	Low	8.4
Low	13	Low	8.5
Low	13.3	Low	9
High	17.6	High	23.6
High	17.7	High	24.1
High	17.7	High	24.1
High	17.9	High	26.3
High	17.9	High	26.3
High	17.9	High	26.4
High	18	High	26.5
High	18.3	High	27.2
High	18.3	High	27.8
High	18.5	High	27.8
High	18.7	High	28.3
High	20.3	High	31

3.2 Sampling process

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 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 seconds 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, labelled with the ewe ID and breed and then immediately placed in a -20^{o}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 driven to the laboratory and immediately transferred from the -20^{o}C portable freezer into a -80^{o}C freezer until analysis.

Total nucleic acid was extracted from the vaginal swabs from individual ewes, by a modification of a South Australian Research and Development Institute (SARDI, Adelaide, Australia) proprietary method (Stirling et al. 2004; Torok et al. 2008; Torok et al. 2014). Diversity profiling analysis of vaginal bacterial communities was done using 16S rRNA amplicon sequencing with the 341F and 806R primers on the Illumina MiSeq platform using the 300 bp paired end protocol (AGRF, 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. Sequencing reads were mapped back to operational taxonomic units (OTU) with a minimum identity of 97%, and taxonomy assigned using the Greengenes database in QIIME.

3.3 Statistics

Community structure of the ewe vaginal populations were analysed against breed and production group (high or low for breed measures stated above). IBM SPSS statistics 24 was used for both alpha bacterial diversity (S, H' and J') and ASBV treatment analysis (Table1), using an Unianova with type 3 sums of squares, with both treatment and breed as fixed effects or treatment (within breed) for the ASBV analysis. The vaginal 16S rRNA bacterial sequencing data were analysed using multivariate statistical techniques (PRIMER6, PRIMER-E Ltd., Ivybridge, UK). These analyses were used to examine differences in vaginal bacterial communities associated with ASBV and breed. 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. Bray–Curtis measures of similarity (Bray and Curtis 1957) were calculated to examine similarities between vaginal bacterial communities of ewes from the 16S rRNA profiling data matrices, following standardisation and fourth-root transformation. Analysis of similarity (ANOSIM) (Clarke 1993) was used to test if vaginal bacterial communities were significantly different between ASBV groups and breed. Similarity percentages (SIMPER) (Clarke 1993) analyses were done to determine which individual OTUs (bacterial taxa) contributed most to treatment groups. Unconstrained ordinations were done to graphically illustrate relationships between treatments using nonmetric multidimensional scaling (nMDS) (Shepard 1962a, 1962b; Kruskal 1964)

4 Results

4.1 Vaginal bacterial communities

The V3-V4 region of the 16S rRNA was sequenced from 48 vaginal ewe swab samples. Following quality control, there were on average 120,481 reads per sample with a median of 125,139 reads per sample. There were no significant differences in ewe vaginal bacterial communities associated with breed, although there was a trend towards significance at the bacterial genus and species levels (Table 2). This is graphically demonstrated at the genus level in Figure 1.

Table 2: One-way ANOSIM of vaginal bacterial taxa associated with ewe breed.

Taxonomic level	**Breed**
Phylum	Global R=-0.008, P=0.629
Class	Global R=0.014, P=0.218
Order	Global R=0.012, P=0.211
Family	Global R=0.013, P=0.190
Genus	Global R=0.032, P=0.062
Species	Global R=0.035, P=0.061

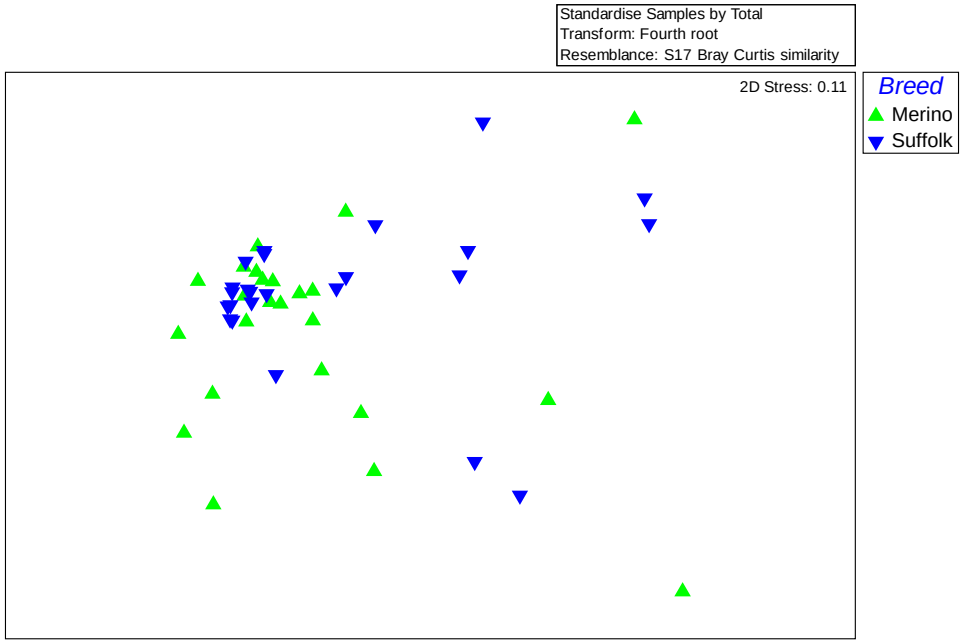

Figure 1: nMDS of vaginal bacterial genera from Poll Merino and White Suffolk ewes regardless of ABV trait.

There were no significant differences in vaginal microbiota associated with the investigated high and low ASBV traits in either the Poll Merino or White Suffolk ewes (Table 3).

Table 3: One-way ANOSIM of vaginal bacterial taxa associated with ASBV traits in Poll Merino and White Suffolk ewes.

Taxonomic level	Poll Merino	White Suffolk
Phylum	Global R=-0.022, P=0.675	Global R=-0.012, P=0.542
Class	Global R=-0.013, P=0.587	Global R=-0.038, P=0.778
Order	Global R=-0.015, P=0.616	Global R=-0.054, P=0.949
Family	Global R=-0.002, P=0.431	Global R=-0.054, P=0.964
Genus	Global R=0.002, P=0.374	Global R=-0.056, P=0.988
Species	Global R=0.000, P=0.407	Global R=-0.057, P=0.988

The lack of treatment related differences observed within breed are shown in Figure 2.

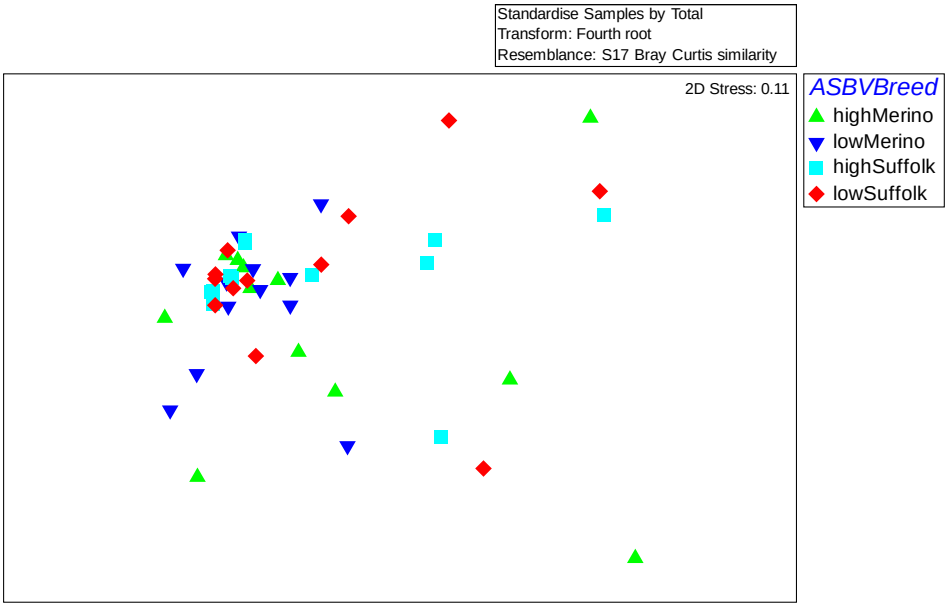

Figure 2: nMDS of vaginal bacterial genus from high and low performing Merion and Suffolk ewes.

The dominant bacterial phyla found in the vagina of ewes (in decreasing order) were Proteobacteria, Fusobacteria, Firmicutes, Bacteroidetes, Tenericutes and Actinobacteria, accounting for over 95% of the bacterial population. Figure 3 shows the vaginal bacterial phyla found in the various groups of ewes.

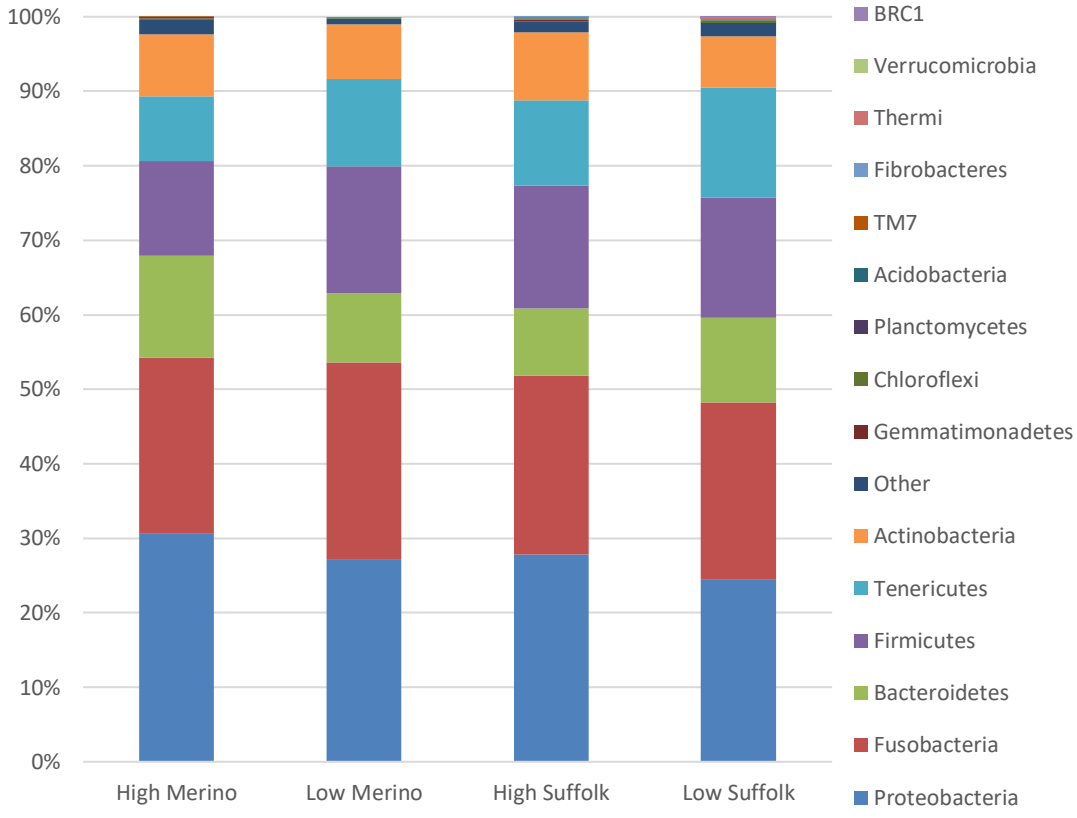

Figure 3: Bar chart of vaginal bacterial phyla of ewes. Order of bacterial phyla seen in bars is as indicated in the legend.

Dominant bacterial class contributing to the ewe vaginal microbiota (in decreasing order) were Fusobacteria, Gammaproteobacteria, Clostridia, Moliicutes, Bacteroidia, Actinobacteria, Bacilli, Betaproteobacteria, Erysipelotrichi and Alphaproteobacteria, accounting for over 90% of the bacterial population (Figure 4).

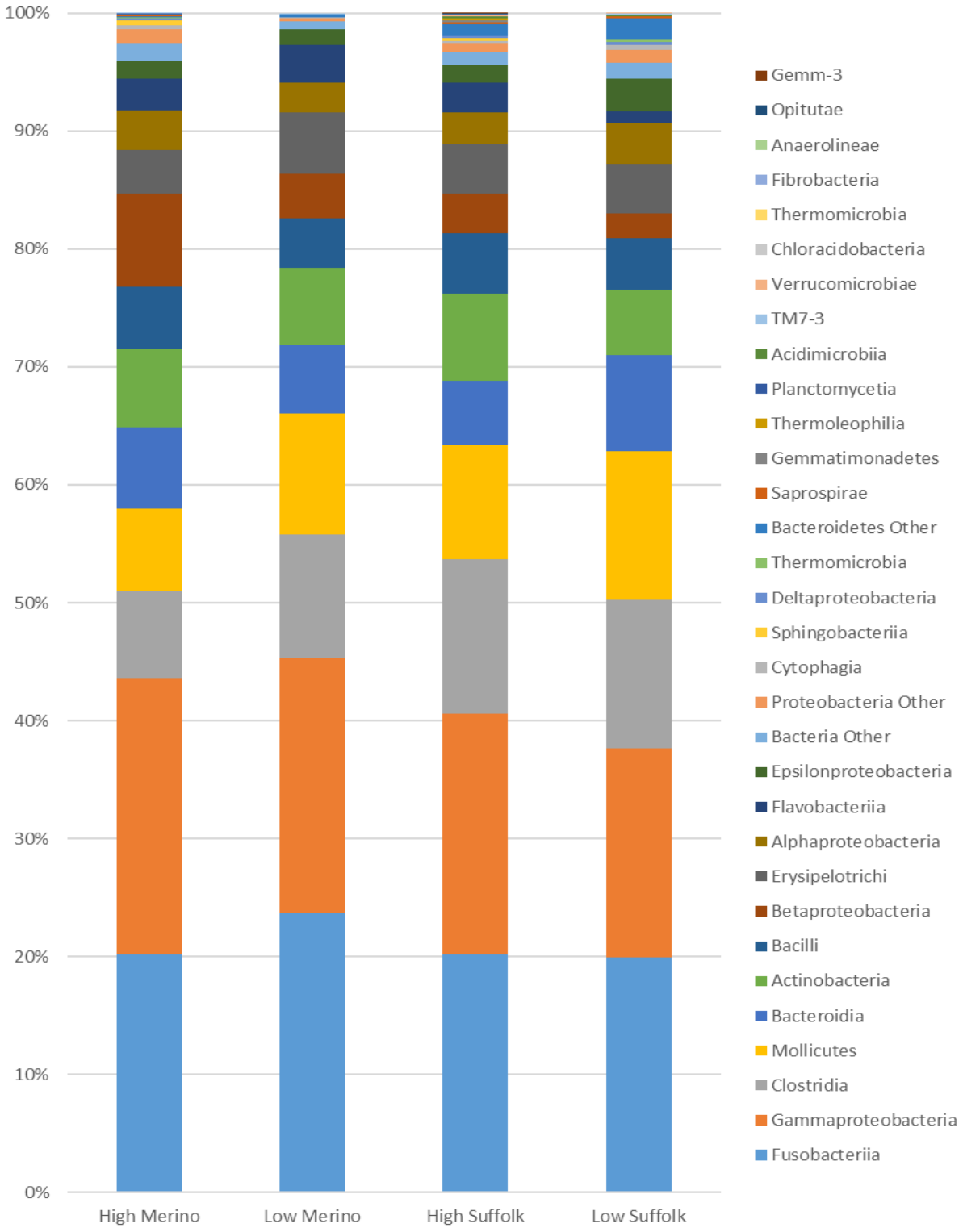

Figure 4: Bar chart of vaginal bacterial class of ewes. Order of bacterial class seen in bars is as indicated in the legend.

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, accounting for approximately 90% of the bacterial population.

Table 4 shows the dominant vaginal bacteria identified in all ewes, regardless of breed or ASBV production trait in this experiment.

Table 4: Bacteria detected within the vaginal of Poll Merino and White Suffolk ewes.

Phyla	**Class**	**Order**	**Family**
Proteobacteria	Gammaproteobacteria	Pasteurellales	*Pasteurellaceae*
	Alphaproteobacteria	Rickettsiales	
	Betaproteobacteria	Neisseriales	*Neisseriaceae*
	Epsilonproteobacteria	Campylobacterales	*Campylobacteraceae*
Fusobacteria	Fusobacteriia	Fusobacteriales	*Leptotrichiaceae*
			Fusobacteriaceae
Firmicutes	Clostridia	Clostridiales	*Ruminococcsaceae*
			Lachnospiraceae
			Tissierellaceae
	Erysipelotrichi	Erysipelotrichales	*Erysipelotrichaceae*
	Bacilli	Gemellales	*Gemellaceae*
Bacteroidetes	Bacteroidia	Bacteroidales	*Bacteroidaceae*
			Porphyromonadaceae
	Flavobacteriia	Flavobacteriales	*Weeksellaceae*
Tenericutes	Mollicutes	Mycoplasmatales	*Mycoplasmataceae*
Actinobacteria	Actinobacteria	Actinomycetales	

Table 5 shows the bacterial taxa which could be identified to the species level within the vagina of each group of ewes, and the overall similarity of microbial communities within treatment group. Although, there were no significant differences between groups and common bacterial species were observed across groups, it appeared that the White Suffolk ewes had a more complex vaginal microbiota. This observation was supported by the alpha diversity metrics, number of taxa (S) and Shannon diversity index (H'), although they were not significant ($P > 0.05$) (Figure 5). Evenness (Pielou's J') was also not significantly different among treatment groups.

Table 2: Bacterial species detected in high- and low-ASBV Poll Merino and White Suffolk ewe.

High Merino (Av sim 34.5%)	Low Merino (Av sim 49.6%)	High Suffolk (Av sim 43.4%)	Low Suffolk (Av sim 42.6%)
Actinobacillus seminis	*Actinobacillus seminis*	*Actinobacillus seminis*	*Actinobacillus seminis*
Escherichia coli	*Alysiella filiformis*	*Alysiella filiformis*	*Campylobacter ureolyticus*
Alysiella filiformis	*Streptococcus minor*	*Campylobacter ureolyticus*	*Alysiella filiformis*
Pseudomonas stutzeri	*Bacteroides fragilis*	*Bibersteinia trehalosi*	*Streptococcus minor*
Bacteroides fragilis	*Campylobacter ureolyticus*	*Escherichia coli*	*Escherichia coli*
Streptobacillus moniliformis	*Escherichia coli*	*Streptobacillus moniliformis*	*Clostridium perfringens*
Acinetobacter schindleri	*Streptobacillus moniliformis*	*Streptococcus minor*	*Bibersteinia trehalosi*
Acinetobacter lwoffii	*Acinetobacter lwoffii*	*Streptococcus luteciae*	*Streptobacillus moniliformis*
Propionibacterium acnes	*Peptostreptococcus anaerobius*	*Pseudomonas stutzeri*	*Bacteroides fragilis*
Pseudomonas veronii	*Bacteroides ovatus*	*Bifidobacterium pseudolongum*	*Streptococcus luteciae*
Actinomyces hyovaginalis	*Pseudomonas stutzeri*	*Bacteroides fragilis*	*Actinomyces hyovaginalis*
	Corynebacterium lubricantis	*Acinetobacter schindleri*	*Ruminococcus gnavus*
	Pseudomonas veronii	*Acinetobacter lwoffii*	*Selenomonas ruminantium*
	Acinetobacter schindleri	*Fibrobacter succinogenes*	*Eubacterium dolichum*
	Bacillus firmus	*Nitrosovibrio tenuis*	*Blautia producta*
		Ruminococcus gnavus	*Suttonella indologenes*
		Defluviitalea saccharophila	*Clostridium neonatale*
		Clostridium perfringens	*Ruminococcus flavefaciens*
		Propionibacterium acnes	*Corynebacterium stationis*
		Actinomyces hyovaginalis	*Pseudomonas stutzeri*
		Corynebacterium lubricantis	*Bifidobacterium pseudolongum*
		Corynebacterium stationis	*Sporosarcina ginsengi*
		Virgisporangium ochraceum	*Faecalibacterium prausnitzii*
		Bacteroides nordii	*Acinetobacter lwoffii*
		Bacteroides ovatus	
		Anaerophaga thermohalophila	
		Porphyromonas endodontalis	
		Prevotella ruminicola	
		Capnocytophaga ochracea	
		Flavobacterium succinicans	
		Sphingobacterium multivorum	
		Bacillus cereus	
		Bacillus firmus	
		Bacillus halodurans	
		Sporosarcina ginsengi	
		Gracilibacillus halotolerans	
		Lactobacillus mucosae	
		Streptococcus agalactiae	
		Streptococcus infantis	
		Clostridium bowmanii	
		Clostridium neonatale	
		Blautia producta	
		Clostridium aminophilum	
		Coprococcus catus	
		Peptostreptococcus anaerobius	
		Butyricicoccus pullicaecorum	
		Faecalibacterium prausnitzii	
		Ruminococcus flavefaciens	
		Selenomonas ruminantium	
		Veillonella dispar	
		Clostridium saccharogumia	
		Eubacterium dolichum	
		Azospirillum amazonense	
		Constrictibacter antarcticus	
		Peredibacter starrii	
		Suttonella indologenes	
		Actinobacillus parahaemolyticus	
		Aggregatibacter segnis	
		Haemophilus parainfluenzae	
		Acinetobacter rhizosphaerae	
		Pseudomonas veronii	
		Pyramidobacter piscolens	

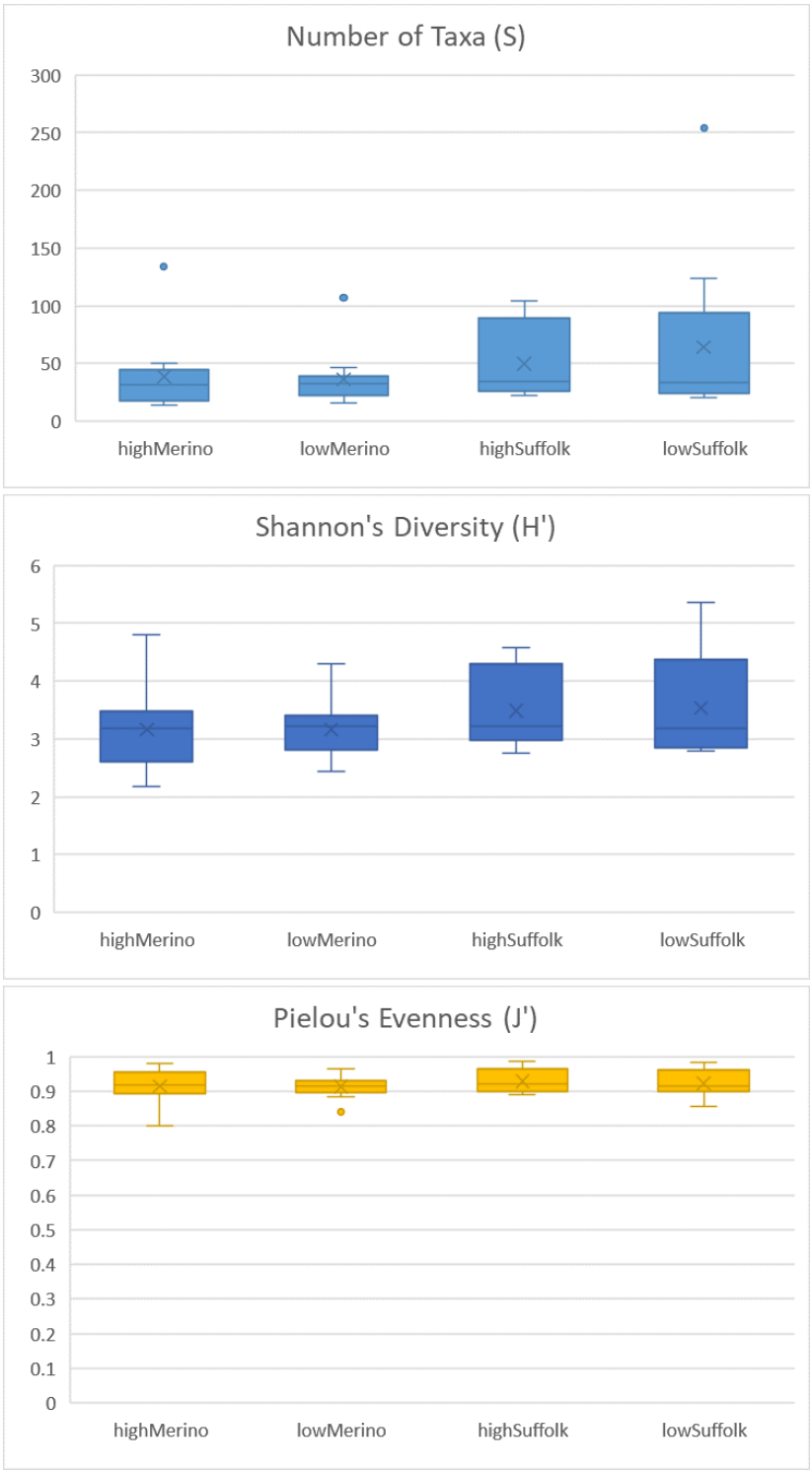

Figure 5: Number of taxa (S), Shannon's diversity (H') and Pielou's evenness (J') of vaginal bacterial genera of ewes ($P > 0.05$).

5 Discussion

5.1 Overall discussion and links to previous research

5.1.1 Characterisation of sheep vaginal bacteria

Various vaginal bacteria were detected and able to be characterised in ewes at the phyla, class, order, family, genus and species level. Most studies to date in sheep have focused on microbial changes due to use of intra-vaginal sponges to bring about oestrus synchronisation, rather than an interest in microbial populations and production linkages. The vaginal microbiota has been studied in regards to vaginal infections associated with this process. There has been a reasonable amount of work done in characterising the vaginal microbiota in goats and some in sheep, all done with breeds not commonly used in Australia. Olivera et al (2013) assessed the change in bacterial populations in goat vagina after oestrus synchronisation with progestogen sponges, using bacterial culture techniques. They found the most prevalent bacteria belonged to the genus *Staphylococcus* spp., except at the time of sponge withdrawal, when the most prevalent bacterium was *Escherichia coli*. The results of this study demonstrated that in goats subjected to a short-term protocol of oestrus induction and synchronisation, the vaginal microbe populations changed due to the protocol, with a rapid re-establishment of the normal microbiota after the sponges were removed. Interestingly, in our ewes *E. coli* was also identified, but only contributed 0.17-1.41% of the vaginal microbiota. Furthermore, *Staphylococcus* spp. were also identified in the vaginal microbiota of our sheep regardless of breed, but again contribution to the overall microbiota was low at 0.01-0.03%.

In earlier research, in which a few studies assessed sheep vaginal microbiota outside Australia, the method of analysis was generally using culture based techniques, as opposed to 16S rRNA analysis. Manes et al (2010) analysed the vaginal mucosal microbiota in Texel ewes in Argentina around the point of oestrus synchronisation and found the predominant microbes were mostly (90%) gram positive bacteria (*Bacillus* spp., *Staphylococcus* spp. and *Corynebacterium* spp.). These bacterial were also identified in our study although the contribution of these bacterial species 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 bacterial taxa between these prior studies and our investigation is most likely due the different methodologies employed, that is microbiological culture based versus molecular 16S rRNA profiling. Swartz et al. (2014) characterised Rambouillet sheep and cross-bred beef cattle vaginal microbiota using 16S rRNA profiling and found that both ewes and cows were predominately colonised by members of the Proteobacteria (almost exclusively gammaproteobacteria), Fusobacteria, and Bacteroidetes phyla. This is consistent with our findings with these three phyla amongst the most prevalent. *Aggregatibacter* spp. and *Streptobacillus* spp. were typically the most abundant genera in both ewes and cows (Swartz et al., 2014). Both genera were identified in our ewes but with a low overall percentage contribution to the overall vaginal microbiota.

5.1.2 Bacterial diversity between breed

Despite the fact that the difference between the ASBV of our allocated treatment groups was found to be significantly different, there were no significant treatment or breed differences associated with the vaginal microbiota, including alpha and beta diversity measures. However, there was a trend towards significance associated with breed observed at the genus and species levels. Furthermore, a greater number of genera and diversity was observed in Suffolk as compared to Merino ewes, although not significant. It may be possible that the small sample numbers are responsible for this result and that a higher sample size may have further teased apart these trends between breeds.

Increased bacterial diversity in the gastrointestinal tract is generally linked to health and improved digestive efficiency, largely based on human research (Claesson et al., 2012). Alterations and disturbances in gut microbiota, along with a reduction in diversity, are linked to increased risk of development of allergies, inflammatory bowel diseases (such as Crohn's disease and ulcerative colitis), autoimmune diseases (type 1 diabetes) and other inflammatory related problems in humans (Sekirov et al., 2010). In ruminants, the presence of rumen microbes and diversity of the population is vital to rumen efficiency. Fonty and colleagues (1988) observed the level of rumen fermentation in gnotobiotic (germ-free) lambs inoculated with 182, 106, 32 and 16 non-cellulolytic bacterial strains isolated from the rumen. The more strains in the inoculum, the higher the volatile fatty acids levels observed. In animals inoculated with 182 strains, the volatile fatty acids concentration was similar to conventional control lambs, but lambs inoculated with only 16 strains demonstrated almost no fermentation. When it comes to vaginal populations, again the focus of this research (regardless of the species studied) is the health of the vagina and not a focus on the inoculation of the young. However, it is generally supported that a dysbiosis of the vaginal microbiota increases the risk of bacterial vaginosis and contributes to an overall decrease in vaginal heath (Barrientos-Durán et al., 2020). Also, a study in pregnant women showed that there is an increase in 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). Therefore, a conclusion may be drawn that in increased diversity in vaginal microbiota is positive for vaginal health, which may show that the Suffolk ewes have a healthier vaginal microbe population.

5.1.3 Treatment separation (ASBV)

A ruminant animal is very efficient from a production standpoint, whether the product be meat, wool or dairy. 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). In Suffolks and Merinos, genetic selection has ensured that nutrient usage goes to either muscle production (in meat breeds) or mainly wool production (in wool breeds). Therefore, we believe that the production parameters by which we separated the two breeds are very relevant when discussing production efficiencies of each breed and there is research supporting the hypothesis that the chosen ASBV are likely to be affected by rumen microbiota. The two values chosen (yearling fleece weight for Merinos and post weaning weight for Suffolks) were chosen because these single traits best describe the production targets of each individual breed. For ease of selection of the animals to sample, we did not create a matrix with several ASBV, rather selected one simple value which we believed most accurately determined the production efficiency of that breed. Due to selection for very different production traits in the White Suffolk and Poll Merino, it did not seem relevant to separate the animals by production by the same trait (for example, weight). There are still, however, no treatment effects. This is further discussed in the below section (5.2). It may also be argued that although, our treatment effects "high vs low" were significant, they may be artificial. Was our "low" group really representative of an industry "low", for example, given the high quality stock from which our experiment animals were selected? It is possible that we didn't get as much of a spread of ASVB that would be seen across the industry, or even in a single but more middle ranking production farm. Perhaps despite the fact that our ASBV groups are significantly divergent, we didn't get as much variance as is needed to see microbial differences within our sampled mob.

5.2 Practical implications for industry and unanswered questions

In a recent study investigating ewe vaginal microbiota and the effects of oestrus synchronisation, Quereda et al (2020) used intravaginal sponges soaked in probiotics. This area of study is where we

envision the next phase of our research, but with a production as opposed to overall vaginal health focus. Quereda et al (2020) used culture based methods in order to analyse the microbial communities making direct comparison with our results difficult. The probiotic used was a mixture of *Lactobacillus* spp. (60% *Lactobacillus crispatus*, 20% *Lactobacillus brevis* and 20% *Lactobacillus gasseri*). In our study we found the prevalence of *Lactobacillus* spp. to be low within the normal ewe vaginal environment. Hence, such a probiotic would likely target maintenance of vaginal health, rather than affecting the neonates health and production. It should be noted that the vaginal probiotic did not affect the general health status of the ewes and did not interfere or have negative effects on ovine fertility during natural mating, which is a promising result for the use of vaginal probiotics. The above-mentioned research shows that there is a great deal of interest in the vaginal microbiota at the point of synchronisation, but that production efficiency and its link to vaginal microbiota is overlooked, especially in ovine research.

Serranto et al (2020) and Deng et al (2019) investigated the genital microbiota of sheep and cattle, respectively, and the impact on artificial insemination (AI) outcomes. Both these studies used 16S rRNA analysis and linked vaginal microbiota with production. Differences in microbiota abundance between pregnant and non-pregnant ewes, and between ewes carrying progesterone-releasing intravaginal devices with or without antibiotic were investigated and *Mageebacillus, Histophilus, Actinobacilllus and Sneathia* genera were found to be significantly less abundant in pregnant ewes (Serranto 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 indicating the presence in ewes' vagina could be due to prior transmission by natural mating with rams reared in that flock. This is a promising finding, regards inoculation of the ewe's vagina with 'designer' microbes, as inoculation from rams upon previous mating was able to colonise the vaginal and affect later pregnancy rates. This may seem backwards, as transmission from rams with the particular bacteria was associated with AI failure (i.e. a decrease in production), but it does show us that an inoculation of bacteria into the vagina can colonise the vagina and affect production down the track. The trial by Serranto et al (2020) also supports the idea that vaginal microbiota does play a part in production targets, in this case affecting pregnancy rates.

Our data showed that there were no significant vaginal microbiota differences associated with the two particular ASBV production traits targeted or sheep breed, although there was a trend for significance associated with breed. This may be due to our small sample size (n=24/breed), which was further reduced due to the two different ASBV traits investigated (n=12/treatment). Although, the sheep selected for this study showed significant differences between our assigned "high" and "low" ASBV grouping, it should be noted that this distinction may not be representative of an industry relevant segregation in ASBV. We believe that there is merit in further investigation into the difference in breed vaginal microbes, due to the differences in diversity (although not-significant) between Suffolk and Merino ewes. It is generally agreed that an increase in bacterial diversity equates to an increase in population efficiency (Claesson et al., 2012; Fonty et al., 1988). Another research question which could be posed is, is there a gestational increase in bacterial diversity in ewes throughout pregnancy (as found in human), and if so is the increase in diversity around parturition a mechanism to assist in inoculation of the neonate or simply to ensure maintenance of vaginal health.

The two ASBV measures (yearling fleece weight for Poll Merinos and post-weaning weight for White Suffolks) were chosen to represent performance, as these traits best describe the production targets of each breed. There could be an argument that post-weaning weight would have been a better measure for other breeds and potentially more comparable across breeds. However, high-production Merinos are not necessarily the heaviest animals, and often the highest production Merinos (for their selected breed trait of fleece production) are lighter, due to diversion of protein from the diet for fleece production rather than muscle growth. This is why the breeds were

separated by two different traits. It could still be argued that weight is more likely to be a gut and microbial related factor than fleece production. There are a number of other ASBV types, such as birth weight, weight, fat depth, eye muscle depth, wool weight, fibre diameter, reproductive ASBV and worm egg count. It could be argued that some of these may be more relevant ASBV parameters to investigate vaginal microbiota against. It would be interesting to investigate the relationship between the vaginal microbiota and birth weight, weight, reproductive ASBV and worm egg count, as these are more likely to be linked to gut health.

5.3 Discussion of the extent to which each specific project objective was met

5.3.1 Objective 1

Objective: To characterise the vaginal microbes of domesticated sheep.

Various vaginal bacteria were detected and able to be characterised in two breeds of ewe commonly used in Australia. This is the first report to our knowledge, which characterises the vaginal microbiota of any domesticated Australian sheep.

5.3.2 Objective 2

Objective: To determine if vaginal microbes differ across sheep breeds with differing growth and wool growth potentials.

No significant differences in ewe vaginal bacterial communities associated with ewe breed or ewe production parameters were detected. Suffolk sheep tended to have a great number for taxa and Shannon's diversity, although not significantly different from Merino sheep.

5.3.3 Objective 3

Objective: To determine a link between vaginal microbes and high- and low-producing animals

No significant differences in ewe vaginal bacterial communities associated with ewe breed or ewe production parameters were detected.

6 Conclusions/recommendations

6.1 Summary of key findings

No significant differences in ewe vaginal bacterial communities associated with ewe breed or ewe production parameters were detected. However, there was a trend towards breeds having different populations at the genus and species levels. Suffolk sheep tended to have a great number for taxa and Shannon's diversity, although not significantly different from Merino sheep.

6.2 Future research directions / practical application of the projects insights

We envisioned that this research would take us in the direction of vaginal probiotics or use of microbiological 'seeds' in order to alter the ewe vaginal microbial populations just before lambing, with a production as opposed to overall vaginal health focus. The above mentioned research shows that there is an interest into the vaginal microbiota at the point of synchronisation, but that production efficiency and the link to vaginal microbiota is currently overlooked, especially in sheep research. However, despite the apparent relevance of a vaginal probiotic aimed at increasing

production, our data shows that there was no significant vaginal microbiota differences associated with the two limited ASBV production traits investigated or breed. This may be due to our low sample size.

However, we still believe that there is merit in further investigation into the difference in breed vaginal microbes, due to the differences in alpha diversity (although not-significant) between the Suffolk and Merino ewes and trend towards significance in beta diversity between the breeds. We foresee that an area of future research would be an investigation of cross inoculation with different breed microbial populations. Perhaps we may be able to increase the growth or Merino lambs quickly and without having to use cross breeding or genetic selection, by using Suffolk vaginal microbe inoculations before lambing? If seeds (like the sponges used for oestrus synchronisation) were used then they could possibly be placed before lambing and expelled when lambing takes place. This area of research has significant possibilities for manipulation.

Future research:

- Revisit ASBV study using larger animal numbers with industry relevant divergence in ASBV in Suffolks only (focusing on carcass growth rather than wool).
- Investigate other ASBV (birth weight, weight, reproductive ASBV and worm egg count) likely linked with gut health. In light of recent research reproductive ASBV might be interesting from a production point of view.
- Utilise real life production records to analysed microbial data against, rather than ASBV data.
- Investigate pregnant ewes (which have been shown to have an altered vaginal microbiota in other studies) and impact on production of offspring
- Vaginal probiotics or use of microbiological 'seeds' in order to alter the ewe vaginal microbial populations just before lambing
- Difference in breed vaginal microbes, and cross inoculation with different breed microbial populations.

7 Key messages

Results:

No significant differences in ewe vaginal bacterial communities associated with ewe breed or ewe production parameters were detected. There was a trend towards breeds having different populations at the genus and species levels. Suffolk sheep tended to have a great number for taxa and Shannon's diversity, although not significantly different for Merino sheep.

This projects objectives were to:

Objective 1: To characterise the vaginal microbes of domesticated sheep. Various vaginal bacteria were detected and able to be characterised in two breeds of ewe commonly used in Australia. This is the first report to our knowledge, which characterises the vaginal microbiota of any domesticated Australian sheep.

Objective 2: To determine if vaginal microbes differ across sheep breeds with differing growth and wool growth potentials. No significant differences in ewe vaginal bacterial communities associated with ewe breed or ewe production parameters were detected.

Objective 3: To determine a link between vaginal microbes and high- and low-producing animals. No significant differences in ewe vaginal bacterial communities associated with ewe breed or ewe production parameters were detected.

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