final report

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Improved ruminant health and productivity through neonatal microbiome manipulation

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Abstract

This final report documents the successes and achievements of this project. This project's objectives were to:

a) Define the characteristics of the rumen microbiome of sheep of different breeds and with different growth characteristics,

b) Determine the extent to which phenotypic differences in growth and health can be attributed to differences in the maternally-derived microbiome in the early post-natal period,

c) Determine the extent to which the immune system is affected by early life manipulations,

d) Determine the potential for early-life microbiome manipulations for sheep (and potentially cattle).

To date, there have been three experiments conducted which allowed us to respond to these objectives. A pilot trial imposed two different diets to White Suffolk and Poll Merino ewes (24 Poll Merino and 24 White Suffolk ewes). This trial was the first to show that there are differences in microbial profiles induced by both genotype and diet in these breeds of commercially utilised sheep. Secondly, there were two subsequent lambing trials, which looked at the effects of natural maternal inoculation, compared to an imposed inoculation, of known rumen microbial populations of lambs at birth. These trials ran over separate lambing blocks, both involving 49 lambs grown out to a week 18 slaughter date, with both Merino and White Suffolk lambs. Lamb Trial 1 examined the differences between naturally-inoculated lambs (Maternal Control) and those given rumen fluid, either from ewes fed a Roughage type diet or ewes fed a High Spec. (effectively high grain) diet. Lamb Trial 2 employed a similar experimental design, except the lambs were either naturally-inoculated, or given fluid from ewes fed a High Spec. diet (sickness in the Roughage inoculation group in Lamb Trial 1 forced removal of the Roughage treatment in Lamb Trial 2). Briefly, the inoculation of lambs with 10ml of mature ewe rumen fluid daily, for one week following birth, resulted in significant differences in rumen bacterial communities at all phylogenetic levels (phyla, class order, family, genus and species) to those of un-inoculated (Maternal treatment) lambs. These differences were evident in rumen samples at weaning (10 weeks of age), and in rumen and intestinal samples at slaughter (18 weeks of age). Both the efficiency and the health of the inoculated animals appears to have been detrimentally affected by artificial inoculation, with the effects differing across inoculation and breeds. While this appears to be a negative result, we have demonstrated that lifelong changes (up until slaughter at week 18) in the rumen and intestinal microbiome can be induced by early intervention in microbial establishment.

The questions raised by the results of this project include;

a) Is this lifelong effect seen with other early life interventions, such as manipulation of the Maternal diet to alter the natural inoculation of the lamb?

b) Can beneficial effects be seen by 'assisting' the colonisation of the lamb through pre and probiotics, rather than via inoculation?

c) Can a beneficial effect be demonstrated after manipulating the neonatal microbiome or is the system of Maternal inoculation and natural colonisation of the lamb rumen close to optimum?

Executive summary

This projects objectives were to:

a) Define the characteristics of the rumen microbiome of sheep of different breeds and with different growth characteristics,
b) Determine the extent to which phenotypic differences in growth and health can be attributed to differences in the maternally-derived microbiome,
c) Determine the extent to which the immune system is affected by early life manipulations,
d) Determine the potential for early-life microbiome manipulations for sheep and cattle.

Three trials were completed to respond to these objectives

1) Pilot trial imposed two different trial diets to White Suffolk and Poll Merino ewes (24 Poll Merino and 24 White Suffolk ewes).
2) Lamb Trial 1 used naturally-inoculated lambs, and lambs given rumen fluid from ewes fed either a Roughage type diet or ewes fed a High Spec. diet.
3) Lamb Trial 2 used lambs either naturally-inoculated, or given fluid from ewes fed a High Spec. diet. (Sickness in the Roughage inoculation group in Lamb Trial 1 forced removal of the Roughage treatment in Lamb Trial 2).

Results

1) The pilot trial is the first to show that there are differences in microbial profiles induced by both genotype and diet in these breeds of commercially-utilised sheep.
2) Inoculation of lambs with 10ml of mature ewe rumen fluid daily, for one week following birth, resulted in significant differences in rumen bacterial communities at all phylogenetic levels (phyla, class order, family, genus and species) to those of un-inoculated (Maternal treatment) lambs. These differences were evident in rumen samples at time of weaning (10 weeks of age) and in rumen and intestine samples at slaughter (18 weeks of age), from both lamb trials.
3) Efficiency and health of the inoculated animals (through assessment of growth and analysis of production, health and immune measures) appears to have been detrimentally affected by the inoculations.

TRIAL SUMMARIES

PILOT TRIAL: This trial investigated the interesting idea that some of the genetic differences in growth potential and productivity between breeds of sheep or cattle, may be generated by differences in their ruminal microbiota. It is assumed that diet has the largest effect on the ruminal microbial composition and that differences in growth and productivity between breeds are due to metabolic differences alone. This trial is the first to demonstrate that sheep breeds differing widely in growth rate and efficiency have very different ruminal microbiomes at all levels (down to species). Further, the differences between breeds were greatest when the sheep consumed a Roughage diet and on this ration the Merinos performed better than the Suffolks, which in fact lost weight. ***This is a very significant finding as it suggests that at least part of the genetic basis for differences in growth resides in the establishment of different microbes and presumably the effects of this on nutrient yield and composition.*** Given the large differences in the environments in which Merinos and Suffolks were developed it is perhaps not surprising that Merinos are better able to handle high-fibre roughage diets and it appears that a significant part of this ability resides in their ability to establish a 'roughage-type' microbiome. When the breeds were offered a High Spec. ration the microbiota differences remained but were diminished, suggesting a true genotype by environment interaction.

<u>Industry relevance</u>: can we identify the ruminal microbes that are associated with handling High Spec. versus Roughage diets better and can we identify the microbes in Suffolks that contribute to their generally higher growth performance than Merinos? If we can this would be a major step towards dietary manipulation of maternal diets to establish the 'best' microbiota in neonates. We are still analysing our microbial results further and may be able to further pull apart these differences for our papers.

LAMBING TRIALS 1 AND 2

These were ground breaking experiments in that no previous trial has attempted to challenge neonates from birth with such a large inoculation of diet-specific ruminal microbes in crude ruminal fluid. Inoculating newborn lambs with ruminal fluid derived from ewes fed different rations produced long-term changes in the ruminal and intestinal microbiota compared to those inoculated naturally from the ewe. Microbiota remained different between the groups at weaning and at slaughter at 18 weeks, the latter being some 17 weeks after inoculations ceased.

<u>Industry relevance</u>: This is an important finding because it demonstrates that if we can find an 'ideal' inoculation source and method we can be sure that the changes we make to the newborn ruminant will be long-lasting. As for trial 1 above, we need further work to identify microbiomes with beneficial effects on animal health and production. Both analysing our project data further and future studies can be used to further this.

The artificial inoculations had negative effects on both growth efficiency and general health of the lambs. The immune systems of inoculated lambs was clearly activated to fight the unnatural exposure of the developing gastrointestinal epithelia to large doses of microbes and also to some pathogens that entered the systemic circulation. This immune activation in fact may have been the cause of reduce growth. The long term effects of this activation are of interest as they may be beneficial in sensitising the animal to resist future exposure, but they may also be negative in creating a hyper-responsive animal that diverts resources from growth to immune activation.

<u>Industry relevance</u>: the immune system of neonates is sensitive to exposure to ruminal microbes. It is possible that exposure to only desirable microbes might have positive effects on immune response without the negative effects we showed.

OVERALL SUMMARY

Our results show that ruminal microbiota differ between breeds and show a genotype by diet interaction. Microbiota differences are associated with differences in genetic control of growth. Manipulation of immediate postnatal exposure to mixed populations of ruminal microbes produced long-lasting effects on the ruminal and intestinal microbiota and on the immune system and growth. If we can identify populations of microbes that have beneficial effects on the newborn ruminant and means of generating these by manipulation of the maternal diet at parturition, we may be able to generate better ruminal and intestinal microbiomes associated with better growth efficiency and disease resistance.

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1 Background

Recently, the role of the gut microbiome has featured prominently in human medical research, with numerous studies providing evidence that gut microbes are intimately involved in human health and even behaviour (Khiaose-ard and Zebeli, 2014). Few studies have addressed this important interaction in ruminant animals. No previous work has been published with regard to differences in rumen microbes between different sheep breeds. There are results from work in cattle (Hennessy et al., 1995; King et al., 2011; Hernandez-Sanabria et al., 2013) and non-ruminant species, humans (Zoetendal et al 2001; Turnbaugh et al., 2009) and mice lines (Kovacs et al., 2011). Dietary effects on ruminant microbes are fairly well established, so dietary manipulation should allow us to enhance differences in the rumen populations between breeds.

Our study will add to previous work, which is novel research, while also allowing further analysis of inoculation effects, through purposefully inoculating with very different rumen samples, allowing for analysis of breed differences and ultimately furthering the understanding of rumen inoculation in lambs. The most relevant recent publication with links to our trials is De Barbiere et al., (2015a,b), who looked at the consequences of maternal diet and rumen inoculation in a cross over design. Their results were favourable and suggest the value of our approach, but failed to bring about longer-term effects of cross-inoculation with foreign organisms, with changes lasting until weaning but not much after. We inoculated intensively in the first week, when the rumen microbiota was establishing, whereas the previous paper inoculated once weekly until weaning.

This project comprises two parts; part 1 (the Pilot Trial) is designed to determine the effect of diet and genotype on the rumen microbiome of ewes, as a prelude to the manipulation of their offspring. The main purpose of the Pilot Trial was to provide two different rumen microbe populations which could be used in the second part of the project. Part 2 of the trial (Lamb Trials) was designed to identify the extent to which early exposure of ruminant neonates to different maternal microbes (through inoculation with foreign microbes versus naturally-acquired microbes) influences their subsequent health and growth efficiency through immune priming and the establishment of an efficient microbiome.

2 Project objectives

The participant will achieve the following objective(s) to MLA's reasonable satisfaction, by 1st August 2019:

a) Define the characteristics of the rumen microbiome of animals of different breeds and with different growth characteristics.
b) Determine the extent to which phenotypic differences in growth and health can be attributed to differences in the maternally-derived microbiome.
c) Determine the extent to which the immune system is affected by early life manipulations.
d) Determine the potential for early-life microbiome manipulations for sheep and cattle.

3 Methodology

All trials documented herein were 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-2017-076). All animal work was carried out at The University of Adelaide Livestock Research Centre at Roseworthy Campus, Roseworthy, South Australia.

3.1 Pilot Trial

The pilot study utilised 48 animals (24 high Australian Standard Breeding Value White Suffolk and 24 high ASVB Poll Merino ewes). Ewes were housed in a climate-controlled shed, within individual housing pens and free access to drinkers. The White Suffolk and Merino ewes were randomly assigned to either a Roughage type diet or a High Spec. diet (high grain). The High Spec. diet consisted of 80% pellet and 20% chaff, whereas the Roughage diet consisted of 20% pellet and 80% chaff (opposite inclusion levels). The pellet comprised Barley grain, Legume hay, Legume (Fava beans), Pellet Hay, Pellet Straw, Oatmeal, Limestone, Molasses, Vitamin and Mineral Premix, Urea, Acid buf (in order of decreasing inclusion level). The particular batch used was sampled and had a metabolisable energy (ME) of 11.8 MJ/kg and crude protein (CP) content of 15.1% (moisture 8.9%, dry matter (DM) 91.1%, acid detergent fibre (ADF) 19.7%, neutral detergent fibre (NDF) 37.5%, dry matter digestibility (DMD) 72.7%, dry organic matter digestibility (DOMD) 71.9%, ASH 7.3%, fat 0.6%, total digestible nutrients (TDN) 68.7%) (Johnsons Premium Stockfeed, Kapunda, South Australia, Lamb finisher pellet). The chaff was oaten and was 9.1 MJ/kg DM, 6.1% CP (Belvidere Ridge Chaff, Greenock, South Australia). All animals were allowed 2.5 kg feed per day with the full ration renewed daily (0800 hrs). The animals were acclimatised to the diet over 11 days, after which the diet remained stable for the next 22 days.

Table 1: Feeding schedule for White Suffolk and Poll Merino ewes on either High Spec. or Roughage diets of 2.5 kg daily.

	Day 1-3		Day 4-5		Day 6-8		Day 9-11		Day 11-33	
	Chaff	Pellet	Chaff	Pellet	Chaff	Pellet	Chaff	Pellet	Chaff	Pellet
High Spec. diet proportion	80%	20%	60%	40%	50%	50%	40%	60%	20%	80%
High Spec. volume	2000g	500g	1500g	1000g	1250g	1250g	1000g	1500g	500g	2000g
Roughage diet proportion	80%	20%	80%	20%	80%	20%	80%	20%	80%	20%
Roughage volume	2000g	500g	2000g	500g	2000g	500g	2000g	500g	2000g	500g

Once the ewes had been on their allocated diets for 21 days, all 48 ewes had a rumen sample taken by the use of a tube and pump (inserted through the mouth and down the throat, with a solid guard tube to stop the ewe chewing and swallowing the other end of the sample tube). From the sample collected, 20 ml was placed into a 70ml plastic beaker with a secure lid and placed on ice during the sampling procedure. The samples were then frozen at -80 ^{o}C, immediately after sampling had been completed. Total nucleic acid was extracted from approximately 20 ml freeze-dried unfiltered rumen fluid from individual ewes by a modification (Torok et al. 2008) of a South Australian Research and Development Institute (SARDI, Adelaide, Australia) proprietary method (Stirling et al. 2004) as previously described by Torok et al. (2014). Diversity profiling analysis of rumen bacterial communities was performed using 16S rRNA sequencing with 341F and 806R primers (AGRF, Melbourne Node).

All ewes were weighed on entry into the shed, and on the last day immediately after the final rumen sample was collected (See Appendix A for all pilot trial ewe weights). The feed intake on the last day before the rumen sample was assessed by measuring the amount of pellets and chaff left for all ewes on the morning before the rumen samples began and before their feeders were filled for the rumen sample day.

The production data were analysed using SPSS (Statistics Package for Social Sciences, IBM), specifically a univariate general linear model using type 1 sums of squares. Entry weight of the ewe was fitted as a covariate; breed, treatment and breed by treatment fitted as fixed effects (all other interactions were removed from the model as they were not found to have a significant effect on any variable

analysed). This analysis allowed understanding of the major variables, but adjusted for the entry weight of the ewe. If data were not normally distributed, they were log-transformed. When transformed data are presented; the adjusted, non-transformed data are presented for reference. Metabolisable energy estimates were calculated by using the MJ ME/DM of the pellets and chaff, and using the consumption data the numbers were placed into the following equation (% chaff consumed x chaff MJ ME/DM) + (% pellet consumed x pellet MJ ME DM) / 100. The same equation was used for % CP.

The rumen 16S rRNA bacterial sequencing data, representing phyla, class, order, family, genus and species, were analysed using multivariate statistical techniques (PRIMER6, PRIMER-E Ltd., Ivybridge, UK). These analyses were used to examine differences in rumen bacterial communities associated with dietary treatment and breed. Bray–Curtis measures of similarity (Bray and Curtis 1957) were calculated to examine similarities between rumen microbial 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 rumen bacterial communities were significantly different between dietary treatments and breed. Unconstrained ordinations were performed to graphically illustrate relationships between treatments using nonmetric multidimensional (nMDS) scaling (Shepard 1962a, 1962b; Kruskal 1964).

3.2 Lamb Trial 1

Artificial insemination of 75 Merino ewes [high Australian Standard Breeding Value (ASBV)) for wool growth and fibre diameter], and 75 Suffolk ewes [high ASBV for growth rate], was completed laparoscopically under sedation with 1ml Xylazine. The ewes were mated to 4 rams per breed (WHITE SUFFOLK: LEAHCIM-130011, LEAHCIM-140326, LEAHCIM-150436, LEAHCIM-160042, POLL MERINO: LEAHCIM POLL-152349, LEAHCIM POLL-162170, LEAHCIM POLL-162929, LEAHCIM POLL-163266), resulting in purebred Poll Merino and purebred White Suffolk lambs. Ewes were fed *ad libitum* hay up until pregnancy checking (ultrasound scanning at day 90 after insemination), and ewes were marked as being either single- or multiple-bearing. Following the scan, the ewes were allocated to six groups based on achieving an even lamb number and breed spread. The pregnant ewes were all managed under the same conditions and were fed a 'ewe and lamb' pellet *ad libitum* with access to additional oaten hay, water and shelter at all times. The pellet was made up of Barley, Cereal Straw, Cereal Hay, Legume (Lentils), Legume hay, Oats, Limestone, Urea, Salt, Vitamin and Mineral Premix, Molafos, listed in order of inclusion levels). The particular batch used and had a metabolisable energy of 11.5 MJ/kg and crude protein content of 14.4 % (moisture 8.7%, DM 91.3%, ADF 21.2%, NDF 38.3%, DMD 72.3%, DOMD 71.6%, ASH 7.1%, fat 1.1%, TDN 68.4%) (Johnsons Premium Stockfeed, Kapunda, South Australia, Ewe and lamb pellet). Non-pregnant ewes were removed from the trial. As the ewes were mated in November, there was a low conception rate and this insemination resulted in 39 Poll Merino lambs and 20 White Suffolk lambs. 34 Poll Merino (Maternal: 13, High spec: 11, Roughage: 10) and 15 White Suffolk lambs completed the trial to week 18 (Maternal: 8, High spec: 3, Roughage: 4) (total n= Maternal: 21, High spec: 14, Roughage:14).

All lambs were kept with their mother until weaning (week 10), with access to the 'ewe and lamb' pellet (as described above) *ad libitum*, with access to additional oaten hay. Upon weaning, the lambs were offered a 50/50 mix of the ewe and lamb pellet (described above) and a premium lamb finisher pellet (the lamb finisher pellet was made up of Barley, Legume Hay, Legume (Lentils), Pellet Hay, Pellet Straw, Oatmeal, Limestone, Molafos, Vitamin and Mineral Premix, Urea, Acid buf (in order of decreasing inclusion level)). The particular batch used and had a ME of 12.3 MJ/kg and CP content of 15.3% (moisture 9.0%, DM 90.8%, ADF 15.9%, NDF 34.0%, DMD 78.2%, DOMD 76.9%, ASH 7.0%, fat 1.0% , TDN 73.3%) (Johnsons Premium Stockfeed, Kapunda, South Australia, Lamb finisher pellet). After the week of acclimatisation to the finisher pellet, the lambs were placed onto 100% premium finisher pellet. The lambs had access to pellets, oaten hay, water and shelter at all times.

3.2.1 Fistulated/cannulated ewes housing and nutrition

Eight non-pregnant (four Poll Merino and four White Suffolk) ewes were fistulated under general anaesthesia (Ketamine/Xylazine then isoflurane) and a rumen cannula inserted (Cannula size 1C, Bar Diamond, USA). Ewes were removed from water 24 hours before the surgery and given a high Roughage diet, made up only of oaten chaff, to avoid a very watery rumen during surgery. The necks of the ewes undergoing the surgery were clipped to allow easy access to the jugular vein. Sheep were sedated and anaesthetised 20 minutes prior to surgery by injection with 1ml of Pamlin (Diazepam), 1.6ml Butorphanal, 8ml Ketamine and 1ml Xylazil. Sheep were then intubated in sternal recumbence with a 9mm endotracheal tube. The sheep were placed in right lateral position and anaesthesia maintained with isoflurane at 1-2 % (Delvet Isoflurane Inhalation Anaesthetic, Ceva Delvet Pty Ltd, Glenorie, New South Wales, Australia) in oxygen. The surgical site, a broad rectangular area extending from the lumbar transverse processes to the ventral abdomen including the left sub lumbar fossa, was prepared by closely clipping the wool followed by repeated scrubbing with an antiseptic (PVP-Iodine Scrub 7.5 %, Apex Laboratories Pty Ltd, Somersby, New South Wales, Australia) and 70 % ethanol. The surgical site was draped with disposable drapes. The skin incision extended approximately 3 cm ventral to the transverse process in the left mid-sub lumbar fossa and extended 15 cm ventrally. The abdominal muscles (internal oblique, external oblique, transverse abdominis) were divided by blunt dissection, before the peritoneum was opened to expose the rumen. The rumen was clamped and a series of anchor horizontal mattress sutures between the rumen and the skin were placed in a clockwise configuration to isolate a pouch of rumen. Within the area covered by the anchoring sutures the rumen was incised dorsoventrally, exposing the ruminal contents. The cannula was placed into boiling water for 5 minutes (to make it more pliable) and was then folded into a spear shape to pass through the fistula. Once the tip of the cannula was in the fistula, the rest of the cannula was pushed through and into the sheep allowing the deep flange of the cannula to flatten on the inside wall of the rumen. Once the rubber cannula was inserted a rubber bung was fitted (Cannula size 1C, Bar Diamond, USA). The analgesic Carprophen (50 mg/ml Rimadyl®, Pfizer Australia Pty Ltd, West Ryde, New South Wales, Australia) was injected at 1 ml/12.5 Kg intravenously and an intramuscular injection of 1 ml/10 kg of antibiotics (Moxylan LA, Jurox, Rutherford, New South Wales Australia) administered. The prepared surgical site was then cleaned again and then sprayed with a topical antibiotic (Cetrigen®, Virbac (Australia) Pty Ltd, Milperra, New South Wales, Australia).

Following surgery, sheep were recovered in their pens. The ewes were injected with pain relief (Rimadyl/Carprofen) for 5 days after the surgery and an antibiotic (Moxylan) for 4 days after surgery. The fistulated area was cleaned weekly and the surgery site inspected to ensure that any problems were noticed early and are treated as soon as possible. Additional antibiotics and pain relief was given if the wound was not healing as expected.

Four White Suffolk and four Merino ewes were assigned to either a Roughage type diet or a High Spec. diet, mirroring the diets fed in the Pilot Trial. The High Spec. diet consisted of 80% pellet and 20% chaff, whereas the Roughage diet consisted of 20% pellet and 80% chaff. The pellet was made up of Barley, Legume Hay, Legume (Fava beans), Pellet Hay, Pellet Straw, Oatmeal, Limestone, Molafos, Vitamin and Mineral Premix, Urea, Acid buf (in order of decreasing inclusion level). The particular batch used for the cannulated ewes and had a ME of 11.8 MJ/kg and CP content of 15.1% (moisture 8.9%, DM 91.1%, ADF 19.7%, NDF 37.5%, DMD 72.7%, DOMD 71.9%, ASH 7.3%, fat 0.6%, TDN 68.7%) (Johnsons Premium Stockfeed, Kapunda, South Australia, Lamb finisher pellet). The chaff was oaten and was 9.1 MJ/kg DM, 6.1% CP (Belvidere Ridge Chaff, Greenock, South Australia). All animals were allowed 2.5 kg feed per day with the full ration renewed daily. Ewes were fed at approximately 0900 hrs daily (when not lambing) for the duration of the trial in individual metal trough feeders, which were hung from the pen bars close to ground level. The animals were acclimatised to the diet over 11 days and then the diet remained stable for the following 22 days.

When lambing began, all ewes were fed at 0730 and 1930 (to reduce variation between the am and pm sample). All cannulated ewes had a rumen sample taken by the use of a tube and pump at 0800 and 2000 hrs from the estimated beginning of lambing until the inoculation process was complete. From the sample collected from each sheep, 20ml was placed into a beaker and pooled with samples from the ewes on the same treatment diet. The ruminal fluid for each treatment group inoculation was then stored in volumes of 25ml in 50ml tubes, in a water bath at 39^{o}C for any inoculations needed in the 4-hourly shifts between fluid collection times. The 50ml tubes had screw-on lids which were left slightly loosened to allow gases to escape. The water bath method was used rather than sampling the cannulated ewes every four hours, because this better ensured the health of the cannulated ewes, allowing them to rest between samples and also allow for a more normal light cycle (lights remained on from the start of the morning samples, at feeding and until completion of the pm samples).

3.2.2 Treatments and lambing measures

Lamb treatment groups consisted of two inoculation groups, High Spec and Roughage, and one control group, referred to as the Maternal Control. Lambs in the High Spec. and Roughage groups were inoculated with rumen fluid, which had been collected from the cannulated ewes on either a High Spec. or High Roughage diet (fistulated/cannulated ewes described in above sub section).

Lambing progress was checked every 4 hours for the lambing period. Upon finding newborn lambs which had been born between each 4 hour shift, the lambs were tagged, the umbilicus sprayed with povidone (Povidone Iodine, Iovone surgical scrub, Jurox Animal Health, Aus), weighed, tagged and returned to the ewe. In the inoculation groups, lambs were inoculated with 10ml of the collected rumen fluid. The inoculation was given from a 10ml syringe with a 9cm plastic tube extension to inoculate behind the teeth of the lambs and reduce sucking reflex (this was done in an effort to reduce the amount of fluid, which would bypass the rumen and go into the abomasum). This inoculation was repeated at the next 0800 shift (regardless of birth time) and at all subsequent 0800 shifts until 7 inoculations had been given to each inoculated lamb.

After all the lambs were born, the average lambing date was calculated and all measurement events were timed from that average date. Lambs were weighed at birth and then reweighed and body condition scored every fortnight until slaughter. Condition scores in weeks 2 to 8 were done using a score of 1 to 3 (as they were still young lambs). A score of 1 was a ‘skinny lamb’, with no fat and very little muscle on the backbone and ribs, or a small amount of muscle. A score of 2 was a ‘medium lamb’ with a good level of fat and muscle and rounded ends of ribs and top of backbone. A score of 3 was a ‘fat lamb’, being round across the backbone and with lots of muscle and fat. Post-weaning, week 10 to 18, the score used was a scoring system of 1 to 5 (Kenyon et al. 2014).

Blood was collected every 14 days from 2 weeks of age and going through to week 18, via jugular venepuncture into serum clot activator vacutainer tubes (red top) and ethylenediaminetetraacetic acid (EDTA, lavender top) vacutainer tubes (BD precision glide needle 18 gauge x 1 inch, USA; BD Vacutainer Serum Blood Collection Tubes and ethylenediaminetetraacetic acid EDTA vacutainer tubes, 9ml, USA). Samples were stored on ice until processing. EDTA tubes were taken to the Roseworthy Veterinary Diagnostic laboratory and analysed for total blood counts using a Siemens Advia 2120i haematology analyser (including haemoglobin, haematocrit and total cell counts). A manual differential count of 100 blood smears was carried out and compared to the machine count for the same animal at the same time point. It was determined that the two methods were correlated, hence machine count analysis was undertaken for all subsequent samples. The full bloods were then left overnight and then centrifuged at 3000rpm for 10 minutes (2,012 x *g*) plasma pipetted off and frozen (-20^{o}C) in 1.5ml Eppendorf tubes. The samples in the serum clot activator vacutainer tubes were spun down immediately after sampling at 3000rpm for 10 minutes (2,012 x *g*) plasma pipetted off and frozen (-20^{o}C) in 1.5ml Eppendorf tubes. Saliva was collected at the same time points as blood, via use of a cotton plug (Salivettes, Sarstedt Australia, South Australia, Australia). Each lamb was allowed to

chew on the Salivette for a maximum of two minutes to obtain the sample. Samples were centrifuged at 2012 x *g* for ten minutes at room temperature and stored at -20^{o}C until analysis. Plasma samples were used for analysis of IgG and saliva samples for analysis of IgA using single radial immunodiffusion, carried out by Dr. Greenwood, using the University of Adelaide veterinary diagnostic laboratory. The antibody concentration in agar used was 150um, with standards of 1:2, 1:4 and 1:8 and a sample dilution of 1:80 (Reagents used were Purified sheep IgG P130-100, Bethyl and Rabbit anti-sheep IgA A130-111, Bethyl). The rings which formed following the reaction were measured 48 hours following creation of the plate, using an eye piece with mm markers.

The feed intake of lambs following weaning (removing the effects of ewes on feed eaten) was measured from weaning until slaughter. This was done by calculating the feeder volume and feed density and then by measuring the depth (mm) of feed consumed per day and converting to kg eaten per day.

Ruminal fluid was collected at weaning (week 10) from 12 lambs from each treatment group, with an even spread across sex, twins and singletons and breed per treatment (36 lambs sampled). The ruminal fluid was also collected from the maternal ewes and a total of 31 ewes were sampled. An 8mm diameter clear vinyl pipe was lubricated with Glycerine/Glycerol and passed through a protective poly-pipe (to stop the animal clamping on the soft sample pipe). The protection pipe was placed into the mouth and moved towards the back of the throat, the sample pipe was then pushed through the protection pipe and towards the animal's oesophagus until the animal started to swallow the tube. The pipe is then pushed until in the rumen or reticulum. Once the pipe was in place, a hand pump was worked to suck fluid up the tube into a container and then the tube was gently and quickly withdrawn. The pH of all samples was taken with a pH meter immediately after sampling. There was 20ml of fluid collected from each lamb and placed into sample pots and immediately chilled before being frozen in -80^{o}C as soon as possible. Total nucleic acid was extracted from approximately 20 ml freeze-dried unfiltered rumen fluid from individual ewes by a modification (Torok et al. 2008) of a South Australian Research and Development Institute (SARDI, Adelaide, Australia) proprietary method (Stirling et al. 2004) as previously described by Torok et al. (2014). Diversity profiling analysis of rumen bacterial communities was done using 16S rRNA sequencing with 341F and 806R primers (AGRF, Melbourne Node).

Lambs were kept in their treatment groups and grown until 18 weeks of age before being sent to slaughter. At slaughter**,** intestines were collected and cable ties were placed at the proximal end of the small intestine and under the rumen at the distal end (to stop contents moving between sections) and the whole digestive tract was brought back for sampling. Also at slaughter, hot carcass weight was measured immediately after the slaughter of all lambs was complete. The intestines were sampled from 1m from the proximal end and 1m from the distal end and two 5cm sections were collected from each point. The sections were flushed and the first section was snap frozen and the second placed in 10% PBS buffered formaldehyde. A third section was taken 1m from the proximal end, following the removal of the 10cm of tissue. This section was not flushed and care was taken to maintain all contents in the section and this was frozen in -80^{o}C for analysis of the microbial populations. Ileum samples taken at slaughter were set in paraffin wax and cut into 5µm sections and mounted on a slide, following staining with Hemoxalin and Eosin stains, cover slides were added and the sections were viewed under a microscope. Photos were taken of each Peyer's patch (at the same scale and using a calibration slide) in every section for all lambs and these were then assessed using image analysis software to assess the intestinal histology for the number, area and presence and morphology of Peyer's patches. A example image for reference on the variation of patch shape and size is below (there were an estimated 800 images analysed with a varied number of patches in each image).

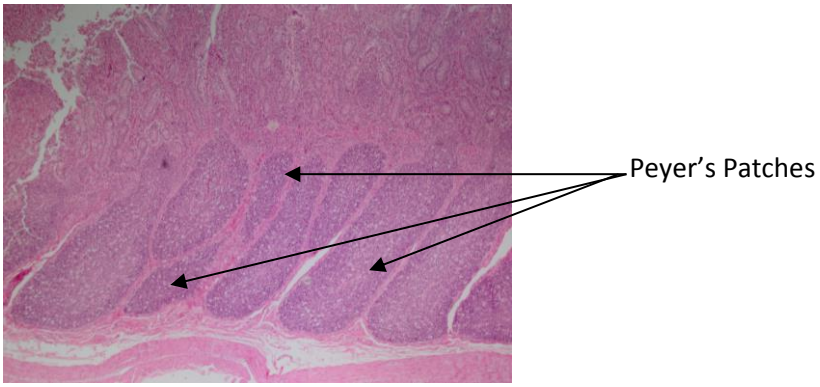

Figure 15: Picture of a histological section from lambing trial 1, of the ileum of a White Suffolk lamb at 18 weeks of age, following Hematoxylin and Eosin staining, showing several Peyer's patches of varying shapes and sizes.

Before analysis of the data all image data was cleaned using the software Reference Value Advisor, in order to eliminate outliers from the data set (Geffré et al., 2011). All data were analysed using the Statistics Package for the Social Sciences (IBM Corp. Released 2011. IBM SPSS Statistics for Windows, Version 20.0. Armonk, NY, USA) using a linear mixed model with type 1 sums of squares. Data were checked for normality by examining the distribution of residual plots, which resulted in some data requiring transformation (specified when data are presented and non-transformed means are also presented for reference). A lamb was used as the statistical unit and was fitted as a random effect by day. Day of measure, treatment, breed, sex, if a lamb was born and then raised a single or twin (SS, TT, TS) were fitted as fixed effects. Pairwise comparisons were used to determine significant differences.

The rumen 16S rRNA bacterial sequencing data, representing phyla, class, order, family, genus and species, were analysed using multivariate statistical techniques (PRIMER6, PRIMER-E Ltd., Ivybridge, UK). These analyses were used to examine differences in rumen bacterial communities associated with maternal and inoculation microbes, breed, sex and treatment. Bray–Curtis measures of similarity (Bray and Curtis 1957) were calculated to examine similarities between rumen microbial 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 rumen and intestinal bacterial communities were significantly different among treatments and breed. Unconstrained ordinations were performed to graphically illustrate relationships between treatments using non-metric multidimensional (nMDS) scaling (Shepard 1962a, 1962b; Kruskal 1964).

3.3 Lamb Trial 2

The second Lamb Trial was run with similar methodology to Trial 1, albeit with some exceptions. Firstly, during Lamb Trial 1, there was some illness in the lambs associated with the Roughage treatment (which can be seen in the results section for Trial 1 to follow), and this inoculation was thus removed from Trial 2. Secondly, due to the large workload and low available labour, the lambs were only checked every 12 hours during the inoculation and lambing period (0800 and 1000 hrs), and the number of measurements after weaning was decreased, with key measurement times maintained. Also, the slaughter collections and intestinal samples were exchanged for a final rumen pump before slaughter. So that Trial 1 had intestinal measures and Trial 2 has rumen measures for analysis of long term effects. Insemination of 85 non-pregnant ewes (remaining from the first trial) was completed through laparoscopic artificial insemination, under sedation. Again, the ewes were mated to 4 high ASVB rams per breed (the same as Trial 1), resulting in pure bred Poll Merino and purebred White Suffolk lambs. The ewes and lambs were maintained under the same conditions as Lamb Trial 1. The

same fistulated ewes from Lamb Trial 1 were used. However, all fistulated/ cannulated ewes were fed with the High Spec. diet to create one single pooled inoculation of 8 ewes.

Treatment groups consisted of one inoculation group from ewes on High Spec. diets (inoculation treatment), and one control group, referred to as the Maternal Control. The methodology for Lamb Trial 2 during lambing and the inoculation phase was the same as with Lamb Trial 1, except that lambing checks were carried out only every 12 hours. After all the lambs were born, the average lambing date was calculated and all measurement events were timed from that average date. Lambs were weighed at birth and weighed and body condition scored every fortnight until slaughter.

Blood was collected every 14 days from birth starting at 14 days and going through to week 10 and then a final measure at week 18, via jugular venepuncture into serum clot activator vacutainer tubes (red top) and ethylenediaminetetraacetic acid (EDTA, lavender top) vacutainer tubes (BD precision glide needle 18 gauge x 1 inch, USA; BD Vacutainer Serum Blood Collection Tubes and ethylenediaminetetraacetic acid EDTA vacutainer tubes, 9ml, USA). Samples were stored on ice until processing. EDTA tubes were taken to the Roseworthy Veterinary Diagnostic laboratory and analysed for total blood counts using a Siemens Advia 2120i haematology analyser (incuding haemoglobin, haematocrit and total cell counts). A manual differential count of 100 blood smears was carried out and compared to the machine count for the same animal at the same time point. It was determined that the two methods were correlated, hence machine count analysis was undertaken for all subsequent samples. The full bloods were then left overnight and then centrifuged at 3000rpm for 10 minutes (2,012 x *g*) plasma pipetted off and frozen (-20^{o}C) in 1.5ml Eppendorf tubes. The samples in the serum clot activator vacutainer tubes were spun down immediately after sampling at 3000rpm for 10 minutes (2,012 x *g*) plasma pipetted off and frozen (-20^{o}C) in 1.5ml Eppendorf tubes. Saliva was collected at the same time points as blood, via use of a cotton plug (Salivettes, Sarstedt Australia, South Australia, Australia). Each lamb was allowed to chew on the Salivette for a maximum of two minutes to obtain the sample. Samples were centrifuged at 2012 x *g* for ten minutes at room temperature and stored at -20^{o}C until analysis. Plasma samples were used for analysis of IgG and saliva samples for analysis of IgA using single radial immunodiffusion, carried out by Dr. Greenwood, using the University of Adelaide veterinary diagnostic laboratory. The antibody concentration in agar used was 150um, with standards of 1:2, 1:4 and 1:8 and a sample dilution of 1:80 (Reagents used were Purified sheep IgG P130-100, Bethyl and Rabbit anti-sheep IgA A130-111, Bethyl). The rings which formed following the reaction were measured 48 hours following creation of the plate, using an eye piece with mm markers.

Ruminal fluid was collected from 18 lambs from both treatment, at week 10 (weaning) and week 18 (day before slaughter) with an even spread across sex, twins and singletons and breed per treatment (36 lambs sampled). The ruminal fluid was collected also from the maternal ewes and a total of 29 ewes were sampled. The same sample method as Lamb Trial 1 was used. Data for rumen sample analysis was carried out as explained under the subheading of Lamb Trial 1, with explanation of analysis of the non-microbial data.

4 Results

4.1 Pilot trial

4.1.1 Diet consumption

Comparison of the ewes consumption on the final day of the trial allow us to estimate their daily consumption. There were no significant differences in the diets consumed between breeds (kg consumed per day, the percentage of pellets consumed per day or the energy consumed per day, $P > 0.05$) or the interaction between breed and treatment ($P > 0.05$). Overall the animals on the High Spec.

diet ate significantly more than animals on the Roughage diet (Table 2). The analysis also shows us that the diets were in fact different in both pellets consumed and also the estimated MJ ME DE and % CP in each treatment diet (Table 2).

Table 2: Estimation of diet consumed per ewe per day, following acclimatisation of ewes on High Spec. and Roughage diets.

	High Spec.	Roughage	P value
Kg consumed	12.1 ± 0.7 (2.3kg)	6.5 ± 0.8 (1.8 kg)	< 0.0001
% pellets consumed per day	85.0 ± 1.7	26.6 ± 1.7	< 0.0001
MJ/ME DM	10.4 ± 0.03	9.5 ± 0.03	< 0.0001
% CP	14.5 ± 0.2	8.74 ± 0.2	< 0.0001

4.1.2 Rumen microbiota

Both diet and breed had a significant effect on the rumen microbial populations present, with diet having a much stronger effect (Figure 2). The rumen profiles of sheep on the High Spec. diet are more variable than those on the Roughage diet (Figure 2), regardless of breed. The differences associate with breed are more pronounced when the sheep are on the Roughage diet (Figure 2). Also, more variation is evident in the bacterial population in the sheep on the High Spec. diet against those on the Roughage diet.

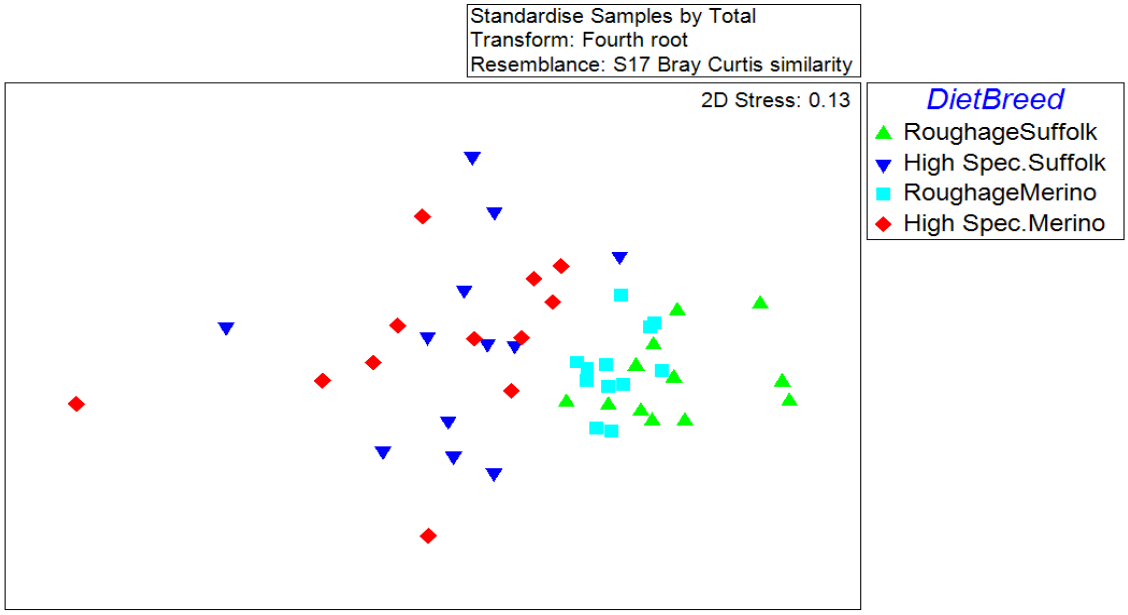

Figure 2: nMDS of rumen bacterial profiles (species) of all sheep identified by diet and breed.

The table below (Table 3) shows the significance of diet and breed effects for each bacterial groups investigated. Due to the manner of visual presentation of the data (which cannot be presented with an associated p value), we have also provided tables of the results of the ANOSIM analysis for understanding of the significance.

Table 3: Two-way ANOSIM of rumen microbial communities associated with breed and diet in pilot trial Merino and Suffolk ewes fed either High Spec. (high grain) or high Roughage diets for each of the bacterial genera investigated

Parameter	*R* and *P* values[a]					
	Phyla	Class	Order	Family	Genus	Species
Diet	**0.543**, *0.001**	**0.628**, *0.001**	**0.654**, *0.001**	**0.661**, *0.001**	**0.590**, *0.001**	**0.594**, *0.001**
Breed	**0.077**, *0.017**	**0.176**, *0.001**	**0.162**, *0.001**	**0.153**, *0.001**	**0.164**, *0.001**	**0.172**, *0.001**

[a] The global R statistic (indicated in boldface) and significance (P value - indicated in italics) are shown for each of the factors bacterial genera. The Global *R* value describes the extent of similarity between each pair in the analysis of similarity (ANOSIM), with values close to unity (1) indicating that the two groups are entirely separate and a zero value indicating that there is no difference between the groups. $P < 0.05$ is significant (* have been used to highlight the significances also).

4.1.3 Weight and weight change

There was no significant difference between the starting weights of ewes in the two treatment groups ($P > 0.05$). However, there were differences in the starting weights between breeds, ($P< 0.001$), with the Suffolks (76.32 ± 0.70) weighing more than the Merinos (61.13 ± 0.70). The final weights were also affected by breed (Suffolk 79.02 ± 1.08 and Merino 66.57 ± 1.10, $P < 0.001$) and by treatment diet (High Spec. 75.13 ± 1.10 and Roughage 70.46 ± 1.10, $P < 0.005$). The interaction between treatment diet and breed did not affect end weights overall. However, weight change (calculated by subtracting the start weight from the end weight) over the treatment duration was affected by breed by treatment, with weight loss seen in the Suffolk breed when they were kept on the Roughage diet (Figure 1). Both Merino diet groups and the Suffolk on the High Spec. diet gained weight over the duration of the trial.

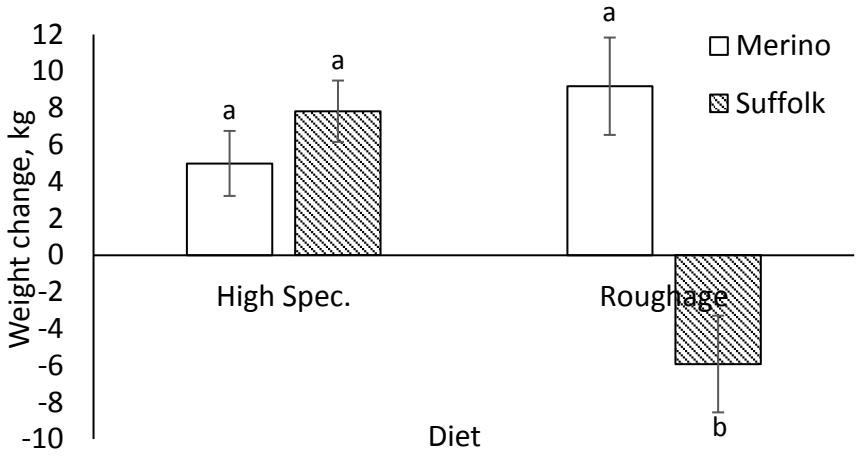

Figure 1: Weight change in White Suffolk and Poll Merino ewes given two different diets over 33 days. Different superscripts denote significant differences $P^{a,b,} < 0.0001$.

4.2 Lamb Trial 1

4.2.1 Microbial analysis

Rumen microbial profiling data sets were analysed after being broken down to phyla, class, order, family, genus and species level. There were significant differences associated with treatment on all the rumen microbial populations of lambs at week 10 (Table 4). Although, breed did not significantly

impact the lamb rumen microbial communities (Table 4). Significant pairwise (P < 0.05) difference were observed between Roughage inoculated and Maternal control lambs for all microbial genera investigated. In addition, there were significant (P < 0.05) differences between High Spec. inoculated and Maternal control lambs in class, order, family, genus and species rumen microbial communities. Significant (P < 0.05) differences between the High spec and Roughage inoculated lambs was only observed in the genus and species rumen microbial communities. These differences are graphically shown for the lamb rumen bacterial order and species communities in Figure 3 and 4, respectively. This suggests that there are greater differences between those which have been inoculated and not, than between the two inoculants. This may be caused due to the response to the mature rumen inoculation itself, rather than the specific microbial populations within the inoculant.

Table 4: Two-way ANOSIM of rumen microbial communities of lambs associated with breed and inoculation treatment in Trial 1 , in Merino and Suffolk lambs investigated at time of weaning (week 10).

Factor	*R* and *P* values[a]					
	Phyla	Class	Order	Family	Genus	Species
Inoculation	**0.131** *0.011**	**0.157** *0.009**	**0.189** *0.005**	**0.174** *0.003**	**0.213** *0.002**	**0.208** *0.002**
Breed	**-0.014** *0.52*	**-0.108** *0.84*	**-0.066** *0.72*	**-0.003** *0.48*	**0.013** *0.44*	**0.041** *0.35*

[a] The global R statistic (indicated in boldface) and significance (P value – indicated in italics) are shown for each of the factors bacterial genera. The Global *R* value describes the extent of similarity between each pair in the analysis of similarity (ANOSIM), with values close to unity (1) indicating that the 2 groups are entirely separate and a zero value indicating that there is no difference between the groups. *P* < 0.05 is significant (* have been used to highlight the significances also).

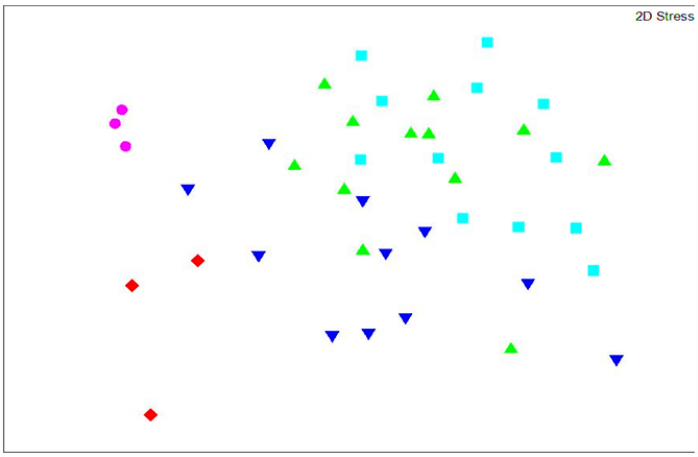

Figure 3: nMDS of rumen bacterial profiles (order) from all lambs (regardless of breed) and inoculum identified by treatment.

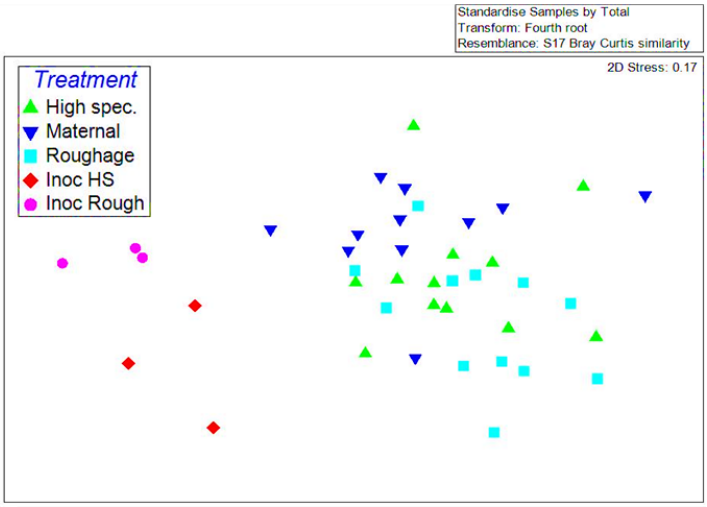

Figure 4: nMDS of rumen bacterial profiles (genus) from all lambs (regardless of breed) and inoculum identified by treatment.

In this trial were no significant differences (P < 0.05) between the rumen microbial profiles of ewes or lambs based on breed. However, the population within this trial was heavily weighted on the Merino side, due to the low pregnancy rates in the Suffolk ewes in this replicate particularly, so numbers may not have allowed us to see these differences.

There were no significant differences between the inoculum fluid itself for high spec (red diamonds on Figures 3 & 4) and Roughage microbial profiles (pink spots on Figures 3 & 4) as there were too few data points (n=3/treatment). However, observation of the nMDS showed there was a separation

between the two groups with closer clustering i.e. similarity/uniformity within the High Spec. inoculum group. Findings from the pilot trial support this observation. It was also observed that the inoculum samples grouped quite far outside of the sheep rumen sample profiles, which could possibly be due to the processing of the inoculation before treating the lambs.

The second microbial samples taken for this trial were intestinal samples, taken at slaughter (week 18). Intestinal microbial profiling was also broken down to phyla, class, order, family, genus and species level. The intestinal bacterial order, class, genera and species were significantly affected by the imposed inoculation treatments (Table 5). Significant pairwise difference were observed between Roughage inoculated and Maternal Control lambs for these intestinal bacterial genera. In addition, significant ($P < 0.05$) differences were observed between the High spec and Roughage inoculated lambs in the intestinal bacterial order. This is graphically displayed in Figure 5.

Table 5: Two way ANOSIM of rumen microbial communities in intestinal samples, associated with breed and inoculation treatment in Trial 1 in Merino and Suffolk lambs at the point of slaughter (week 18).

Parameter	*R* and *P* values[a]					
	Phyla	Class	Order	Family	Genus	Species
Inoculation	**0.039** *0.163*	**0.071** *0.048**	**0.079** *0.028**	**0.664** *0.054*	**0.064** *0.037**	**0.071** *0.039**
Breed	**0.035** *0.306*	**-0.037** *0.652*	**-0.044** *0.650*	**-0.065** *0.730*	**-0.07** *0.727*	**-0.065** *0.724*

[a] The global R statistic (indicated in boldface) and significance (P value - indicated in italics) are shown for each of the factors bacterial genera. The Global *R* value describes the extent of similarity between each pair in the analysis of similarity (ANOSIM), with values close to unity (1) indicating that the 2 groups are entirely separate and a zero value indicating that there is no difference between the groups. $P < 0.05$ is significant (* have been used to highlight the significances also).

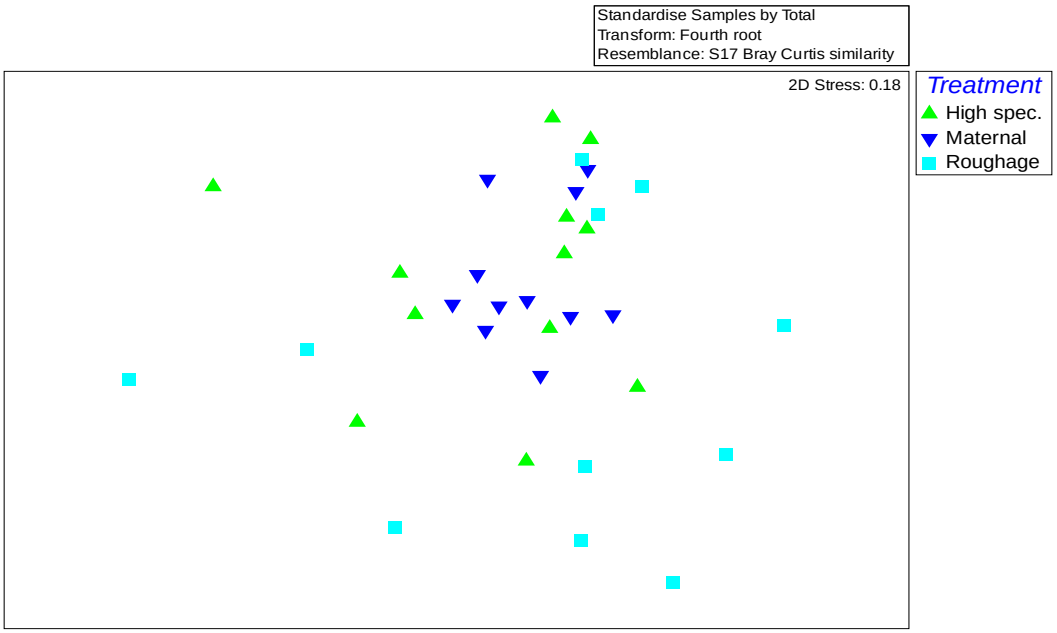

Figure 5: nMDS of intestinal bacterial profiles (order) from all lambs at slaughter identified by treatment.

There were no significant differences between intestinal microbial profiles of lambs at slaughter based on breed (Table 5). It is noted again, however, that Merino sheep dominated. There were significant differences in lamb intestinal microbial profiles associated with treatment.

4.2.2 Lamb mortalities

There were losses of 6 White Suffolk lambs and 5 Poll Merino lambs over the course of the trial. All of the deaths were within the first two weeks after birth. All animals which were treated for an illness with antibiotics resulted in mortality (no animals in the remaining cohort were treated with antibiotics). The main reason for White Suffolk mortality (Table 6) was the udder conformation of the ewes (in particular the older and larger ewes, with large and low teats, which made it hard for lambs to feed.

Table 6: Lamb mortalities in White Suffolk lambs in Lambing Trial 1.

Records	**lamb 1**	**lamb 2**	**lamb 3**	**lamb 4**	**lamb 5**	**lamb 6**
Treatment	Roughage	High Spec.	High Spec.	Roughage	High Spec.	High Spec.
Breed	Suffolk	Suffolk	Suffolk	Suffolk	Suffolk	Suffolk
Birth type	Singleton	Twin	Twin	Singleton	Twin	Singleton
Sex	F	F	F	M	F	F
Birth weight (kg)	5.55	4.87	5.34	5.30	5.06	6.59
Ewe	Suffolk 1	Suffolk 2	Suffolk 2	Suffolk 3	Suffolk 4	Suffolk 5
Symptoms	None noted	Udder conformation poor and ill joints in the lamb	Udder conformation poor	Udder conformation	Ewe sore feet	Udder conformation poor
Medications/ treatments given	No	Ewe and lamb antibiotic and anti-inflammatory, powdered colostrum and milk replacer	Ewe antibiotic and inti inflammatory, powdered colostrum and milk replacer	Powdered colostrum and milk replacer	Ewe antibiotic and anti-inflammatory, separated into shelter with lamb and close feed and water	Powdered colostrum and milk replacer
Mortality cause noted	Exposure	Starvation (poor ewe conformation) and ill joints following	Starvation (poor ewe udder conformation-not mastitic)	Starvation (poor ewe udder conformation-not mastitic)	Exposure (escape and separation from ewe)	Starvation (poor ewe udder conformation-not mastitic)
Caused by treatment?	Not likely	Not likely	No	No	No	No

The mortalities in the Merino group were more suspicious, in that they could have possibly been related to the treatments. We did not have the same udder conformation problems with the Merino ewes and the only deaths in Merino lambs were in the Roughage treatment group, in relatively small birth weight lambs (Table 7).

Table 7: Mortality in Poll Merino lambs in Lambing Trial 1.

Records	**lamb 7**	**lamb 8**	**lamb 9**	**lamb 10**	**lamb 11**
Treatment	Roughage	Roughage	Roughage	Roughage	Roughage
Breed	Merino	Merino	Merino	Merino	Merino
Birth type	Singleton	Singleton	Twin	Twin	Twin
Sex	F	M	F	F	M
Birth weight (kg)	5.28	4.50	4.35	4.00	4.28
Ewe	Merino 1	Merino 2	Merino 3	Merino 3	Merino 4
Symptoms	None before death noted	Swollen joints and ill thrift	Swollen joints and ill thrift	Ill thrift and cold to the touch, deteriorated quickly	No
Medications/ treatments given	No	Lamb antibiotics, several courses	Lamb antibiotics, several courses	Warm milk replacer and placed in shelter with hot water bottle and covers	Previously ill thrift and treated with antibiotics prior to exposure
Mortality cause noted	Humanely euthanised- diagnosed septicaemia - high levels of Fusobacterium in joints and liver abscesses	Humanely euthanised - ill joints with puss in joints and likely septicaemia	Humanely euthanised , ill joints, necropsy suggests septicaemia	Died, necropsy was inconclusive (lamb placed on hot water bottle to warm up shortly before death)	Exposure (storm overnight)
Caused by treatment?	Likely	Likely	Likely	Possible	Possible

4.2.3 Lamb weights, condition and growth

There was a significant effect of treatment by breed by week on lamb weight. In week 18 specifically, in White Suffolk lambs the Roughage treatment was not significantly different to the Maternal treatment, with the High Spec. treated lambs weighing significantly less. In Merino lambs, the Roughage treatment was not significantly different to the Maternal treatment but the High Spec treated lambs weighed significantly more (Figure 6). If we look at weeks before slaughter, the Merino Roughage lamb weighed less than other Merinos in several weeks over the duration of the trial. The High Spec. treatment is the only treatment where no difference between the breeds was seen at any point over the measurement period, as the Suffolks in the High Spec. treatment were significantly smaller than the others, making them comparable to the Merinos of the same treatment.

There was a significant effect of treatment and breed on condition score ($P < 0.0001$), with lower condition scores seen in Roughage treated Merinos in the weeks immediately following the inoculation treatment (week 2-6). On the week of weaning (week 10), the Roughage Suffolks were in significantly higher condition than all other groups. However, by week 18 the condition score within breed had evened out, with no significant difference with treatment within breed, but a significant difference at week 18 between the two breeds, with the Suffolks ending the trial with higher scores than the Merinos (Figure 7).

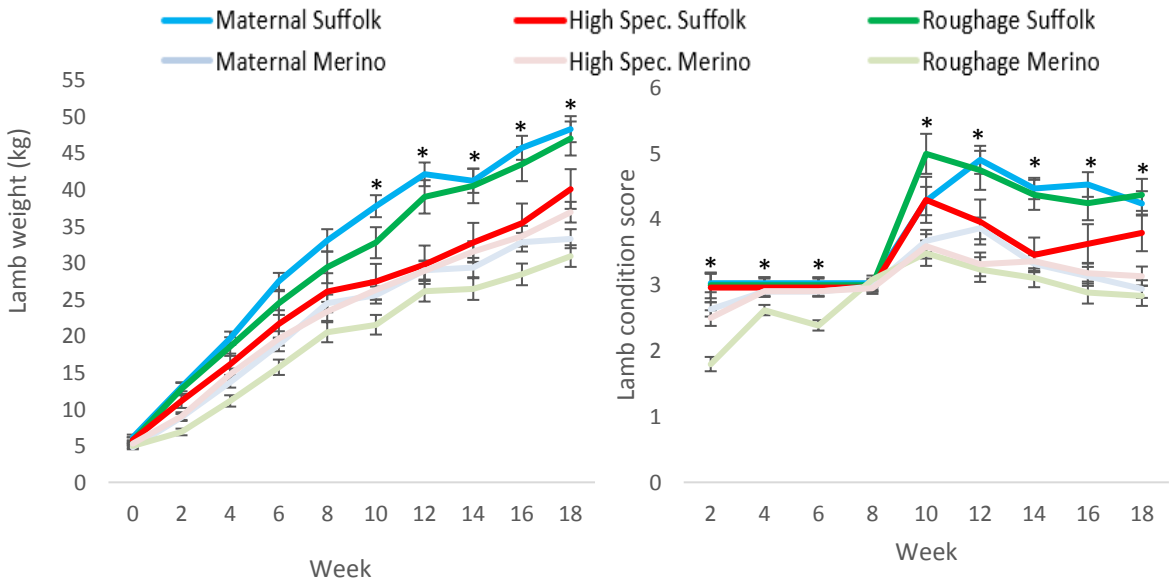

Figure 6: The effect of treatment and lamb breed on live weight over 18 weeks in lambs treated with inoculations of known rumen fluid populations from ewes treated with High Spec. and Roughage diets and Maternal Control lambs. P < 0.001 (* indicates significant differences)

Figure 7: The effect of treatment and lamb breed on live weight over 18 weeks in lambs treated with inoculations of known rumen fluid populations from ewes treated with High Spec. and Roughage diets and Maternal Control lambs. P < 0.001 (* indicates significant differences)

Figure 8 presents the relationship between breed, treatment and week for grams of growth per day. The High Spec. Suffolk lambs were decreasing in growth per day significantly in the weeks before weaning, gaining significantly less than the animals of the same breed in other treatments. The growth of the High Spec. Suffolk treatment seem to be picking up again upon slaughter measures. Also, the two Roughage treatments gained significantly more weight after weaning than the others of the same breed but then drop back as quickly as they gained, with week 14 (Figure 8, P = 0.039).

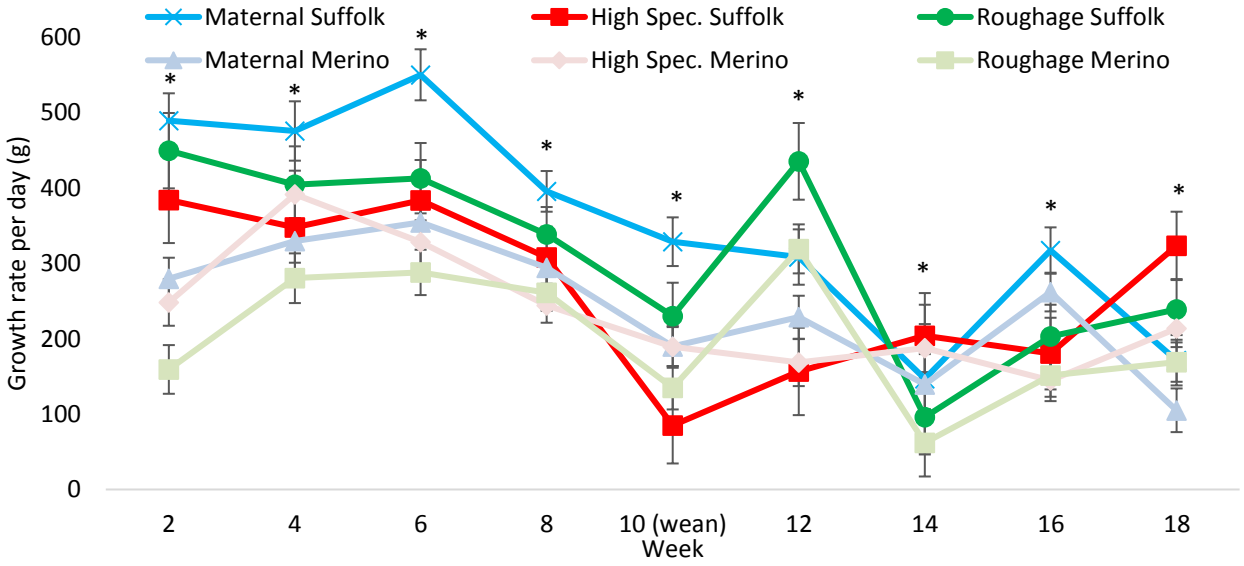

Figure 8: Lamb growth rate (g/day) of White Suffolk and Poll Merino lambs from birth until slaughter at week 18, in lambs treated with inoculations of known rumen fluid populations from ewes treated with High Spec and Roughage diets and Maternal Control lambs (*P=0.039).

The final weight of the lambs after slaughter (carcass weight) was significantly affected by treatment (P = 0.032) and breed (P < 0.001) and there was an interaction between the two (P = 0.011, Figure 9).

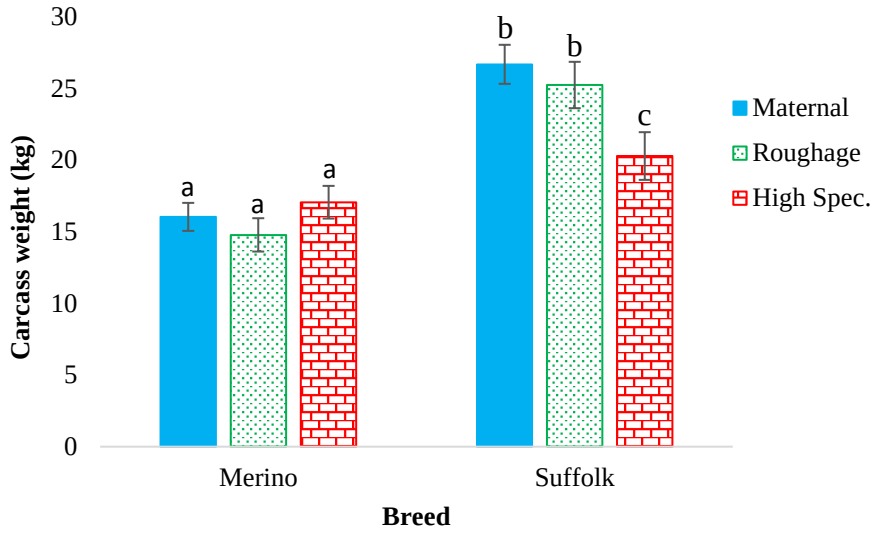

Figure 9: Final carcass weights in White Suffolk and Poll Merino, in lambs treated with inoculations of known rumen fluid populations from ewes treated with High Spec. and Roughage diets and Maternal Control lambs ($P^{a,b}$ = 0.011).

4.2.4 Feed conversion efficiency (FCE)

Feed conversion efficiency was affected by breed (P = 0.006), week (P = 0.007) and treatment by week (P < 0.001). The FCE was significantly increased in the White Suffolk lambs (7.81 g ± 0.74 g of feed for every g in weight gained), compared to the Poll Merino lambs (10.00 g ± 0.88 g, P = 0.006). When analysed separate of treatment, week was also a significant factor affecting FCE (week 10-12: 7.71 g ± 0.90 g, week 12-14: 10.89 g ± 1.30 g, week 14-16: 7.78 g ± 0.87 g, week 16-18: 9.23 g ± 0.91 g, P = 0.007). Figure 10 shows the changes in FCE with treatment over the weeks measured (P < 0.001). Just following weaning from week 10-12 the Roughage treatment had lower FCR then both other treatments. Also in week 14-16 the Maternal group was significantly more efficient and then in week 16-18 was significantly less efficient than both treatment groups (Figure 10).

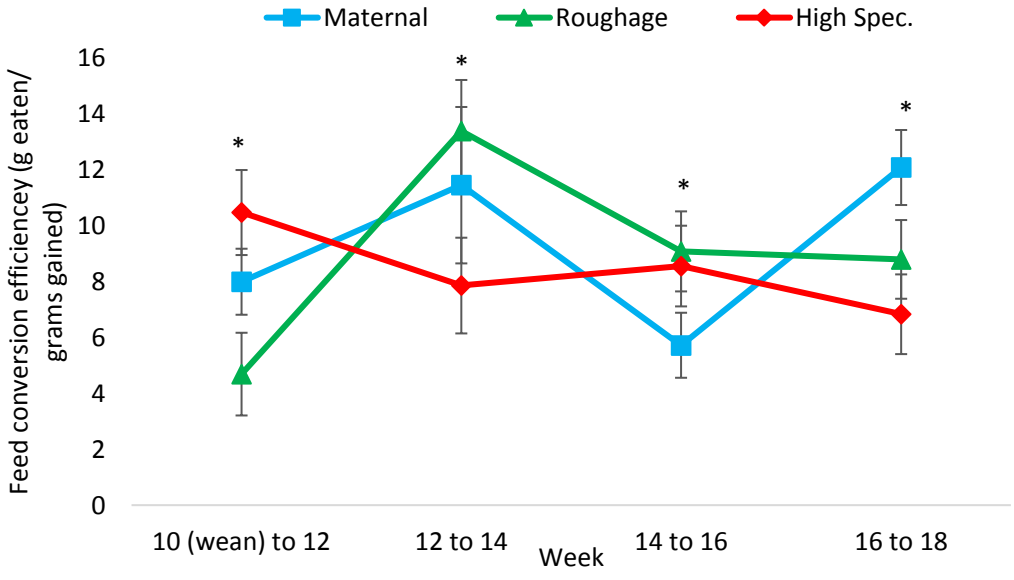

Figure 10: Feed conversion efficiency in grams (grams of feed eaten for grams of weight gained) in lambs treated with inoculations of known rumen fluid populations from ewes treated with High Spec. and high Roughage diets and Maternal Control lambs (*P < 0.001).

4.2.5 Haematology

Haemoglobin concentration in blood was affected by treatment (P = 0.026), breed (P < 0.001) and treatment by breed (P = 0.002, Figure 11). Haematocrit was affected by treatment (P = 0.008) and treatment by breed (P = 0.004, Figure 12). Red blood cell count was affected by treatment (P < 0.03), breed (P < 0.001) and treatment by breed (P < 0.001, Figure 13). The 'breed by treatment' effect shows a significant difference in the red blood cell count in Suffolk only, with a significantly lower count in the Suffolk lambs treated with High Spec. inoculation, compared to both other treatments. Mean cell volume (MCV) was also affected by treatment (P < 0.001) and breed (P = 0.031). Regarding the effects of treatment, each treatment was significantly different to the other, with High Spec. having the highest MCV (33.19 ± 0.42), then Maternal (31.52 ± 0.40) and then Roughage (30.81 ± 0.46, P < 0.001).

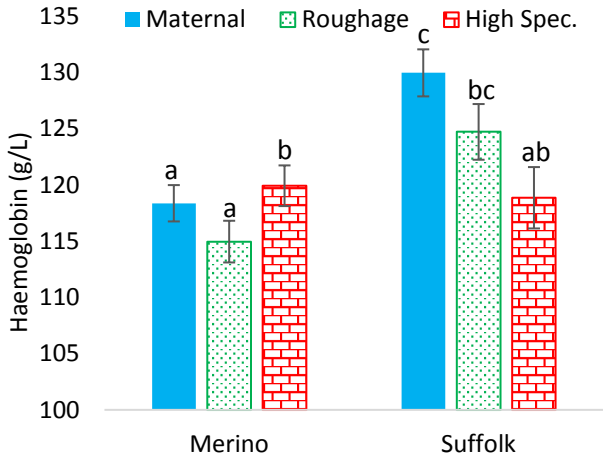

Figure 11: Haemoglobin (g/L) in Poll Merino and White Suffolk lambs treated with inoculations of known rumen fluid populations from ewes treated with High Spec. and Roughage diets and Maternal Control lambs.

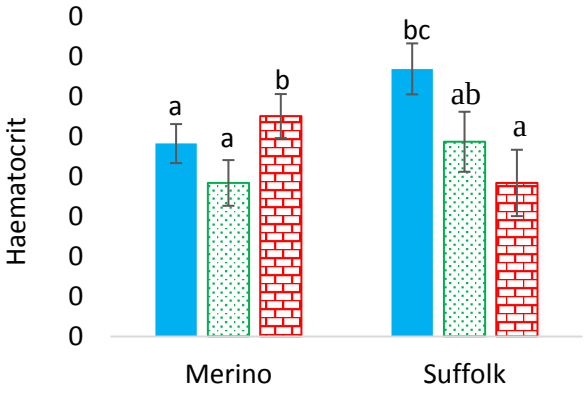

Figure 12: Haematocrit in Poll Merino and White Suffolk lambs treated with inoculations of known rumen fluid populations from ewes treated with High spec. and Roughage diets and Maternal Control lambs.

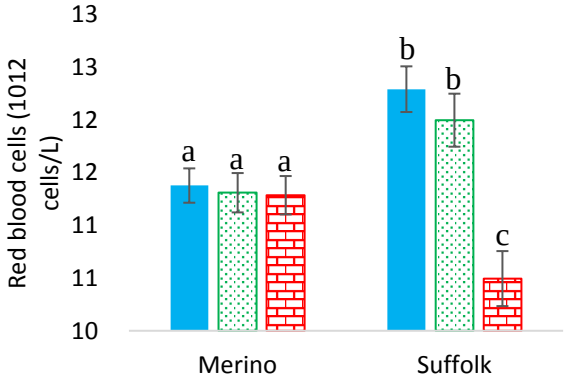

Figure 13: Total red blood cell count (cells 10^{12}/L) in Poll Merino and White Suffolk lambs treated with inoculations of known rumen fluid populations from ewes treated with High Spec. and Roughage diets and Maternal Control lambs.

Eosinophils (EOS - cell count 10^9/L) were significant affected by treatment (P = 0.046), breed (Merino: 0.103 ± 0.013, Suffolk: 0.133 ± 0.014, P=0.005) and treatment by week (P = 0.031, Figure 14). Basophils were affected by breed (Merino: 0.088 ± 0.004 cell count 10^9/L, Suffolk: 0.077 ± 0.005, P = 0.042).

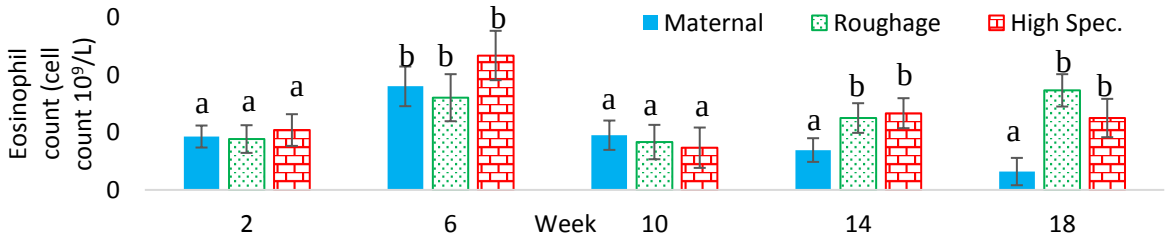

Figure 14: Eosinophil cell count (cells x 10^9/L) in Poll Merino and White Suffolk lambs from birth until slaughter at week 18, in lambs treated with inoculations of known rumen fluid populations from ewes treated with High Spec. and Roughage diets and Maternal Control lambs.

4.2.6 IgG and IgA

There were no significant effects of treatment, breed, birth type or sex on IgG concentrations in serum or IgA concentrations in saliva (P < 0.05). There was a significant effect of week by salivary IgA and serum IgG concentrations, which was expected as the animal aged.

4.2.7 Histology (Peyer's patch measurements)

Multiple measures of the morphology of Peyer's patches were taken (a single section can be seen in figure 1%). Of these measures, the Feret measure (measure of the longest part of each patch) was significantly altered by treatment (P = 0.048), specifically the proportion of patches in the 4th quartile (longest patches measured), was lower in the Maternal Control, compared to both treatment groups (Table 8).

Table 8: Measures of Peyer's patches from Maternal Control lambs and those inoculated with donor (Roughage or High Spec.) rumen fluid at birth

Patch measure (mm)	Measures analysed	Maternal	Roughage	High Spec.	P value
Area	Average	275.7 ± 39.5	301.5 ± 45.6	336.6 ± 41.3	0.165
	Average Quartile	2.3 ± 0.2	2.4 ± 0.2	2.6 ± 0.2	0.170
Perimeter	Average	2.1 ± 0.1	2.2 ± 0.2	2.3 ± 0.2	0.012
	Average Quartile	2.3 ± 0.2	2.3 ± 0.2	2.6 ± 0.2	0.174
Feret diameter (longest point)	Average	0.8 ± 0.1	0.9 ± 0.1	0.9 ± 0.1	0.077
	Average Quartile	2.4 ± 0.2	2.3 ± 0.2	2.6 ± 0.2	0.095
	Proportion of patches in Q4	11.8 ± 6.0^a	20.9 ± 7.0^b	25.8 ± 6.3^b	0.048*

Superscripts^{a,b} highlight significant differences between treatments (*P < 0.05).

4.2.8 Rumen pH at weaning

Rumen pH, measured at weaning, was significantly affected by treatment and sex of the lamb. Females had a significantly higher (7.34 ± 0.05) rumen pH than the male lambs (7.15 ± 0.05, P = 0.001). Treatment also affected rumen pH, with significantly lower rumen pH in the Roughage treatment (7.10 ± 0.06) than the Maternal Control (7.34 ± 0.06) or the High Spec. inoculation (7.29 ± 0.06, P = 0.015).

4.3 Lamb Trial 2

4.3.1 Microbial analysis

Bacterial data were separated into phyla, class, order, family, genus and species level and each level analysed separately. For this trial, breed and sex of the lambs did not have a significant ($P < 0.05$) effect on the rumen bacterial populations at weaning or at slaughter. The post birth inoculation with mature rumen contents had a significant effect on the lamb rumen bacterial populations at weaning (week 10), despite lamb and ewe rumen bacterial populations being significantly different (Table 9). The relationship and lamb treatment effects in rumen bacterial phyla at weaning are shown in Figure 16 and 17, respectively.

Table 9: Two-way ANOSIM of rumen bacterial communities associated with lamb treatment (inoculations vs control) and relationship (lamb vs maternal ewe) at lamb weaning (week 10)

Factor	*R* and *P* values[a]					
	Phyla	Class	Order	Family	Genus	Species
Treatment	**0.070** *0.027**	**0.071** *0.028**	**0.401** *0.001**	**0.422** *0.001**	**0.139** *0.001**	**0.129** *0.001**
Relationship	**0.336** *0.001**	**0.328** *0.001**	**0.115** *0.003**	**0.111** *0.001**	**0.470** *0.001**	**0.488** *0.001**

[a] The global R statistic (indicated in boldface) and significance (P value - indicated in italics) are shown for each of the factors bacterial genera. The Global *R* value describes the extent of similarity between each pair in the ANOSIM, with values close to unity (1) indicating that the two groups are entirely separate and a zero value indicating that there is no difference between groups. P values of < 0.05 are deemed significant (* have been used to highlight the significances also).

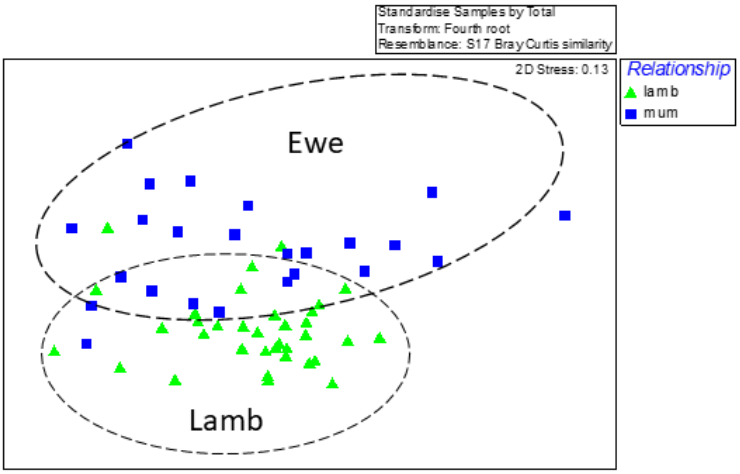

Figure 16: nMDS of rumen bacterial phyla from lambs and ewes at time of lamb weaning (week 10)

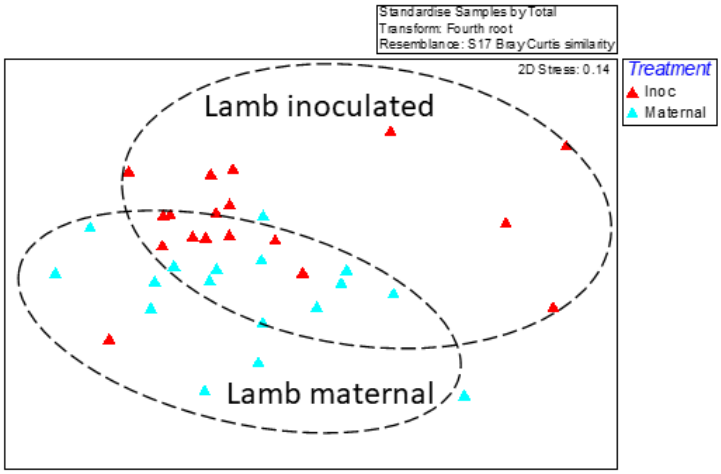

Figure 17: nMDS of rumen bacterial phyla from lambs at time of weaning (week 10) identified by treatment.

The lamb rumen bacterial communities were significantly different at weaning (week 10) and at slaughter (week 18) regardless of treatment, although the impact of post birth inoculation with mature rumen fluid could still be observed at slaughter (Table 10). This is an outstanding result, as maintaining long term effects from an inoculant has not yet been achieved in any ruminant species, to the best of our knowledge. Differences in rumen bacterial phyla and genera associated with treatment at slaughter (week 18) are shown in Figure 18 and 19, respectively.

Table 10: Two-way ANOSIM of rumen bacterial communities associated with treatment (inoculations vs control) at slaughter and age (week 10 vs week 18) of lambs.

Factor	*R* and *P* values[a]					
	Phyla	Class	Order	Family	Genus	Species
Treatment	**0.098** *0.003**	**0.120** *0.001**	**0.173** *0.001**	**0.195** *0.001**	**0.230** *0.001**	**0.222** *0.001**
Age	**0.277** *0.001**	**0.274** *0.001**	**0.396** *0.001**	**0.429** *0.001**	**0.451** *0.001**	**0.464** *0.001**

[a] The Global R statistic (indicated in boldface) and significance (P value - indicated in italics) are shown for each of the factors bacterial genera. The Global *R* value describes the extent of similarity between each pair in the ANOSIM, with values close to unity (1) indicating that the two groups are entirely separate and a zero value indicating that there is no difference between groups. P values of < 0.05 are deemed significant (* have been used to highlight the significances

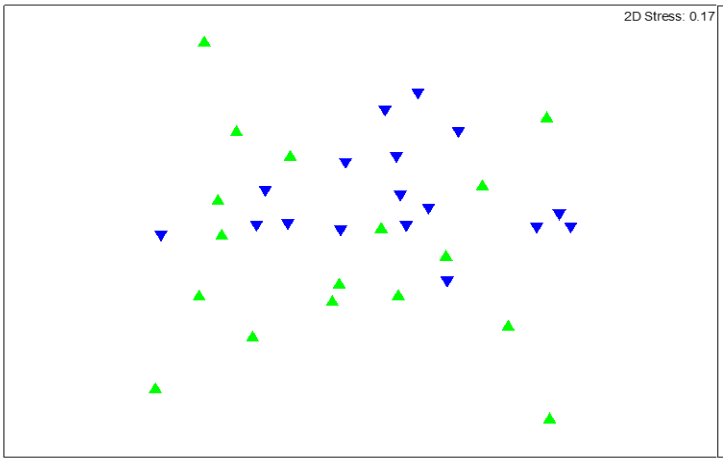

Figure 18: nMDS of bacterial phyla from lambs at slaughter (week 18) identified by treatment.

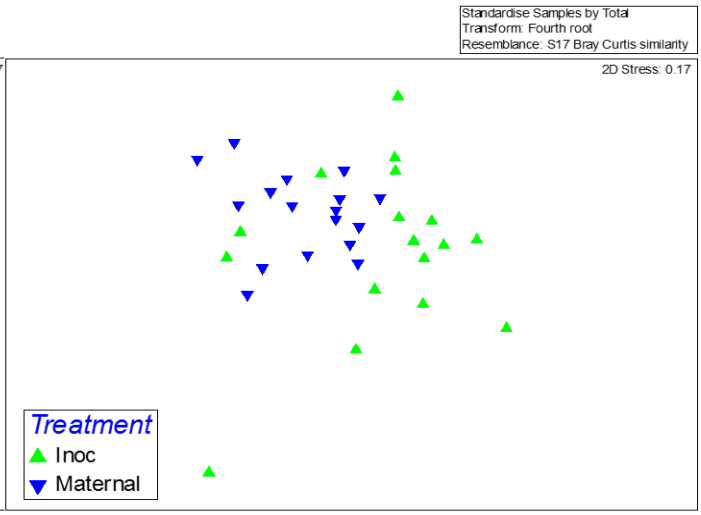

Figure 19: nMDS of bacterial genera from lambs at slaughter (week 18) identified by treatment.

By comparing lamb weaning and slaughter rumen bacterial communities identified by treatment (Figure 20 and 21) it is evident that not only does the inoculation cause lasting effects through population shifts, but also that it causes changes in how each population matures and shifts as the animal grows.

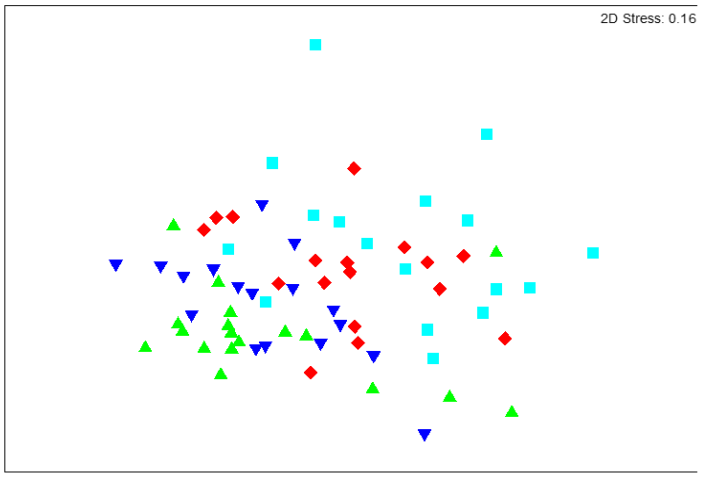

Figure 20: nMDS of lamb rumen bacterial phyla associated with sampling time (week 10 and 18) and treatment

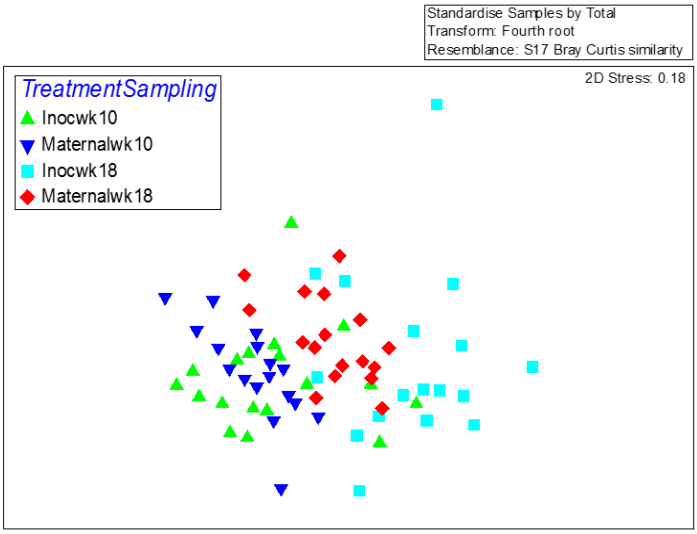

Figure 21: nMDS of lamb rumen bacterial genera associated with sampling time (week 10 and 18) and treatment

4.3.2 Lamb weights, condition, growth and FCE

Regarding treatments there was a significant effect of treatment by breed by week ($P < 0.0001$). Figure 22 shows this relationship, with clearly different weights throughout the trial in Suffolk and Merino lambs.

There was a significant effect of treatment and breed across weeks on lamb condition score ($P < 0.0001$, Figure 23). Differences in condition score in the Merino lambs was dependent on treatment, with the inoculation group having slightly higher condition score at week 18.

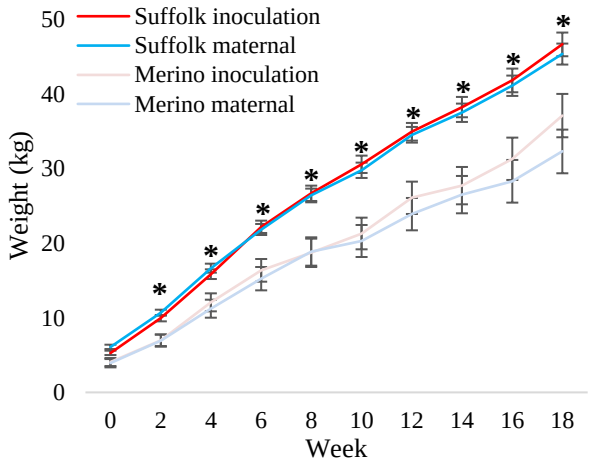

Figure 22: The effect of treatment and lamb breed on live weight over 18 weeks in lambs treated with inoculations of known rumen fluid populations from ewes treated with High Spec. or Maternal Control lambs. (*P < 0.0001)

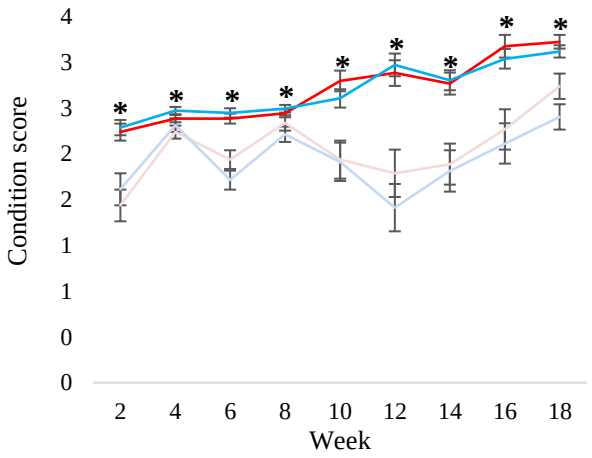

Figure 23: The effect of treatment and lamb breed on live weight over 18 weeks in lambs treated with inoculations of known rumen fluid populations from ewes treated with High Spec. or Maternal Control lambs (*P < 0.0001).

Lamb growth (analysed as the average number of grams gained per day averaged between the fortnightly weights) was significantly affected by breed (Merino: 210.19g/day ± 16.73, Suffolk: 311.93g/day ± 8.61, $P<0.001$) but not by treatment ($P > 0.05$). Feed conversion efficiency was not affected by any fixed factor, including treatment ($P = 0.26$) or breed ($P = 0.20$).

4.3.3 IgG and IgA and haematology

There were no significant effects of treatment or breed on serum IgG or salivary IgA concentrations ($P > 0.1$). No blood parameters (total blood cell counts) were affected by treatment ($P > 0.05$).

5 Discussion

5.1 Pilot Trial

The Pilot Trial experiment clearly showed breed and diet effects on the resident rumen bacterial populations, as well as, impacts on production data. These are discussed further below.

5.1.1 Consumption and creating different diets

There was a clear preference for the pellet proportion of the diets over the chaff, which can be seen in the amounts eaten. We fed the Roughage group 20% pellet and 80% chaff and their diet ended up being 26% pellet, showing that the roughage proportion of the diet was not completely consumed. This preference for pellets did not differ with breed, with both Suffolk and Merino eating more grams of the High Spec. diet per day. Anecdotally, the Suffolks were much more excited by the feed drops than the Merinos appeared to be, suggesting that there would be a significant difference in amounts consumed between breeds. Perhaps, Suffolks ate the majority of the food at the drop, whereas Merinos may have grazed throughout the day, resulting in similar consumption levels. Despite this, due to the methodology of the feeding provisions and renewed rations each morning, the experiment managed to maintain significantly different diets.

5.1.2 Individual animal differences

Rumen microbiota has been found to vary among individual animals (Li et al., 2009a), most likely with a stable core population within a herd or flock (Jami and Mizrahi, 2012). Our microbial data does show that there is variation within the population and within breeds, but also a core stability within breed and diet. Jami and Mizrahi (2012) found 82% similarities in the rumen microbiota among 16 Holstein Friesian lactating cows fed the same diet. Weimer et al., (1999) found greater differences between individuals than were created with dietary changes. However, in a large study (with 742 rumen samples, from 32 species and 35 countries) Henderson et al., (2015) suggested that diet caused the greatest variation among samples, with the host being less influential, which aligns more with the results of our current study.

5.1.3 Diet differences

We found not only that diets created divergent microbial populations in the ewes, but that the rumen populations are more variable on the High Spec. (high grain) diet compared to the Roughage diet. The components of the fermentation process will vary depending on the composition of the feed and, therefore, microbial populations will be impacted (de Menezes et al., 2011). This in turn may allow for different and varied niches to be filled within the rumen, possibly leading to our more varied population.

5.1.4 Breed differences

Despite the difference between treatments there was no significant effect on the ewe weights when assessed by treatments. However, ewe weight was affected when assessed by treatment and breed as an interaction, showing a weight loss in the Suffolk ewes on the Roughage diet. This group of ewes was the only one to lose weight, with the Suffolks on High Spec. and all of the Merinos, gaining weight over the duration of the trial. The differences between the breed microbial populations are more pronounced with animals fed the Roughage diet, as seen with the effects of diet on their weight. The bacterial rumen populations of the Roughage treated animals are quite tightly clustered, suggesting that there is lower variation in these the rumen populations.

It may be the case that these animals lost weight on the Roughage diet as Suffolks are a British breed and therefore may be genetically programmed to respond to a higher quality diet. Whereas the Merinos performed the same on the high quality High Spec. diet and the Roughage diet, as they have become accustomed to having to perform in the harsh, relatively low quality forage environments in which the breed has been developed. A review by Hegarty (2004) may support this idea, and suggests that between breed dietary preferences and, therefore, eating habits differ greatly. Barbado sheep have a higher intake of browse species across seasons and across pasture types than Rambouillet and Karakul (Warren et al., 1984). Also, Dorper sheep are less-selective grazers, utilise shrubs and bushes more and grass less, utilise a larger number of different plant species, walk less to select food, have shorter grazing time and less separate grazing periods than Merino, when kept under the same conditions (Brand, 2000). Differences in how breeds utilise the same feeds were highlighted by Wilkes et al (2012). This trial was conducted to quantify differences in the efficiency of feed utilisation and growth performance of Damara and Merino sheep under two contrasting dietary regimes ('low-quality' followed by a 'high-quality' diet). This experiment found many breed differences in diet utilisation, culminating in the Damara carcasses being 22% heavier than Merinos. The Damaras achieved higher total digestible energy intakes than the Merinos on both diets. On the low-quality feed this was achieved through higher feed digestibility and on the high-quality feed through greater voluntary feed intake. The authors of the paper speculated that this difference could come from variation between the breeds in rumen volume, particle flow and/or the site of digestion (Wilkes et al., 2012).

5.2 Lamb Trial 1 and 2

5.2.1 Lasting rumen microbial effects and lasting intestinal changes

Lambing trial 1 resulted in changes in rumen populations with both inoculants in week 10 (weaning), which was 9 weeks following the completion of our inoculation treatments. The results show that inoculated animals differed from non-inoculated animals, but also that there were differences between animals inoculated with the two donor fluids as well. Literature in the area of inoculating lambs with rumen populations to create microbial change is fairly sparse. In previous papers, which do use rumen fluid inoculations, there have been varied results. For example, the closest study to ours is that of De Barbieri et al., (2015 a, b) who explored manipulation of neonatal rumen microbes through inoculation of Merino lambs with foreign Merino hogget ewe rumen microbes, from ewes fed a coconut oil diet or a protected fat diet. De Barbieri et al., (2015b) found that lambs inoculated just with water as a control had lower dry matter intake after weaning and lower concentrations of rumen acetate, total VFA and protozoa by week 8, which shows that the inoculum enhanced rumen microbial activity. Their inoculant differed from ours in that it was less focused at birth and was given weekly from 1 -8 weeks. However, lamb production (body weight and condition score throughout the trial) was not altered by inoculum, so although there were changes to the microbiome there were no resulting changes in production. This trial did find lasting differences to weaning (week 8) in butyrate and protozoal populations, although inoculations had also been given up to week 8 (De Bariberi et al., 2015b). De Barbieri et al 2015a found that bacterial differences did last to weaning (week 8) but that

2 months after weaning the differences were no longer seen. We suggest that our inoculation caused lasting effects to the rumen microbial populations for 18 weeks (4.5 months), which is much longer than seen in the referenced trials, as the inoculation which we imposed was intense around birth and prior to any stabilisation of the gastrointestinal microbiota.

Not only did the inoculants result in changes in rumen populations until weaning, they also brought about intestinal and rumen microbiota changes at slaughter age at week 18. This is interesting, as this means that changes in the rumen microbial populations have changed the intestinal microbial population also, with lasting effects until week 18. There are no trials like this, showing lasting effects in rumen and intestinal microbiota for such a long duration. The interesting carry on of effects on intestinal microbiota is that these changes are more likely to be linked to changes in immune system functioning.

One challenge faced with the question of inoculation is which microbiome is superior in the first place, or which microbiome is likely to lead to production gains. This seems to be the problem which we have faced with our inoculants, regardless of the fact that they have simply come from ewes fed fairly standard pellet and chaff feeds, our inoculations created illness or decreased production. Turnbaugh and colleagues (2006) found that there is an 'obese microbiome' in mice, which has an increased capacity to harvest energy from the diet. Furthermore, this trait is transmissible: colonization of germ-free mice with an 'obese microbiota' results in a significantly greater increase in total body fat than colonization with a 'lean microbiota'. This idea that a microbiome with certain production traits could be transmissible is an interesting area for future study, especially in production animals, where the microbiome plays a role in affecting production efficiency. Our trial has undoubtedly proven that long term microbial changes and long term production effects can be brought about by neonatal inoculation. However, one of the next steps is to isolate a microbiome that will result in superior production, not decreases in production.

5.2.2 Roughage inoculant main health and production effects highlighted

The Roughage inoculant was imposed in lambing trial 1 only, due to some sickness in Merino lambs on this treatment. We believe that cases of septicaemia noted in the Roughage treatment and Merino lambs were only due to a breed specific response to this treatment. The Merinos were lower birth weight on average than the Suffolk lambs, so this illness may be caused by the Merino lambs decreased maturity at birth. It may also be caused due to the ml/kg of lamb inoculated, as all lambs were given 10mls of inoculant each day, regardless of birth weight. The gastrointestinal tract is a major interface between the host and its environment (Stokes, 2017) and is the site with the highest load of microorganisms (Lalles, 2016). In order for microbial populations to exist within the host, the immune system must tolerate those organisms, whilst simultaneously remaining vigilant against the potential threats posed by them (Brown et al., 2013). There are benefits accompanying this balancing act. We think that in this group of animals our Roughage inoculant may have tipped the scales in favour of the microbial population.

5.2.3 High spec inoculant

The main effects of note from the High Spec inoculation are decreased weight, condition score and carcass weights in Suffolk lambs. This is always a possibility when manipulating the microbial populations of animals, as the relationship between the animal and its commensal microbes is a balance which can swing either way. To clarify, despite the many benefits of the microbiota, it can also negatively affect the animal in many ways. These include, immune costs, competition for nutrients, toxic amino catabolites, decreased fat digestibility and altered intestinal morphology and function (Richards, et al., 2005). For example, the microbiota does play a key role in the development of the host animal's immune system, but there is also in efficiency in the system, brought upon by the

constant immune activation due to the presence of commensal bacteria (Richards, et al., 2005). Therefore, if we could reduce the additional response of the immune system to changes in the microbiota, this would mean that the several hundred grams of protein which is directed towards the secretion of microbiota specific IgA (humans secrete 5g of IgA a day, of which most binds to intestinal microbiota and dietary antigens) can be directed to growth of the animal instead (Macpherson et al., 2000). Also, the same mechanisms which decrease the immune systems response to pathogens, decreasing the permeability of the intestine, may also reduce nutrient uptake, due to thicker walls (Gaskins et al., 2002). We believe that this decrease in growth seen with the High Spec inoculant could be linked to an increase in immune activity and/or decreased nutrient uptake brought on by the inoculation.

5.2.3.1. Timing of the inoculant compared across lambing trials

The treatment differences seen in production measures in lambing trial 1 were not seen in trial 2, despite having more lambs per treatment and more Suffolk lambs. This is interesting, as there were very few changes between the two lambing trials. The main difference which may have resulted in changes in one trial and none in the other, is the extending of the lambing catching and inoculation window from 4 hours from birth, to a window of 12 hours from birth. This is an important change. Both trials resulted in changes in microbial populations, but only one resulted in changes to production markers, such as weight and FCE. Manipulation of the mature microbiota is likely to be more difficult and specifically less binding, than manipulation of the neonatal population (Malmuthuge and Guan, 2016). In mature animals the microbiota has a strong host dependency, so that even when following near total exchange of microbiota between animals or dietary changes in ruminants, the animal's rumen environment will revert to its previous state over a few days (Weimer et al., 2010; Khiaosa-ard and Zebeli, 2014; Malmuthuge and Guan, 2016). Weimer and colleagues aimed to examine the stability and host specificity of a cow's rumen bacteria, following a massive challenge with rumen microbiota from another cow. They found that rumen bacterial community composition displays substantial host specificity that can re-establish itself with varying success, even after a challenge with a microbial community optimally adapted to rumen conditions of a different host animal. In a healthy rumen contamination of the eco-system does not occur, in spite of constant introduction of environmental microbes, through drink, feed and air (Kamra 2005) which may explain why making a lasting change to the adult microbe population is difficult. Adult microbes are adapted to survive in a set of constraints and any contaminant which does not fit into these constraints is eliminated (Kamra 2005). It is difficult to achieve significant modification in the adult animal once the microbial ecosystem is established (Abecia et al., 2014). There is more promise in experiments which manipulate neonatal rumen bacteria populations. Early postnatal life is a period in which bacterial colonisation of the gut is occurring, when a practically sterile environment becomes inhabited by microorganisms that are likely to determine the lifetime microbiota of the host (Sudo, et al., 2004). Perhaps, in order to bring about production change with microbial manipulation the manipulation needs to be very close to birth, not just focusing on neonatal changes but focusing on 'immediate' manipulations. This could be researched further by altering the timing of inoculants in order to determine its effects.

5.2.4 Breed differences in response to different inoculum sources

There has been a reasonable amount of research on the differences in digestion and rumen microbial populations in different breeds of cattle (and less so in sheep). As early as the 1960s comparisons of Bos taurus and Bos indicus cattle suggested that Bos indicus had faster fermentation rates and shorter rumen retention time (Phillips et al., 1960). More recently King and colleagues (2011) looked at breed differences in rumen methanogen populations of lactating Jersey and Holstein dairy cows, fed the same diet. They used 365 sequences, while 85% were common to both breeds, there were several sequences found only in the Jersey (n=18) and several only in the Holstein (n=36), showing increased diversity in the Holstein library. As many other variables were controlled, the animals were from the

same herd, housed under the same environmental conditions and fed the same diet, the differences observed may well be due to differences in host breed genetics. Bacterial profiles have been found to be more clustered within a certain breed, supporting the argument that host genetics may play a part in the rumen microbiome (Guan et al., 2008).

It is interesting that the two inoculations had different effects depending on which breed they were imposed on. With the Roughage inoculant causing illness in neonatal Merinos, with no long term effects in the remaining population, and the High Spec inoculation affecting the production efficiency of the Suffolk lambs in particular. This has not been seen in previous manipulation trials, as they focus on single breeds. This effect is important, as it must be noted that the eventual development of one single universal inoculant with production benefits may not be reasonable to expect for differing production breeds, as there are obvious breed differences in how the animals respond to the imposed manipulation.

5.2.5 Immunology and haematology effects

The microbiota of the gastrointestinal tract has several important functions, which include enhancement of the intestinal epithelial barrier, development of the immune system and nutrient acquisition (Kamada et al., 2013). In a review focusing on human gut microbiota, Marchesi and colleagues (2016) suggest that it might be more accurate to think of the commensal microbiota as an immune system in itself, with a collection of cells that work in unison with the host and promote health, or initiate disease. Therefore, it was reasonable for us to assume that manipulation of the microbiota of the ruminant neonate may result in changes to immune development and function. Induction of the host immune response can also be caused by commensal bacteria. In studies using microbiota-lacking mice, obvious immune impairment was seen, ranging from abnormal numbers of several immune cell types and products, deficits in lymphoid structures, poorly formed spleens and lymph nodes, reduced number of IgA-producing cells, reduced secretion of immunoglobulins and irregularities in cytokine levels and profiles (Bauer et al., 1963; Macpherson and Harris, 2004; O'Hara and Shanahan, 2006). Also, germ-free mice, and those with low-diversity microbiota, develop elevated serum IgE levels in early life, which play a central role in atopic allergic disease (linking in with the increased allergy symptoms with altered micobiota which was discussed briefly in the previous sub section) and immunity to parasites (Cahenzli et al., 2013).

There were several effects of our inoculations on immunological measures, although IgA and IgG were not affected. The proportion of Peyer's patches in the 4^{th} quartile for length were significantly more in both inoculations than in the Maternal group. Peyer's patches are part of the gut-associated lymphoid tissue and are composed by aggregated lymphoid follicles surrounded by a specialized epithelium and are more prominent in the ileum (which is why this particular tissue was sampled). Experiments on animals raised in a germ-free environment have suggested that the number of patches found is fixed at, or shortly after, birth, and the presence of bacteria is necessary for their full development (Miyakawa, 1959). In dogs, ileac Peyer's patches expand rapidly after birth, reaching maximal size by 6 months of age (HogenEsch and Hahn, 2001). Upon reaching sexual maturity, the follicles of the canine ileac Peyer's patch become markedly reduced in size (HogenEsch and Hahn, 2001). This difference in length between inoculated and non-inoculated lambs may show us that the lymphoid associated tissue in the inoculated animals has matured more quickly than in the Maternal control animals.

Also, there were effects in the number of eosinophils, which are a type of disease-fighting white blood cell. Drastic increases in this cell type can indicate a parasitic infection, an allergic reaction or cancer. In week 14 and 18 the inoculated lambs (regardless of which inoculation) had significantly higher eosinophil counts than the controls. There are two possibilities, 1) that the inoculation has caused in increase in eosinophil counts as a result of the inoculation itself and the introduction of a high burden

of foreign microbes at a young age, resulting in a differently functioning immune system in the inoculated lambs as they grew or, 2) that the inoculated lambs are more susceptible and therefore have a higher pathogen load (either a 'sensitised' immune system or a real pathogen load). As we did not measure pathogen loads in the lambs we cannot say which of these hypothesis is more likely. In hindsight, possibly further immune measures, such as IL6 would have been good to measure, to further analyse the changes in the immune system. However, due to poor conception rates and splitting the trial into two lambing blocks our budget would no longer allow for this.

The treatments also changed haemoglobin, haematocrit and red blood cell counts, a highlight of these results is how significantly different the High Spec. Suffolks are to the other Suffolks. This result is in line with this treatment also being significantly smaller than the other two Suffolk treatment groups. However, all blood results are within normal levels for lambs aged 9-16 weeks (Lepherd., et al, 2009: Eosinophils 10^9/L, 0.1 ± 0.03, Haemoglobin g/L 122 ± 1.2, Red Blood cell count, 10.7 x 10^{12}/L, Haematocrit L/L 0.33 ± 0.004). This makes the changes across treatment a little difficult to discuss, as the cell count changes are not suggestive of any form of anaemia. It is possible that the difference between breed haemoglobin (HGB) and haematocrit (HCT) is linked to breed genetic differences. The treatment differences are more interesting. We can see that the treatments affect HCT and HGB in the same way, which may imply something dilutional. It is possibly that the breeds are genetically programmed to take more or less water from the feed, resulting in different reactions to the inoculation, or that the inoculation itself causes different water movements within different breeds.

6 Conclusions/recommendations

The following sections summarise the findings of the trials and report recommendations to MLA managers on potential applications of the findings for industry or for further research needed to apply the findings on-farm (the 'next step').

6.1 Pilot trial

This trial investigated the idea that some of the genetic differences in growth potential and productivity between breeds of sheep, may be generated by differences in their ruminal microbiota. It is generally assumed that diet has the largest effect on the ruminal microbial composition and that differences in growth and productivity between breeds are due to metabolic differences alone. This trial is the first to demonstrate that sheep breeds, differing widely in growth rate and efficiency, have very different ruminal microbiota at all levels (down to species). Furthermore, the differences between breeds were greatest when the sheep consumed a Roughage diet and on this ration the Merinos grew faster than the Suffolks, which in fact lost weight. ***This is a very significant finding as it suggests that at least part of the genetic basis for differences in growth resides in the establishment of different rumen microbes and presumably the effects of this on nutrient yield and composition.*** Given the large differences in the environments in which Merinos and Suffolks were developed it is perhaps not surprising that Merinos are better able to handle high-fibre roughage diets and it appears that a significant part of this ability resides in their ability to establish a 'roughage-type' microbiome. When the breeds were offered a High Spec. (high-grain) ration the breed microbiota differences remained but were diminished, suggesting a true genotype by environment interaction.

<u>Industry relevance</u>: The next step may be to identify the ruminal microbes that are associated with handling High Spec. versus Roughage diets better and to identify the microbes in Suffolks which contribute to their generally higher growth performance as compared to Merinos. If we can, this would be a major step towards dietary manipulation of maternal diets to establish the 'best' microbiota in neonates for different environments/diets. With some further analysis of our microbial

data results we may be able to answer this question partials, but further research in his area would further answer this question.

6.2 Lamb Trials

This was a ground breaking experiment, in that no previous trial has attempted to challenge neonates from birth with such an enormous inoculation of diet-specific ruminal microbes in crude ruminal fluid. Inoculating newborn lambs with ruminal fluid derived from ewes fed different rations produced long-term changes in the ruminal and intestinal microbiota compared to those inoculated naturally from the maternal ewe. Rumen microbiota remained different between the groups at weaning and at slaughter at 18 weeks, the latter being an impressive 17 weeks after inoculations ceased.

<u>Industry relevance</u>: This is an important finding because it demonstrates that if we can find an 'ideal' inoculation source along with our method we can be optimistic that the changes we make to the newborn ruminant will be long-lasting. As for trial 1 above, we need further work to identify microbiota with beneficial effects on animal health and production.

The artificial inoculations had negative effects on both growth efficiency and general health of the lambs. The immune systems of inoculated lambs was clearly activated to fight the unnatural exposure of the developing gastrointestinal epithelia to large doses of microbes and also to some pathogens that entered the systemic circulation. This immune activation in fact may have been the cause of reduced growth. The long term effects of this activation are of interest as they may be beneficial in sensitising the animal to resist future exposure, but they may also be negative in creating a hyper-responsive animal that diverts resources from growth to immune activation.

<u>Industry relevance</u>: The immune system of ovine neonates is sensitive to exposure to ruminal microbes. It is possible that exposure to only desirable microbes might have positive effects on immune response without the negative effects observed.

6.3 The next possible steps

The results from this trial can lead into several areas of new research, these include:

1. Can we identify microbial population cohorts using DNA technology which are associated with better performance?

This project shows us that longer-term change can be brought about through our method of neonatal inoculation. However, we have not had positive production changes (when looking at animal growth measures mainly). Therefore, the question which this result poses is, can we isolate and understand which populations of microbes are likely to result in positive production effects upon inoculation?

- This might be answered by an in-depth study into the natural populations of high and low producing animals from different flocks and genetics and kept under the same conditions. This would likely necessitate choosing one breed to focus on, in order to keep animal numbers in the trail reasonable. This would allow separation of the microbes from high and low producing animals of different genetics, which could then be studied for differences. This would be a primary scientific trial which would then lead into new inoculation studies.
- Experiments to determine optimum populations would be needed, keeping in mind that there will be varied optimums depending on breeds and genetics. This is why in the above

point we suggest different genetics all brought together to compare the microbial populations. This would ensure that answers relevant to the whole Australian flock would be closer to being reached, rather than to a single genetics.
- Examination of microbial populations from sheep with varying growth and wool growth and quality would be beneficial to get a rounded view.

2. Can we change the rumen profile through lower labour methodology, such as manipulating the ewe using simple dietary manipulations to replicate the beneficial population needed?

Our inoculation method has proven itself in altering the microbial populations of the neonates. However, it was a very high labour method and was never intended to be suggested as in industry method, but rather to see if microbial populations could be affected long term. The only way to do this was with a high 'challenge' with the inoculation fluid, and so our methodology was designed. However, it may now be relevant to look into less laborious methodologies to manipulate the neonatal lamb microbial population, which may become more relevant for industry use.

a) The success of manipulation of the ewe microbial population before laming and therefore manipulation of the lamb inoculant could be examined.
    - The reasoning behind this suggestion is that the neonatal lamb inoculant may be changed through manipulation of the ewe, which would likely require significantly less intervention.
    - This could be done through manipulation of the ewe diet prior to lambing, provision of pre and pro-biotics or population transfers from high value ewes into low value ewes before lambing (for example).
b) The success of other and lower labour manipulations of the neonatal lamb at birth could be examined.
    - Do lower labour methods have the same effect if not as intensive?
    - Can fewer inoculations be given?
    - Can we move the inoculations away or closer to birth?

3. Do these changes (defining positive populations and then using varied inoculation methods) transfer to the lambs and confer a benefit?

4. Other smaller ideas stemming from the project:

    a) How long do the changes in populations last following our inoculations?

    Unfortunately, due to the need to sell the lambs in order to pay for sample analysis for the project, we were not able to keep any lambs following the slaughter date. However, it would have been interesting to keep the ewe lambs and follow them for longer. Perhaps there would be less concern with the laborious nature of the inoculation process if results are very long lasting.
    Also, it would be interesting to observe the ewe lambs through to their first lambing and to note if there are any differences in inoculation animals in their reproductive efficiency and in their progeny's microbial populations.

    b) This research all leads to creating a more efficient ruminant and lowering the feed input needed for the same results. However, when looking at the blood results we seem to have had some interesting results with the inoculations on water movement and

retention. It would be relevant to see if microbial populations might be manipulated in order to create a more water efficient rumen using microbial populations.

c) Understanding the breed differences
- What effects are seen with pure cross inoculation from different breeds on production targets, growth and wool?
- Are there breed differences that are driven predominantly by microbial populations?
- Can we alter British breed sheep to be more effective under Australian conditions by inoculation with Australian breed microbiota? And does this affect wool growth?

7 Key messages

Results:

1) Pilot trial: This trial is the first to show that there are differences in microbial profiles induced by both genotype and diet in these breeds of commercially used sheep.
2) Inoculation of lambs with 10ml of mature ewe rumen fluid daily, for one week following birth, resulted in significant differences in rumen bacterial communities at all phylogenetic levels (phyla, class order, family, genus and species) to those of un-inoculated (Maternal treatment) lambs. These differences were evident in rumen samples at time of weaning (10 weeks of age) and in rumen and intestine samples at slaughter (18 weeks of age), from both lamb trials.
3) Efficiency and health of the inoculated animals (through assessment of growth and analysis of production, health and immune measures) appears to have been detrimentally affected by the inoculation.

This projects objectives were to:

a) Define the characteristics of the rumen microbiome of sheep of different breeds and with different growth characteristics,
   This objective has been met. We have highlighted the differences between breed and diet in sheep rumen populations. We hope to further pull apart any differences in these populations for publications.
b) Determine the extent to which phenotypic differences in growth and health can be attributed to differences in the maternally-derived microbiota,
   Our experiments show that there are significant changes to growth and health upon the manipulation of the microbiota which lambs are inoculated with.
c) Determine the extent to which the immune system is affected by early life manipulations,
   We have shown that manipulation of the rumen microbiota at birth does effect the immune system, through changes in ileac Payer's patch morphology and eosinophil counts.
d) Determine the potential for early-life microbiome manipulations for sheep and cattle.
   Our project is the first to create changes in lamb microbiota which lasted until slaughter at week 18. This shows that sustained changes (with production effects) can be created through manipulation of the neonatal ruminant microbiota.

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Appendix A**: Pilot trial ewe weights**

Breed	Pilot Treatment	Ewe ID	Start Weight	End weight
Merino	High Spec.	Y26	64	72
Merino	High Spec.	Y33	60.5	66
Merino	High Spec.	Y18	59.5	65.5
Merino	High Spec.	Y34	64.5	74.5
Merino	High Spec.	Y37	66.5	75.5

Merino	High Spec.	Y52	57.5	65.5
Merino	High Spec.	Y57	59.5	64
Merino	High Spec.	Y63	64.5	68
Merino	High Spec.	Y69	61	68.5
Merino	High Spec.	Y71	63.5	69.5
Merino	High Spec.	Y72	60	flighty, jumped from scales
Merino	High Spec.	Y75	60.5	66
Suffolk	High Spec.	B8	71.5	82
Suffolk	High Spec.	B10	82	84
Suffolk	High Spec.	B11	74	87.5
Suffolk	High Spec.	B12	79	80
Suffolk	High Spec.	B25	79.5	85
Suffolk	High Spec.	B31	74.5	81
Suffolk	High Spec.	B33	75.5	81.5
Suffolk	High Spec.	B45	80.5	81
Suffolk	High Spec.	B46	74.5	87.5
Suffolk	High Spec.	B68	78.5	84.5
Suffolk	High Spec.	B70	64	70
Suffolk	High Spec.	B71	71	75.5
Merino	Roughage	Y2	58.5	59
Merino	Roughage	Y17	58	64.5
Merino	Roughage	Y19	58.5	63.5
Merino	Roughage	Y23	64	67.5
Merino	Roughage	Y31	62.5	67
Merino	Roughage	Y39	58.5	61
Merino	Roughage	Y40	59	64
Merino	Roughage	Y53	62	64
Merino	Roughage	Y54	60	66
Merino	Roughage	Y56	60	63
Merino	Roughage	Y65	61	66
Merino	Roughage	Y68	63.5	68.5
Suffolk	Roughage	B1	80.05	83.5
Suffolk	Roughage	B4	76	76
Suffolk	Roughage	B14	80	87
Suffolk	Roughage	B15	74	68
Suffolk	Roughage	B17	78.5	86.5
Suffolk	Roughage	B19	79	78
Suffolk	Roughage	B30	80.5	78.5
Suffolk	Roughage	B38	74	65.5
Suffolk	Roughage	B39	79	79
Suffolk	Roughage	B53	81	76
Suffolk	Roughage	B58	73.5	77.5
Suffolk	Roughage	B69	71.5	61.5