

METABOLIC AND MICROBIAL IMPACTS ON REPRODUCTION IN LIVESTOCK

by

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A thesis submitted in partial fulfillment
of the requirements for the degree

of

Master of Science

in

Animal and Range Sciences

MONTANA STATE UNIVERSITY
Bozeman, Montana

April 2014

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DEDICATION

To my dear wife Colleen and daughter Anna.

ACKNOWLEDGEMENTS

I want to thank my wife Colleen Swartz for her sacrifices and encouragement as I pursued my education. I thank my daughter Anna for always being happy to see me even though I have not always been able to spend much time with her. In addition, I thank my parents, Jeff and Ruth Swartz, for giving me the education I needed to succeed in higher education, and for their encouragement in my pursuits. I also thank the other graduate students who have helped and encouraged me these couple of years. In addition, I would like to thank my major professor Dr. Carl Yeoman for giving me this opportunity. I have really appreciated your willingness to proofread, mentor, and provide whatever materials I needed to succeed. I would also like to thank the other members of my committee, Dr. Jennifer Thomson, Dr. Patrick Hatfield, and Dr. James Berardinelli, for the support and mentoring they have provided throughout my project and the thesis writing process. Support for this project was provided by the Montana Agricultural Experiment Station through project number MONB00113, and is a contributing project to Multistate Research Project, W-3177, Enhancing the Competitiveness of U.S. Beef through project number MONB00195. In addition, I want to thank Dr. Dennis Hallford, New Mexico State University, Las Cruces, NM for assisting in assaying samples, and Dr. Andrew Roberts and Dr. Thomas Geary of Fort Keogh, Miles City, MT for their assistance in collecting cow samples.

TABLE OF CONTENTS

1. INTRODUCTION	1
References Cited	5
2. LITERATURE REVIEW	8
Factors that Affect Reproduction in Domestic Sheep.....	8
Metabolites and Hormones	12
Vaginal Microbiota of Livestock	16
References Cited	27
3. TEMPORAL PATTERNS OF INTAKE, ENERGY-RELATED METABOLITES, METABOLIC HORMONES, PROGESTERONE CONCENTRATIONS, AND LAMBING RATES IN RAMBOUILLET EWES SELECTED FOR HIGH OR LOW REPRODUCTIVE RATE.....	34
Contribution of Authors and Co-Authors	34
Manuscript Information Page	36
Abstract	37
Introduction.....	39
Materials and Methods.....	40
Animals, Nutritional Management, Breeding.....	41
Blood Sampling and Assays	42
Normalization of Ewes and Determination of Cyclicity.....	44
GrowSafe Data Processing	44
Statistical Analysis.....	45
Results and Discussion	45
Literature Cited	57
4. THE VAGINAL MICROBIOTA OF EWES AND COWS ARE UNIQUE AND DISTINCT FROM HUMANS AND NON-HUMAN PRIMATES.....	59
Contribution of Authors and Co-Authors	59
Manuscript Information Page	61
Abstract	62
Introduction.....	63
Materials and Methods.....	65
Ethics Statement.....	65
Sampling	65

TABLE OF CONTENTS - CONTINUED

pH Analyses	66
Sequencing Microbiota	66
Data Analyses	67
Results	69
Sequencing Overview	69
α -Diversity	69
β -Diversity	70
General Taxonomic Compositional Traits	70
Livestock Vagina Maintains a Near-Neutral pH	72
Discussion	72
Acknowledgements	76
References	85
5. SHEEP VAGINAL MICROBIOTA EXHIBIT CORRELATIVE CHANGES WITH PROGESTERONE CONCENTRATIONS AND VAGINAL MUCUS SECRETIONS, BUT NOT REPRODUCTIVE SUCCESS	88
Contribution of Authors and Co-Authors	88
Manuscript Information Page	90
Abstract	91
Introduction	92
Materials and Methods	94
Ethics Statement	94
Sampling	94
Sequencing Microbiota	96
Data Analyses	97
Results	99
Sequencing Overview	99
Temporal Changes in α -Diversity of Ewe Vaginal Microbiota	99
Compositional Changes	100
Discussion	102
Supplementary Material	109
References	110
6. CONCLUSION	114
References Cited	118

TABLE OF CONTENTS - CONTINUED

REFERENCES CITED.....119

LIST OF TABLES

Table	Page
2.1 Bacteria that have been identified as occurring in the vaginal or uterine tract of lab animal and livestock hosts.....	25
3.1 Lambing rates, and least squares means for litter size, lamb birth weight (LBW), total LBW per ewe lambing, total intake, total TDN intake, and kg of TDN • total kg of lamb born-1 • ewe-1 of Rambouillet ewes selected for high (HL) and low (LL) reproductive rate.....	52
3.2 Least squares means of metabolic hormones and metabolites before gestation for Rambouillet ewe lines selected for high (HL; n = 20) and low (LL; n = 15) reproductive rate	53
3.3 Least squares means of metabolic hormones and metabolites during gestation for Rambouillet ewes selected for high (HL; n = 20) and low (LL; n = 15) reproductive rate	53
3.4 Least squares means of metabolic hormones and metabolites before gestation shown temporally for Rambouillet ewes selected for high (HL; n = 20) and low (LL; n = 15) reproductive rate	54
3.5 Least squares means of metabolic hormones and metabolites during gestation shown temporally for Rambouillet ewes selected for high (HL; n = 20) and low (LL; n = 15) reproductive rate	54
4.1 Pregnancy status of animals used in this trial	77
4.2 Sum of occurrence of Lactobacillus spp. identified in cow and ewe ectocervicovaginal lavages	78
4.S1 Mean Shannons diversity index values for cow, ewe, human, and non-human primate vaginal microbial communities.....	84
5.1 Ewe ear tag, line, age, weight at breeding, rams exposed to for breeding (Rambouillet/Suffolk ram), and number of lambs born from each ewe sampled by ectocervicovaginal lavage for vaginal microbiota.....	105
5.2 Shannon's Diversity Index values, and relationships between open and pregnant ewes and current and preceding time points within pregnancy status	106

LIST OF TABLES – CONTINUED

Table	Page
5.3 Vaginal microbial genera whose relative abundance correlates with systemic progesterone concentration. All reported correlative relationships were found to be significant ($p \leq 0.05$).....	107

LIST OF FIGURES

Figure	Page
3.1 Least squares means of thyroxine (T4) concentrations before gestation in Rambouillet ewes selected for high (HL; n = 20) and low (LL; n = 15) reproductive rate. Error bars represent \pm SE of each mean. Line by d of gestation interaction; P = 0.05. Means with different superscripts differ ($P \leq 0.05$).....	55
3.2 Least squares means of progesterone (P4) concentrations during gestation in Rambouillet ewes selected for high (HL; n = 20) and low (LL; n = 15) reproductive rate. Error bars represent \pm SE of each mean. Line by d of gestation interaction; P = 0.04. Means with different superscripts differ ($P \leq 0.05$).....	56
4.1 Shannon's diversity of livestock vaginal microbiota as compared to humans and non-human primates. Boxplots showing the median, quartiles and extremities of Shannon's diversity index values calculated for individual ewes, cows, humans, and non-human primates compared in this study.....	79
4.2 Bray-Curtis relationship among vaginal microbiota of livestock, humans, and non-human primates. Non-metric multi-dimensional scaling plot of Bray-Curtis dissimilarity measures of vaginal microbiota determined from individual ewes, cows, humans, and non-human primates compared in this study.....	80
4.3 Phylum-level composition among livestock vaginal microbiota. Bar chart showing the proportional distribution of the six most abundant phyla.....	81
4.4 Genus-level composition among ewe vaginal microbiota. Heat map showing the relative abundances of the most abundant genera identified in individual ewe vaginal microbiota. Color breaks in heatmap are adjusted to show genera seen at less than 1% (blue shades), 1-10% (white shades), and greater than 10% (red shades) proportionate abundance	82
4.5 Genus-level composition among cow vaginal microbiota. Heat map showing the relative abundances of the most abundant genera identified in individual ewe vaginal microbiota. Color breaks in heatmap are adjusted to show genera seen at less than 1% (blue shades), 1-10% (white shades), and greater than 10% (red shades) proportionate abundance	83

LIST OF FIGURES – CONTINUED

Figure	Page
5.1 A NMDS plot of open and pregnant ewe microbial communities using Bray-Curtis dissimilarity matrix. The ANOSIM provided weak support for clustering by ewe pregnancy status at time of sampling (pregnant or not pregnant; $R = 0.07$; $P \leq 0.05$) and day of trial ($R = 0.11$, $P \leq 0.05$), but not for final pregnancy outcome (pregnant, open, or aborted; $R < 0$, $P > 0.05$) of ewes	108
5.S1 Hierarchic clustering of samples on average bray-curtis dissimilarity. Dendrogram showing the average linkage clustering relationships among temporal samples of ewe vaginal microbiota.....	109

ABSTRACT

Variations exist in the reproductive outcomes of livestock animals impacting the financial sustainability of livestock operations. The objectives of this study were to determine if temporal variations in intake, metabolites, hormones, and/or the vaginal microbiota co-varied with reproductive outcomes using two lines of Rambouillet ewes selected for high or low reproductive rates. Ewes were fed from GrowSafe bunks and blood sera were collected before and during gestation. Sera were assayed for glucose, NEFA, cortisol, triiodothyronine, thyroxine, IGF-I, and progesterone concentrations. The vaginal tracts of Rambouillet ewes and crossbred cows were sampled by vaginal lavage. Microbes present in vaginal samples were determined by sequencing of their 16s ribosomal RNA gene and taxonomy was assigned by naïve Bayesian classification using the 16s rRNA gene database of SILVA. Average total intake did not differ ($P > 0.05$) between lines of ewes throughout the study. However, kg of TDN • total kg of lamb born⁻¹ • ewe⁻¹ was greater ($P \leq 0.05$) for LL ewes than for HL ewes. We observed a line and day of gestation effect for progesterone and thyroxine concentrations. Cow and ewe vaginal microbiota did not differ ($P > 0.05$), although cow microbiota exhibited greater ($P \leq 0.05$) diversity compared to ewe microbiota, and both differed ($P \leq 0.05$) from other described vaginal ecosystems. Temporal microbial compositions of open, pregnant, and aborted ewes did not vary by individual, by line, or with age. Microbial compositions did cluster by ewe pregnancy status at time of sampling and by the day of trial. Several microbial taxa were found to co-vary with mucus secretions, P4 concentrations, and pH. Rambouillet ewes that became pregnant and those that remained open did not exhibit significant differences in genera composition of their microbial communities. Future research is needed to determine if there are differences in species, strains, or metabolites produced by these communities.

INTRODUCTION

All livestock enterprises, directly or indirectly, rely on reproduction for their long-term production and economic sustainability. Although nutrition is an important factor in determining costs of maintaining a livestock enterprise, reproduction is the largest factor affecting profitability (Hess et al., 2005). In the U.S. sheep industry, profitability is primarily determined by the ratio of ewes mated to lambs weaned (Schoenian and Burfening, 1990). Failure to produce and deliver live young represents the single largest economic cost to the sheep and cattle production industries in the U.S. (Bellows et al. 2002; Camacho et al., 2012).

Bellows et al. (2002) attempted to determine the costs of reproductive disorders in U.S. beef and dairy cattle herds. Dystocia and infertility were found to be the most expensive disorders at \$220 million and \$137 million, respectively, for dairy cattle; and \$185 million and \$249 million, respectively, for beef cattle. The cost of abortions/stillbirths was \$27 million and \$64 million for the dairy and beef industries, respectively (Bellows et al., 2002). However, costs associated with decreased productivity associated with these disorders, such as reduced weaning weight and delayed breeding of beef cows after parturition, were not determined and were not included. Even so, the total costs of reproductive disorders in the U.S. were estimated to be \$473 to \$484 million for dairy cattle industry, and \$441 to \$502 million for the beef cattle industry in 2002 (Bellows et al., 2002).

Increased reproductive efficiency has clear implications for economics and sustainability of animal agriculture. Determining the origins and possible remediation of

decreased reproductive efficiency warrants thorough investigation. Mechanisms for increasing reproductive efficiency in sheep production systems include but are not limited to: 1) increasing opportunities for more offspring by increasing ovulation rates; and, 2) decreasing early embryonic loss and fetal survival (Willingham et al., 1986); 3) increasing litter size; 4) fertility; and, 5) growth rate of lambs (Bradford, 2002).

Genetic factors have been extensively studied with respect to reproduction in livestock. Genetic impacts on reproduction are multifactorial, and include genetic, transcriptional, translational, and epigenetic variation. Furthermore, reproductive traits are thought to have low heritabilities, which can limit genetic progress (Snowder and Fogarty, 2009). Conception rates and the numbers of lambs born per ewe lack uniformity across populations, and repeatability of these traits are quite low (Vinet et al., 2012).

Nutrition impacts reproduction by providing energy directly to the ewe and developing fetus, as well as modulating hormonal signals (for reviews, see, Robinson et al., 2006; Boland et al., 2000). Concentrations of metabolic and reproductive hormones, and metabolites are affected by plane of nutrition (Parr et al. 1987; Verbeek et al., 2012; Vonnahme et al., 2013). Nutrition also has direct effects on reproduction, affecting factors such as ovulation rate (Forcada and Abecia, 2006).

There are other, less studied, factors that can affect reproduction. It has been shown that the vaginal microbiota play an important role in the reproductive success and health of humans (Li et al., 2012). Bacteria that produce and secrete lactic-acid, known as lactobacilli, are largely responsible for maintaining homeostasis in the human vagina by

maintaining low vaginal pH (O'Hanlon et al., 2013). A common morbidity of reproductive age women is bacterial vaginosis (BV); the signs of which include elevated pH, clue cells, dis-colored discharge, and an amine odor (Li et al., 2012). The incidence of BV always occurs in association with a notable perturbation of the 'normal' vaginal bacterial community (Zhou et al., 2007). The condition of BV predisposes a pregnant woman to infectious morbidity, pre-term delivery, and powerfully predisposes her for mid-trimester miscarriage (Leitich and Kiss, 2007). Vaginal tract bacteria are the first colonizers of human neonates. It has been proposed that this initial colonization acts as a defense mechanism by limiting the growth of potentially pathogenic species until the development of the climax community (Dominguez-Bello et al., 2010). The passing of pathogenic vaginal bacteria to neonates during birth has been documented in sheep (Davies et al., 2003), which suggests that livestock neonates may have the ability to be colonized by commensal bacteria from the vaginal tract of their dams, similar to that observed in humans. Furthermore, the bacterial communities of the vagina in rats and sheep appear to play an important role in the sexual attractiveness of the female (Merkx et al., 1987; Ungerfeld and Silva, 2005) that could have an effect on the likelihood of mating. While there may be several contributing factors that determine the occurrence and relative abundance of bacterial communities in the vagina, vaginal pH appears to be of primary importance, and in humans, this appears to be associated with lactate producing lactobacilli (Ravel et al., 2011; Yeoman et al., 2013). Interestingly, canine and bovine sperm motility has been shown to be affected by intracellular pH and lactate in vitro (Carr et al., 1985). This implies that the vaginal microbiota may affect fertility

through its influence on viability and fertility of sperm cells. In addition, certain bacteria, present in the uterus during the puerperium period have been associated with major disease problems and resulting financial losses, especially in cattle (Sheldon et al., 2008).

While there is strong evidence for the importance of vaginal microbiota in humans, and there are known interactions between reproduction and the vaginal microbiota in livestock, research focusing on livestock vaginal microbiota to date has been limited. Furthermore, studies that have been performed on livestock species have been confounded by the use of culture-dependent methods for bacterial identification and quantification. Analyses of communities previously described by culture-based techniques using culture-independent molecular approaches have revealed that culture-based methods of microbial identification can exclude the vast majority of microbiota present in these communities (Hugenholtz et al., 1998; White et al., 2011).

Thus the present study was undertaken to evaluate whether nutrient intake, systemic, energy-related metabolites and metabolic hormones, and progesterone concentrations differ between Rambouillet ewes that had been selected for high and low reproductive rate, and if variations in these variables are associated with reproductive failures among these ewes. Likewise, an additional aim of this study was to examine the vaginal microbiota of sheep and cattle with culture-independent, second generation, 16s rRNA gene sequencing to more thoroughly determine species representation, and their potential interaction with reproduction in agriculturally important species.

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LITERATURE REVIEW

Factors that Affect Reproduction in Domestic Sheep

Sheep are primarily seasonal breeders, exhibiting the greatest sexual activity during fall and winter when days are short (Senger, 2012a). It has been suggested this provides offspring with more advantageous environmental conditions, including nutrition to assist in their growth, development, and survival (Forcada and Abecia, 2006). The duration of the anestrus or non-breeding season in the inter-mountain region of Montana is from March through August (Tulley and Burfening, 1983); although, in Mediterranean-type environments it is possible for ewes that have adequate body condition to exhibit estrous activity throughout the year (Forcada and Abecia, 2006). Estrous cycles in ewes are typically 16 to 18 days in length (Forcada and Abecia, 2006).

Genetic factors affect reproductive rate. One factor is the *FecB* gene that has been shown to increase litter size and total lamb weights per ewe from birth through 12 months of age in heterozygous carrier ewes compared to ewes that lack a *FecB* allele (Mishra et al., 2009). In 1968, a selection experiment for reproductive rate was begun in Rambouillet sheep at the Montana Agricultural Experiment Station, Bozeman, MT. Development of these flocks has been described previously (Schoenian and Burfening, 1990; Burfening et al., 1993). Briefly, a flock of random-bred Rambouillet ewes were stratified by age into two breeding groups based on the following selection index (SI) formula: $SI = \frac{\text{total lambs born in lifetime}}{\text{age of ewe} - 1}$. Thereafter, ewes were bred for a reproductive rate that was either high (high line) or low (low line) using the selection index. The flocks were

closed after the assignment of the four highest and lowest selection index value rams to the high line and low line ewes, respectively. A control line was formed in 1972 that comprised the foundation ewes from both lines along with eight randomly selected rams from the high and low lines. Rams were used to breed ewes for one season only, and ewes had the opportunity to lamb the first time at 2 years of age. Ewes were culled if they had bodily ailments, did not produce a lamb in 2 consecutive years, or were 6-years-old (Schoenian and Burfening, 1990). The use of this SI caused a profound difference in reproductive rate between these lines of ewes, indicating a genetic change in reproductive processes between ewes of these lines (Burfening et al., 1993).

Various studies have investigated the impact of this selection index on physiological variables related to reproduction in these high and low line sheep. Two-year-old low line rams were shown to have a greater scrotal circumference than high line rams (Tulley and Burfening, 1983); however, high line ram lambs had a greater scrotal circumference than low line ram lambs from the end of September to mid-January; a period of the year that corresponds to the breeding season for ewe in these lines (Burfening and Rossi, 1992). In both of the aforementioned studies, the authors reported that high line rams performed a greater number of mounts and services using serving capacity tests than low line rams. Furthermore, high line rams (22 months of age) had more mounts and ejaculations using artificial vaginas than low line rams, although no difference was detected 6 years later, when 18- to 24-month-old rams were used in serving capacity tests, nor was there a difference between high and low line rams in sexual preference performance tests (Stellflug and Berardinelli, 2002).

Schoenian and Burfening (1990) reported that the rate of lambing and size of litters were greater in high line ewes than low line ewes. The authors attributed this observation to increased numbers of ovulations between ewes of this line, even though high line ewes also had a lower rate of those ovulations that developed into fetuses than low line ewes (Schoenian and Burfening, 1990). Selection for high or low reproductive rate in these lines has resulted in divergent breeding values between lines (Burfening et al., 1993). Berardinelli et al. (1995) found that high line ewes had greater systemic progesterone concentrations in daily samples during the diestrus phase of the estrous cycle than low line ewes. However, ovarian concentrations of progesterone, total corpora lutea (CL) mass, mean CL weight, and IGF-I and hCG binding capacity per mg CL did not differ between high and low line ewes (Berardinelli et al., 1995). The authors speculated that higher systemic progesterone during the estrous cycle could be due to differences in catabolism of progesterone or binding of progesterone to serum proteins in the blood between high line and low line ewes. Thus, selection, using the aforementioned index, in these Rambouillet sheep has resulted in phenotypically different reproductive rates. However, reproductive traits generally have low heritability, and no gene or genes have been found to control all major aspects of reproduction (Snowder and Fogarty, 2009).

It is well established that nutrition affects reproduction in sheep, often through effects on follicular development and ovulation rate rather than sexual behavior (for review, see Forcada and Abecia, 2006). Regulation of intake in sheep seems to be related to digestive tract capacity, and to fluctuate in relation to metabolic body weight (Blaxter

et al., 1961). Appetite increases in cold temperature, with increased reticulum passage, and decreased dry matter digestibility (Westra and Christopherson, 1976). Body condition and effort required to obtain feed has been described as affecting intake. Ewes with higher body condition exhibit lower intake when even modest increases in effort are required to obtain feed (Verbeek et al., 2012).

Nutrition is known to affect reproductive homeostasis through its influence on hormonal regulatory pathways involved in the reproductive process. One aspect of nutrition that may impact reproduction is its apparent effect on systemic progesterone concentrations. Progesterone has many functions in reproduction, including regulation of estrous cycle length, preparation of the uterus to allow early embryonic development, support and continuation of pregnancy, and preparation of mammary glands for lactation (Senger, 2012b). Increased intake of nutrients has been found to reduce systemic progesterone concentrations (Parr et al., 1987; Parr et al., 1993; Vonnahme et al., 2013). Data from ovariectomized ewes indicates that increased nutrient intake increases metabolic clearance rate of progesterone (Parr et al., 1993). Lowered peripheral progesterone concentrations resulting from intake above calculated maintenance requirements after breeding decreased pregnancy rates unless exogenous progesterone was supplied to the ewe (Parr et al., 1987). Sarda et al. (1973) evaluated progesterone concentrations during estrus and anestrus in intact ewes, and in ovariectomized ewes. The results indicated progesterone during estrus is from a non-ovarian source. In pregnant ewes these authors reported that progesterone concentrations remained relatively constant during the first-trimester of pregnancy, similar to peak progesterone

concentration during the estrous cycle, but they indicated that concentrations as low as 1 mg/mL were able to sustain pregnancy. Between days 80 to 90 of gestation, concentrations of progesterone increased dramatically (Sarda et al., 1973). Placental input of progesterone increases around day 50 of gestation in ewes (Arck et al, 2007). Weight, number, and sex of fetus are not correlated with progesterone concentrations (Sarda et al., 1973).

Besides its essential role in sustaining pregnancy, progesterone modifies the immune system (Arck et al, 2007). Stress has a negative effect on progesterone via the hypothalamic-pituitary-adrenal axis, with gonadotropin releasing hormone negatively affected by corticotrophin releasing hormone, and luteinizing hormone negatively affected by glucocorticoids (Arck et al, 2007).

Metabolites and Hormones

Nutritional metabolites and metabolic hormones are essential to regulation of homeostasis and reproduction. Glucose plays pivotal roles in reproduction. It is a main source of energy used by the fetus for energy and metabolism (Bell and Bauman, 1997; Hay et al., 1983). Fetal utilization rate of glucose compared to maternal utilization remains high, even when the dam is hypoglycemic (Hay et al., 1983); in other words the fetus draws glucose from the maternal circulation at the expense of the dam (Bell and Bauman, 1997). Furthermore, the placenta itself utilizes glucose and as such is a major drain on the maternal supply of glucose, and after birth glucose is a major precursor for production of milk to sustain the neonate (Bell and Bauman, 1997). Thus, disorders in

energy metabolism, such as ketosis, can occur during pregnancy and lactation (Bell and Bauman, 1997).

Ruminants absorb only small amounts of glucose directly from the digestive tract, as dietary glucose is rapidly converted to short-chain fatty acids by the microbiota that reside in the rumen (Bergman, 1963). Instead, glucose available for endogenous metabolism of the ruminant is primarily derived through gluconeogenesis (Bergman, 1963; Bell and Bauman, 1997), that predominantly occurs in the liver (Bell and Bauman, 1997). Adaptions to glucose utilization by the dam during pregnancy or lactation include changes in insulin sensitivity of muscle and adipose tissues, and a concurrent increase in potential for placental glucose transport, allowing for adequate fetal supply (Bell and Bauman, 1997). In late gestation, lipolysis and increased NEFA concentrations appear to be related to decreased insulin concentrations (Moallem et al., 2011). Adipose cells release NEFA, which are oxidized in the liver; these compounds are not metabolized by the fetus and can result in fetal ketosis (Moallem et al., 2011). During gestation in ewes, glucose (Verbeek et al., 2012) and insulin concentrations increase, while NEFA concentrations decrease with increased nutrient intake (Vonnahme et al., 2013). Concentrations of NEFA more than doubled from early to late gestation in ewes fed to NRC estimated nutrient requirements, while those at 140% NRC requirements remained relatively static (Vonnahme et al., 2013).

Cortisol is an important glucocorticoid that has many functions related to maintenance of homeostasis in the body, affecting metabolism and stress response (Akers and Denbow, 2008). Cortisol release is related to resting rhythms, photoperiod, nursing of

young, and stress (Brunet and Sebastian, 1991). Epinephrine, glucose, and lactate increase along with cortisol in response to stress (Apple et al., 1995). Brunet and Sebastian (1991) reported with Manchega ewes in Spain that cortisol concentrations did not differ with photoperiod or reproductive status. Ewes fed above NRC nutritional requirements seem to have higher cortisol concentrations than ewes fed at or below NRC requirements (Verbeek et al., 2012; Vonnahme et al., 2013).

The thyroid gland and its hormones are essential for maintaining and modulating basal metabolic rate (Blaxter et al., 1949; Villanueva et al., 2013), and can change mitochondrial rate of respiration, as well as affect maintenance, proliferation, and development of cells (Villanueva et al., 2013). Triiodothyronine (T3) and thyroxine (T4) regulate metabolism and growth through both genomic and non-genomic mechanisms (Villanueva et al., 2013). Normal maturation of the central nervous system requires thyroid hormones, and when deficient during key periods, the effects are usually permanent (Dussault and Ruel, 1987). Removing the thyroid stunts growth, the extent of this effect is dependent on the age of the animal when these hormones are no longer available (Blaxter et al., 1949). Low levels of hyperthyroidism may increase growth rate of young animals, although high levels generally result in weight loss (Blaxter et al., 1949).

Fluctuations in temperature affect systemic concentrations of thyroid hormones. It has been shown in sheep that blood concentrations of T3 and T4 increase during cold weather, which has been associated with increased oxygen consumption (Westra and Christopherson, 1976). Blaxter et al. (1949) noted that feeding iodinated protein to

stimulate the thyroid of cows at amounts tolerated in cool climates causes distress in cows maintained in warmer climates. Pregnant ewes do not differ in thyroid hormone secretion rates from non-pregnant ewes, but greater thyroid hormone secretion has been reported during the winter months and lactation, as well as differences during part of the year between ages and breeds of ewes (Henneman et al., 1955). Acute stress decreases systemic thyroid stimulating hormone, T3, and T4, although T4 can increase under mild stress (Helmreich and Tylee et al., 2011). Concentrations of T3 and T4 in pregnant ewes are greater if nutrient intake is increased during gestation (Vonnahme et al., 2013).

Thyroid hormones also affect reproduction. Low concentrations of thyroid hormone have been associated with infertility, miscarriage, and ovulatory dysfunction in humans (Poppe et al., 2007; Poppe et al., 2008). Thyroid hormones affect steroid hormone formation (Poppe et al., 2007), and feeding small amounts of iodinated protein to bulls has improved libido, although probably as a result of correcting hypothyroidism (Blaxter et al., 1949). Hypothyroidism also decreases milk production by females (Blaxter et al., 1949).

The functions of IGF-I are very broad. While the liver is generally considered the main source of IGF-I (Liu et al., 1998; Vijayakumar et al., 2011), it appears that systemic IGF-I may be derived heavily from other sources as well (Liu et al., 1998).

Concentrations of IGF-I influence growth of the placenta and may control maternal nutritional effects on the fetus (for review, see Igwebuike, 2010). Signal pathways involving IGF-I, along with growth hormone, glucocorticoids, and T3, appear to regulate skeletal bone growth (for review, see Robson et al., 2002). Concentrations of IGF-I are

also thought to increase during obesity, and possibly contribute to the condition in humans (Vijayakumar et al., 2011). Stunting or death results when the *igf-1* gene is knocked down or out (Liu et al., 1998). Concentrations of IGF-I increase during gestation (Verbeek et al., 2012; Ward et al., 2008) and with increasing nutrient intake in gestating ewes (Verbeek et al., 2012).

Vaginal Microbiota of Livestock

The vaginal microbial ecosystem (microbiota) has been shown to be important in reproductive success of humans. Dysbiotic vaginal microbiota are associated with pre-term birth and mid-trimester miscarriage in women (Leitich and Kiss, 2007). The typical, healthy vaginal microbiota of a reproductive-age woman is dominated by *Lactobacillus spp.* (Ravel et al., 2011). Lactobacilli ferment glycogen, producing large amounts of lactate that creates a highly acidic vaginal environment (Wylie and Henderson, 1969). Commonly, a healthy vaginal microbiota promotes a pH < 4.5. Ravel and colleagues (2011) profiled the 16s rRNA gene content of approximately 400 reproductive age women and identified five definable biotypes that vary in prevalence among ethnic groups. These vaginal biotypes are defined by their dominating bacterial species, which was found to be either *L. crispatus*, *L. gasseri*, *L. iners*, *L. jensenii*, or alternatively by non-*Lactobacillus ssp.*, such as *Atopobium*, *Dialister*, *Megasphaera*, *Prevotella*, and *Sneathia* (Ravel et al., 2011). At birth, Caesarean section and conventionally born human neonates are colonized by skin and vaginal bacteria, respectively (Dominguez-Bello et al., 2010). Infants colonized by vaginal bacteria may be better protected from

colonization by pathogenic bacteria (Dominguez-Bello et al., 2010). A shift in neonatal bacterial community structure, especially of the immunologically important gastrointestinal tract, could possibly affect long-term immune status and predispose these individuals to disease (Neu and Rushing, 2011).

Vaginal microbial communities have been described in several species. In rabbits, *Micrococcus spp.*, *Staphylococcus epidermidis*, and *Staphylococcus spp.* were isolated from vaginal swabs in at least 10 of 35 rabbits (Jacques et al., 1986). *Bacteroidaceae* and streptococci have also been isolated from rabbit vaginas (Noguchi et al., 2003). The most prevalent vaginal bacteria in mice are streptococci, while hamsters are dominated by Gram-negative rods, Gram-positive anaerobic cocci, and *Bacteroidaceae*, and rats by streptococci, *Bacteroidaceae*, and Gram-negative rods (Noguchi et al., 2003).

The vaginal microbiota of the female dog (bitch) has been most widely reported to consist of *Streptococcus spp.* (Laurusevicius et al., 2008; Gunay et al., 2010; Feyen et al., 2004; Noguchi et al., 2003), *Escherichia coli* (Laurusevicius et al., 2008; Gunay et al., 2010; Feyen et al., 2004) and *Staphylococcus spp.* (Feyen et al., 2004; Laurusevicius et al., 2008). Laurusevicius et al. (2008) noted that older bitches had more bacterial isolates, and Feyen et al. (2004) reported that kennels that reported high conception rates in bitches had greater rates of *E. coli* and cultures with over five morphologies.

While less is known about the vaginal microbiota of livestock animals and their potential interactions with neonates, livestock vaginal microbiota are reported to affect reproduction in animals. The vaginal microbiota of the bitch, ewe, and cow can contain opportunistic pathogens that under the right circumstances can cause infection and

reproductive harm (Laurusevicius et al., 2008; Moorthy and Singh, 1982; Amin et al., 1996 respectively). Bacteria can cause a nonspecific infection of the reproductive tract that results in infertility in cattle (Panangala et al., 1978). Additionally, vaginal microflora of sheep and rat appears to affect attractiveness of females to male conspecifics (Ungerfeld and Silva, 2005; Merckx et al., 1987 respectively). Lastly, Gatti et al. (2012) speculated that decreased sexual attractiveness of ewes from insertion of intravaginal sponges for synchronization of estrus is likely due to changes in the vaginal microbiota.

Mice, rats, and hamsters have the highest vaginal microbial populations during estrus (Noguchi et al., 2003). This has also been observed in bitches, with the highest bacterial populations occurring during estrus and proestrus (Laurusevicius et al., 2008; Gunay et al., 2010; Feyen et al., 2004; Noguchi et al., 2003). However, no bacteria could be associated with a specific stage of the estrous cycle of the bitch (Feyen et al., 2004). Vaginal bacterial populations, including lactobacilli, have been reported to be higher during estrus in cattle (Otero et al., 1999), and lactobacilli populations have been shown to increase with sexual maturity in growing heifers (Otero et al., 2000). Amin et al. (1996) reported greater total bacterial numbers in cows than in heifers. This observation is possibly due to breeding or sexual maturation, although these authors were not able to relate the populations to stage of estrus.

Vaginal microbial species have also been reported in goats. *Bacillus spp.*, *E. coli*, and coagulase negative staphylococci have been suggested as normal aerobic bacteria of the vagina in Sahelian does of Nigeria (Bukar-Kolo et al., 2007). *Staphylococcus spp.*

and *Streptococcus spp.* were the predominant isolates in Nigerian Savanna Brown does, while *E. coli* was only found twice (Fasanya et al., 1987). Oliveira et al. (2013), in a study involving estrus synchronization with intravaginal sponges, reported that *Staphylococcus spp.* were most abundant at sponge insertion, and *E. coli* most abundant at sponge withdrawal. A similar study reported that *Bacillus spp.* were more abundant at intravaginal device insertion, and *Arcanobacterium pyogenes* more abundant at withdrawal (Manes et al., 2013). *E. coli*, *Arcanobacterium pyogenes*, and *S. aureus* have been isolated from does after kidding (Ababneh and Degefa, 2006).

Uterine bacteria of postpartum cows, even in the absence of morbidity, inhabit the uterus soon after parturition; however, most are cleared within 14 days after birth (Hussain et al., 1990). *E. coli*, *Clostridium spp.*, *Proteus spp.*, and streptococci have been described in one study as the most common bacteria isolated in healthy dairy cattle with and without retained placenta using culturing methods (Hussain et al., 1990). Santos et al. (2011) sequenced 16s rRNA-pooled clone libraries of metritic and non-metritic uterine lavages of dairy cattle from two farms. Bacteroidetes, Firmicutes, Fusobacteria, Gamaproteobacteria, Tenericutes, and uncultured bacteria formed the major classifications of bacteria isolated. Gammaproteobacteria were most prevalent in non-metritic Holstein cows, while Fusobacteria were most prevalent in metritic Holstein cows (Santos et al., 2011). Machado et al. (2012) found the phyla Firmicutes, Bacteroidetes, Proteobacteria, Tenericutes, Actinobacteria, Spirochaetes, as well as bacteria from the genera *Bacillariophyta* and TM7 *genera incertae sedis*, in 97 Holstein uterine lavages using 16s rRNA sequencing. These authors also reported that *Lactobacillus spp.* had a

decreased predominance, whereas, *Bacteroides spp.*, *Fusobacterium spp.*, *Trueperella spp.*, and *Ureaplasma spp.* had increased predominance in non-pregnant, milked cows 200 days after calving.

Several studies have investigated the bovine vaginal tract. Criolla heifers were sampled from 7 months of age to just before breeding, and Enterobacteriaceae, enterococci, staphylococci, and lactobacilli were isolated, including α -haemolytic *Streptococcus*, *E. coli*, and coagulase-negative *Staphylococcus* (Otero et al., 2000). Enterococci, mainly *E. cecorum*, *E. faecalis*, *E. munafi*, and *E. pseudoavium*, as well as low amounts of lactobacilli, mainly *L. buchnerii* and *L. brevis*, have been isolated from Hereford-Nelore heifers throughout a synchronized estrous cycle (Otero et al., 1999). *Bacillus spp.*, *C. bovis*, coagulase-negative *Staphylococcus*, and *Streptococcus spp.* have been reported to occur during all phases of reproductive development and maturity in cattle of Sahel, Nigeria (Amin et al., 1996). *Arcanobacterium pyogenes*, *Staphylococcus epidermidis*, and *Staphylococcus aureus* were the most prevalent bacteria isolated from healthy, lactating Criollo X Limonero cows (Zambrano-Nava et al., 2011). *Enterococcus*, *E. coli*, *Lactobacillus*, and *Pediococcus* have been reported to occur in metritic and non-metritic Holstein cattle (Wang et al., 2013). *Lactobacillus* numbers remained similar before and after calving in Holsteins with metritis, while other bacteria counts increased by 3 log units, mainly due to *E. coli* and Enterobacteriaceae (Wang et al., 2013). Panangala et al. (1978) reported that repeat breeder and normal, fertile cows from Ontario, Canada had Bacillaceae, *Corynebacterium*, Enterobacteriaceae, Micrococcaceae, and Streptococaceae vaginal bacteria. While present in both classifications of cattle,

Corynebacterium pyogenes and Enterobacteriaceae were present in greater numbers in repeat breeder cows. *Bacillus subtilis*, *Haemophilus somnus*, and *Staphylococcus aureus* were the only isolates found in repeat breeders but not in fertile cows. These authors reported the presence of mycotic flora (predominately *Candida krusei*), and mycoplasmas consisting almost exclusively of *Mycoplasma bovis* or T-strain mycoplasmas (Panangala et al., 1978). Petit et al. (2009) reported the presence of fungi in vaginal tracts of healthy cows, and they did not consider *Bacillus* normal colonizers of the vaginal tract in Austrian cattle.

Rodríguez et al. (2011) found differences in *Lactobacillus* groups between 3-year-old, healthy dairy and beef cows. Beef cows had twice as many strains that produced hydrogen peroxide as dairy cows, as well as all of the most hydrophobic strains. *L. plantarum* and *L. acidophilus* were the two most often isolated strains of *Lactobacillus* spp., although they suggested that the numbers of lactobacilli present in the isolates were low and that they have a restricted role in the vaginal tract (Rodríguez et al., 2011). While concurring with Otero et al. (1999; 2000) that Lactobacilli populations of the vagina are low, Rodríguez et al. (2011) suggested that beef and dairy cows differ in their bacterial populations.

Panangala et al. (1978) concluded that there is a relatively consistent microbial population between repeat breeder and fertile cattle that make up a typical community. However, Wang et al. (2013) suggested, due to inconsistent species composition and minimal bacteria present in the mucus of the vaginal tract, that there is no inherent, healthy microbiome of the bovine vagina. The presence of a normal, healthy microbiome

of livestock vaginal tracts remains to be determined. These studies suggest that the cow vagina is often colonized by enterococci, staphylococci, and streptococci. While lactobacilli are present in low numbers, there may be differences between those colonizing dairy and beef cows. This may indicate that there could be other differences between breeds.

There does not appear to be the same recent interest in lactobacilli and probiotics in sheep as in cattle. Culture-based methods have not revealed strict anaerobes in reproductive tracts of the ewe (Quinlivan, 1970; Adams, 1975; and Sawyer, 1977). Adams (1975) found *Corynebacterium*, *Bacillus*, *Streptococci*, and *Escherichia* in the uteri of slaughtered Merino ewes. Quinlivan (1970) considered the bacteria present at the second estrous in ewes, after sponge removal for estrus synchronization, relatively typical; chiefly composed of various corynebacteria, streptococci, staphylococci, *E. coli*, and micrococci. Sawyer (1977) reported *Bacillus spp.*, *Corynebacterium spp.*, *Achromobacter spp.*, and *Alcaligenes spp.* in lavages of the *os cervix* of Merino ewes. It was also noted that populations remained low and relatively constant over the estrus cycle (Sawyer, 1977). *Bacillus spp.*, Coagulase negative staphylococci, *Corynebacterium pyogenes*, and *E. coli* have been reported in vaginal tracts of ewes from England (Al-Amin, 1988). This author also reported that two ewes that exhibited vaginal discharge had *E. coli* alone and in conjunction with *Bacillus spp.* and *C. pyogenes*. *Escherichia*, *Corynebacterium*, *Staphylococcus*, and *Streptococcus* have been reported as most prevalent bacteria in the vaginas of Indian sheep (Moorthy and Singh, 1982). Inserting sponges into the vaginal tract for synchronization of estrus increased bacterial numbers in

the vagina of ewes (Suarez et al., 2006; Martins et al., 2009; Gatti et al., 2011). In a study investigating estrus synchronization with sponges in the vagina for 7 or 14 days, it was determined that most bacteria were gram positive *Bacillus spp.*, *Corynebacterium spp.*, and *Staphylococcus spp.* at sponge insertion, but were predominately gram negative Enterobacteriaceae when sponges were removed (Manes et al., 2010). At breeding, 82% of 7-day treated and 40% of 14-day treated animals had Gram-negative bacteria, which did not affect pregnancy rate or vaginal pH (pH ranged from 7.2 to 7.6 before treatment, and from 6.8 to 7.4 at breeding; Manes et al., 2010). Suarez et al. (2006) reported that vaginal pH ranged 7.3 to 7.5, 5 to 13 days after sponge removal. Based on these studies, it would appear that *Bacillus spp.*, *Cornyebacterium spp.*, *Escherichia spp.*, *Staphylococcus spp.*, and *Streptococcus spp.* are common isolates of the ewe vaginal tract. Table 1 is a compilation of bacterial species that have been reported to occur in the reproductive tract of the aforementioned host species or breeds.

While studies to date have mostly exploited culture-based analyses of bacterial populations of the vagina of livestock species, there is a lack of DNA sequence-based data on these microbial communities. Culture of many species of bacteria is difficult or impossible, and results are generally biased towards what grows or performs best in the culture medium used to induce bacterial production. Because of this, culture-based methods are known to often overlook abundant phylotypes in microbial communities revealed by more sensitive rRNA gene sequencing methods (Hugenholtz et al., 1998). In addition, the importance of the metabolic ability of bacteria is proving important, as the same species can differ in virulence and metabolic capability (White et al., 2011). Since

studies of sheep and cattle vaginal microbiota have historically relied on culture-based methods, the true major bacterial components of these ecosystems, and their metabolic function, may have escaped documentation.

In summary, reproduction in mammals has many factors that can either individually or interactively, affect reproductive success. It is well established that the biological and physiological processes of reproduction are affected by nutrition and genetics. Furthermore, at least in humans, it is now known that the microbiota colonizing the vagina impact reproductive success. Given the significant impact of reproduction on the financial sustainability of livestock production, it is important to establish if the vaginal microbiota of livestock also play important roles in their reproductive success or failure.

Table 1. Bacteria that have been identified as occurring in the vaginal or uterine tract of lab animal and livestock hosts.

Host Species	Bacterial Species	Reference
Mice Vagina	streptococci	Noguchi et al., 2003
Hamster Vagina	<i>Bacteroidaceae</i> Gram-negative rods, Gram-positive anaerobic cocci	Noguchi et al., 2003
Rat Vagina	<i>Bacteroidaceae</i> , Gram-negative rods, streptococci	Noguchi et al., 2003
Rabbit Vagina	<i>Bacteroidaceae</i> , <i>Micrococcus spp.</i> , <i>Staphylococcus spp.</i> , <i>Staphylococcus epidermidis</i> , streptococci	Jacques et al., 1986 Noguchi et al., 2003
Dog Vagina	<i>Escherichia coli</i> , <i>Staphylococcus spp.</i> , <i>Streptococcus spp.</i>	Feyen et al., 2004 Gunay et al., 2010 Laurusevicius et al., 2008 Noguchi et al., 2003
Goat Vagina	<i>Arcanobacterium pyogenes</i> , <i>Bacillus spp.</i> , <i>E. coli</i> , coagulase negative staphylococci, <i>Staphylococcus spp.</i> , <i>Streptococcus spp.</i> , <i>S. aureus</i>	Ababneh and Degefa, 2006 Bukar-Kolo et al., 2007 Fasanya et al., 1987 Manes et al., 2013 Oliveira et al., 2013
Cattle Uteri	Actinobacteria, <i>Bacteroidetes</i> , <i>Clostridium spp.</i> , <i>E. coli</i> , Firmicutes, Fusobacteria, Gamaproteobacteria, Proteobacteria, <i>Proteus spp.</i> , Spirochaetes, streptococci, Tenericutes, uncultured bacteria,	Hussain et al., 1990 Machado et al., 2012 Santos et al., 2011

Table 1. Continued. Bacteria that have been identified as occurring in the vaginal or uterine tract of lab animal and livestock hosts.

Host Species	Bacterial Species	Reference
Cattle Vagina	<i>Arcanobacterium pyogenes</i> , Bacillaceae, <i>Bacillus spp.</i> , <i>Corynebacterium</i> , <i>C. bovis</i> , Enterobacteriaceae, <i>Enterococcus</i> , <i>E. cecorum</i> , <i>E. coli</i> , <i>E. faecalis</i> , <i>E. munafi</i> , <i>E. pseudoavium</i> , <i>Lactobacillus</i> , Micrococcaceae, staphylococci, coagulase-negative <i>Staphylococcus</i> , <i>Streptococcus spp.</i> , α -haemolytic <i>Streptococcus</i> , <i>Staphylococcus aureus</i> , <i>Staphylococcus epidermidis</i> , <i>Pediococcus</i>	Amin et al., 1996 Otero et al., 2000 Otero et al., 1999 Panangala et al., 1978 Wang et al., 2013 Zambrano-Nava et al., 2011
Sheep Uteri	<i>Bacillus</i> , <i>Corynebacterium</i> , <i>Escherichia</i> , <i>Streptococci</i>	Adams, 1975
Sheep Vagina	<i>Achromobacter spp.</i> , <i>Alcaligenes spp.</i> , <i>Bacillus spp.</i> , <i>Corynebacterium</i> , <i>Corynebacterium</i> <i>pyogenes</i> , <i>Escherichia</i> , <i>E. coli</i> , micrococci, <i>Staphylococcus</i> , Coagulase negative staphylococci, <i>Streptococcus</i>	Al-Amin, 1988 Manes et al., 2010 Moorthy and Singh, 1982 Quinlivan, 1970 Sawyer, 1977

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CHAPTER THREE

TEMPORAL PATTERNS OF INTAKE, ENERGY-RELATED METABOLITES,
METABOLIC HORMONES, PROGESTERONE CONCENTRATIONS, AND
LAMBING RATES IN RAMBOUILLET EWES SELECTED FOR HIGH
AND LOW REPRODUCTIVE RATE

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Manuscript Information Page

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Journal of Animal Science

Status of Manuscript:

Prepared for submission to a peer-reviewed journal

Officially submitted to a peer-review journal

Accepted by a peer-reviewed journal

Published in a peer-reviewed journal

American Society of Animal Science

Temporal patterns of intake, energy-related metabolites, metabolic hormones, progesterone concentrations, and lambing rates in Rambouillet ewes selected for high and low reproductive rate¹

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ABSTRACT: The objectives of this study were to evaluate temporal patterns of intake, metabolites, metabolic hormones, and progesterone concentrations before and during gestation, and lambing traits in Rambouillet ewes from lines selected for high (HL) and low (LL) reproductive rates. Lines of ewes had been selected for reproductive rate for over 44 yr. Twenty-seven HL and 22 LL ewes were co-mingled and fed from GrowSafe bunks to monitor intake. Diet consisted of chopped-grass hay during maintenance, chopped-alfalfa hay during breeding and gestation, and pelleted feed during late gestation. Ewes were single-sire mated within line over an 18-d breeding season in November, followed by 18-d of exposure to Suffolk rams. Jugular venous blood samples were collected from each ewe 22 and 8 d before introduction of rams, within 24 to 48 h of being marked by rams with brisket paint, and on d 30, 60, 90, and 120 of gestation. Lambing rates and litter size (number lambs per ewe) were obtained at lambing. Body weight of each lamb was recorded at birth and before suckling. Serum samples from ewes were assayed for glucose (GLUC), NEFA, cortisol, triiodothyronine (T3), thyroxine (T4), IGF-I, and progesterone (P4) concentrations. Temporal patterns of metabolites, metabolic hormones, and P4 concentrations for 20 HL and 15 LL ewes were normalized to lambing date within \pm 1-wk period of the sampling day. All ewes had at least 1 estrous cycle

before introduction of rams. Lambing rate did not differ ($P > 0.05$) between HL (92.6%) and LL (77.3%) ewes. Litter size was greater ($P \leq 0.05$) in HL ewes than in LL ewes. Even though HL ewes had greater ($P \leq 0.05$) total birth weight per ewe than LL ewes, lambs from LL ewes were heavier ($P \leq 0.05$) than those of HL ewes. Temporal concentration patterns of GLUC, NEFA, cortisol, T3, T4, and IGF-I did not differ ($P > 0.05$) before or during gestation between HL and LL ewes. There was a line by d interaction ($P \leq 0.05$) for T3:T4 ratios before gestation, that was not observed during gestation, due to greater decrease in the T3:T4 ratio 8 d before mating in LL ewes. Concentrations of NEFA, T3, and IGF-I did not differ ($P > 0.05$) on d -22, -8 or at mating. However, GLUC and cortisol concentrations of ewes decreased ($P \leq 0.05$) from d -22 to d -8; whereas, T3:T4 ratio increased ($P \leq 0.05$) between these two days. Cortisol and NEFA concentrations did not differ ($P > 0.05$) among d 30, 60, 90, and 120 of gestation. Glucose, T3, T4, and IGF-I concentrations increased ($P \leq 0.05$) between d 90 and 120; whereas, T3:T4 ratios increased ($P \leq 0.05$) by d 90 and decreased ($P \leq 0.05$) by d 120. There was an interaction ($P \leq 0.05$) of line and d of gestation for P4 concentrations. P4 increased to a greater concentration from d 60 to d 120 in HL ewes than in LL ewes over this period. Total intake did not differ ($P > 0.05$) between HL and LL ewes from breeding through gestation. However, kg of TDN • total kg of lamb born⁻¹ • ewe⁻¹ was greater for LL ewes than for HL ewes. In conclusion, the physiological mechanism(s) for this divergence in reproductive rate in these Rambouillet lines does not appear to be directly related to changes in energy-related metabolites and metabolic hormones, or intake. However, selection appears to have altered systemic P4

concentrations after d 90 of pregnancy between the two lines of ewes. Whether this is caused by a change in luteal and(or) placental P4 secretion, or by a change in catabolism of P4 between ewes of these lines requires further investigation.

Key words: metabolites, metabolic hormones, progesterone, reproductive rate, genetic selection

¹Appreciation is expressed to Dr. Dennis Hallford, New Mexico State University, Las Cruces, NM. This study was supported by the Montana Agric. Exp. Sta., and is a contributing project to Multistate Research Project, W-3177, Enhancing the Competitiveness of U.S. Beef.

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INTRODUCTION

In 1968, Dr. P. J. Burfening of the Montana Agricultural Experiment Station began a selective breeding program to generate two lines of Rambouillet sheep with high (HL) or low (LL) reproductive rates (Schoenian and Burfening, 1990). The selection index (SI) was $SI = \text{lifetime number of lambs}/(\text{age of ewes} - 1)$, with HL and LL sheep bred for high or low SI values, respectively. The selective breeding of these lines was found to have resulted in diverging breeding values after 23 yr of selection (Burfening et al., 1993). Phenotypic outcomes of using this SI in these lines of ewes include that ovulation rate, litter size, and lambs born per ewe exposed had increased in HL ewes compared to LL ewes (Schoenian and Burfening, 1990). The physiological mechanisms that are involved in causing these changes were not clear and hereinto remain to be determined.

Nutrition and metabolism are known to affect reproduction in livestock. It is now established that nutrition affects reproduction not only by providing energy to develop and sustain the fetus or embryo directly, but also through regulation of hormones that control reproduction and impact development of the neonate (for reviews see, Robinson et al., 2006; Boland et al., 2001). Furthermore, nutritional status of ewes has been shown to interact with systemic progesterone (P4) concentrations and influence pregnancy rates (Parr et al., 1987). One could hypothesize that selection for high or low reproductive rate in ewes of these Rambouillet lines has altered nutrient intake, metabolism, utilization of energy-related metabolites, metabolic hormones, and(or) P4 synthesis or catabolism during gestation. The objectives of this study were to compare intake, temporal patterns of metabolites, metabolic hormones, and P4 concentrations during gestation, and lambing traits between HL and LL Rambouillet ewes. The null hypotheses were that: 1) temporal patterns of GLUC, NEFA, cortisol, triiodothyronine (T3), thyroxine (T4), IGF-I, and progesterone (P4) concentrations, and T3:T4 ratios; 2) nutrient intakes; 3) lambing rates; and, 4) $\text{kg of TDN} \cdot \text{total kg of lamb born}^{-1} \cdot \text{ewe}^{-1}$ lambing, before mating and during pregnancy do not differ between Rambouillet ewes from lines selected for high (HL) and low (LL) reproductive rates.

MATERIALS AND METHODS

This experiment was conducted at the Montana State University Bozeman Area Research and Teaching Facility (BARTF). Animal care, handling, and protocols used in

this experiment were approved by the Montana State University Agricultural Animal Care and Use Committee.

Animals, Nutritional Management, Breeding

Thirty HL and 27 LL nulli-, primi-, or multiparous ewes, approximately equally divided among ages 1 to 4 yr, were used in this study. The long-term breeding, care, and management of ewes in these lines has been described by Burfening et al. (1993). Ewes were transported from the Red Bluff Research Ranch near Norris, MT to the BARTF in fall after weaning. Ewes of both lines were co-mingled and housed in open-shed lots equipped with GrowSafe bunks (GrowSafe Systems, Ltd., Airdrie, AB, Canada) throughout the study. Ewes in both lines had access to the same diets fed in the GrowSafe bunks throughout the study. Ewes were weighed on 2 consecutive d at start of study and approximately every 12 d thereafter. Ewes were fed chopped-grass hay for 41 d, beginning Oct. 5, then chopped-alfalfa hay for 106 d, and finally, an alfalfa-, barley-, and molasses-pelleted diet, until lambing (55 d). Ewes had free access to water and a trace-mineral supplement.

Hay was sampled (Penn State Forage Sampler) prior to chopping, and pellets were grab sampled. Partial DM was determined prior to grinding hay samples. Sub-samples were pooled by weight and the nutrient composition analyzed (Dairy One, Inc., Ithaca, NY). As fed feed analysis was corrected with partial DM to determine as fed nutritional composition of hays. The final as fed DM, CP, NDF, and TDN composition of

the grass hay was 91.1, 7.4, 61.2, and 54%, the alfalfa hay was 89.6, 19.4, 34.4, and 54%, and the alfalfa based pellet was 90.8, 18.1, 36.8, and 55%, respectively.

The breeding season began on Nov. 9, and the diet was switched from chopped-grass to chopped-alfalfa hay 1 wk after introduction of rams. Ewes were single-sire mated for 18 d within lines, in groups of 12 to 15, to Rambouillet rams. The brisket region of each ram was painted with raddle powder (Premier 1, Washington, IA) mixed ~3:1 with vegetable oil prior to their introduction to ewes and every 3 d thereafter for detection of breeding behavior. After exposure to Rambouillet rams, ewes were exposed to 1 of 2 Suffolk rams for an additional 18 d. At lambing, lambs were weighed with a hand-held scale immediately at birth before suckling.

Blood Sampling and Assays

Jugular venous samples were collected 22 and 8 d before introduction of the rams, within 24 to 48 h for any ewe marked with brisket paint, and approximately 30, 60, 90, and 120 d thereafter. Samples were stored overnight at 4°C and serum was separated by centrifugation at 1,850 x g, 30 min, at 4°C. Samples were stored at -20°C until assayed.

Glucose concentrations were determined in duplicate using a GLUC assay kit (Infinity Glucose Hexokinase liquid reagent, Thermo Fisher Scientific, Waltham, MA). Intra- and inter-assay CV were < 10% for ewe sera pools that contained 55.5 and 83.7 mg/dL, respectively.

Concentrations of NEFA were quantified with enzymatic-colorimetric assay kits (HR Series NEFA – HR [2]; Wako Diagnostics, Richmond, VA). Intra- and inter-assay

CV were 4.1 and 9.6% for serum that contained 0.291 mEq/L; and, 5.3 and 11.7%, respectively, for serum that contained 0.113 mEq/L.

Concentrations of cortisol were determined in duplicate by solid-phase RIA kits (Siemens Healthcare Diagnostics, Los Angeles, CA). Intra- and inter-assay CV were 3.3 and 10.1% for ewe serum that contained 26.5 ng/mL; and, 3.6 and 11.5%, respectively, for ewe serum that contained 25.5 ng/mL.

Triiodothyronine and T4 were assayed in duplicate using solid phase RIA kits (Siemens Healthcare Diagnostics Los Angeles, CA, USA) validated for sheep serum (Richards et al., 1999; Wells et al., 2003, respectively). Intra- and inter-assay CV for T3 were 4.4 and 14.0 %, respectively, for ewe serum that contained 1.39 ng/mL. Intra- and inter-assay CV for T4 were 5.8 and 6.4 %, respectively, for ewe serum that contained 70.0 ng/mL.

Concentrations of IGF-I were assayed by Dr. Dennis Hallford (New Mexico State University, Las Cruces, NM) in duplicate using double antibody RIA, validated for sheep serum (Spoon and Hallford, 1989). Intra- and inter-assay CV were 8.1 and 16.3%, respectively.

Progesterone concentrations were determined in duplicate by solid-phase RIA kits (Siemens Healthcare Diagnostics, Los Angeles, CA). Intra- and inter assay CV were 8.2 and 14.8% for ewe serum that contained 2.4 ng/mL; and 2.0 and 7.4%, respectively, for ewe serum that contained 11.5 ng/mL.

Normalization of Ewes and Determination of Cyclicity

Ewe samples were normalized to mating dates during gestation. Mating was determined by 1) gestation length (142-152 d); 2) date brisket paint marked; 3) P4 concentration, over 2 ng/mL in diestrus; 4) cycle length of 17-18 d; and 5) pure or cross bred lamb. Thirty-five ewes grouped into consecutive whole weeks for 4 of 5 samples (d 0, 30, 60, 90 and 120). These ewes were considered to have a similar physiological status, and were used for the analysis. Ewes were determined to be cycling when a sample from d -22, -8, or 0 had a concentration of progesterone ≥ 1 ng/mL.

GrowSafe Data Processing

Intake was recorded for 29 d on chopped alfalfa hay during breeding, 76 d on chopped alfalfa hay during gestation, and 30 d on pellets during late gestation. Intake data of normalized ewes was filtered, and 2 d with assigned feed disappearance $\leq 92\%$ and 7 d with scale noise ≥ 12 were removed. Additionally, 4 d were removed because intake was not recorded due to shearing (2 d) and system failure (2 d). Excluded d were given the average intake value for the d before and after excluded d(s). Three high line ewes were excluded for lack of consumption from GrowSafe bunks or morbidity. Assigned feed disappearance was $99.75 \pm 0.76\%$ for days analyzed. Intake from breeding, gestation, and late gestation was summed for each ewe, and intake for the combined periods analyzed by line of ewe.

Statistical Analysis

Data for lambing rates included 27 HL and 22 LL ewes; whereas, data for litter size of ewes that lambled included 25 HL and 17 LL ewes. There were 20 HL and 15 LL ewes that were used to normalize intake and temporal patterns of GLUC, NEFA, cortisol, T3, T4, IGF-I, and P4 concentrations to within a 2-wk period of d 30, 60, 90, and 120 of gestation. Data for BW at the start of the study, lambing rate, birth weight of lambs, metabolites, metabolic hormones, P4, total intake, total TDN intake, and kg of TDN • total kg of lamb born⁻¹ • ewe⁻¹ were analyzed using SAS procedures (SAS Inst. Inc., Cary, NC) and significance was set at $P \leq 0.05$.

Lambing rate was analyzed with contingency chi-square analysis. Litter size, lamb birth weight, total lamb birth weight per ewe, total intake, total TDN intake, and kg of TDN • total kg of lamb born⁻¹ • ewe⁻¹ were analyzed by separate ANOVA using PROC GLM. The model included line of ewe. Temporal patterns of metabolites, metabolic hormones, and P4 concentrations were analyzed by separate ANOVA using the PROC MIXED model for repeated measures. The model included line, d, and the line by d of gestation interaction. Ewe within line was the subject and d of gestation was the repeated measure. Means were separated using Bonferroni's multiple comparison adjustment.

RESULTS AND DISCUSSION

At the beginning of the study, BW of HL and LL ewes used for analyses of temporal patterns of intake, metabolites, metabolic hormones, and P4 concentrations did not differ ($P > 0.05$; 51.3 ± 7.3 kg). All ewes were cycling before introduction of rams.

Lambing rates did not differ ($P > 0.05$) between HL and LL ewes (Table 1). This result is similar to that reported by Schoenian and Burfening (1990) for number of ewes lambing per ewe exposed. Data for lambing rate in the present study may indicate that an additional 23 yr of selection has not influenced this reproductive characteristic in ewes of these lines. However, this conclusion must be qualified in that the numbers of ewes per line were low.

Consistent with the results of Schoenian and Burfening (1990), litter size in the present study was greater ($P \leq 0.05$) for HL ewes than LL ewes (Table 1). These results indicate that an additional 23 yr of selection for high or low reproductive rate using the SI [lifetime number of lambs/(age of ewes – 1)] has not increased the phenotypic divergence between ewes of these lines.

Ewes from the HL had greater ($P \leq 0.05$) total birth weight of all lambs per ewe, although individual lambs born to LL ewes were larger ($P \leq 0.05$) (Table 1). Greater total lamb birth weight per ewe for HL ewes could be due to the greater proportion of twins in HL ewes than in LL ewes. This conclusion is consistent with results in sheep reported by Gardner et al. (2007), in which twinning ewes had lower individual birth weights, but increased total weight of all lambs per ewe lambing.

Intakes did not differ ($P > 0.05$) between HL and LL ewes during maintenance, breeding, or gestation, nor did total TDN consumed from breeding through gestation differ ($P > 0.05$; Table 1). It appears that in addition to not selecting for different systemic metabolites and metabolic hormone concentrations, this SI for reproductive rate

has not affected intake differentially. However, kg of TDN • total kg of lamb born⁻¹ • ewe⁻¹ was greater ($P \leq 0.05$) for LL ewes than HL ewes.

Triiodothyronine, NEFA, and IGF-I did not differ ($P > 0.05$) before gestation (Table 4). Cortisol, GLUC, and T3:T4 exhibited d effects ($P \leq 0.05$) prior to gestation (Table 4). Glucose, and cortisol were higher ($P \leq 0.05$) on d -22 compared to d -8 and d 0. Cortisol and GLUC rise when sheep are under stress (Apple et al., 1995), and cattle have been shown to become accustomed to handling, cortisol concentrations decreasing with subsequent handling (Crookshank et al., 1979). Day -22 was the first sample taken from each ewe, and stress associated with this could explain the higher concentration of cortisol and GLUC on d -22 compared to d -8 and 0.

The T3:T4 ratio before gestation was lower ($P \leq 0.05$) at d -22 compared to both d -8 d and d 0. This was due to increased T4 concentration on d -22, since T3 did not differ ($P > 0.05$) from d -22 to d 0. Concentrations of T3 and T4 have been reported to decrease under stress induced by inescapable tail-shock in rats (Helmreich and Tylee, 2011). However, that study also found that less extreme stimulation, such as handling of rats, increased concentrations of T4 (Helmreich and Tylee, 2011). This second stress level is more comparable to the present study, and agrees with T4 concentrations observed. There was a line by d effect before gestation ($P \leq 0.05$) for T4 (Fig. 1). Concentrations of T4 on d -22, -8, and 0 did not differ ($P > 0.05$) between HL and LL ewes. However, T4 concentrations in LL ewes decreased ($P \leq 0.05$) faster from d -22 to d -8 than in HL ewes. The cause of this remains to be determined. There are various factors

that affect concentration of T4; however, both HL and LL ewes were treated the same, and had concentrations of T4 that did not differ on any other sample d.

Cortisol and NEFA did not differ ($P > 0.05$) temporally during gestation (Table 5). Concentrations of GLUC, T3, and T4 did not differ ($P > 0.05$) among d 30, 60, and 90, but increased ($P \leq 0.05$) by d 120 (Table 5). Concentrations of IGF-I increased ($P \leq 0.05$) linearly throughout gestation (Table 5). The T3:T4 ratio on d 30 did not differ ($P > 0.05$) from those on d 60, 90, or 120, but the ratios on d 60 and 120 were higher ($P \leq 0.05$) than that on d 90 (Table 5).

Glucose was higher ($P \leq 0.05$) on d 120 than on d 30, 60, or 90. Glucose concentrations in the present study were similar to those reported by Verbeek et al. (2012) for pregnant high BCS ewes fed free-choice pellets, except they did not report an increase in GLUC concentration at d 120, and we did not observe a decrease in concentration at d 90. Verbeek et al. (2012) reported increased GLUC concentrations after d 105 of gestation in ewes fed to excess of NRC requirements. Intake increased from an average of 2.228 kg per d on the alfalfa hay diet to 3.171 kg per d on the pelleted diet, possibly explaining the increase in GLUC concentration at d 120.

Concentrations of T3 and T4 were higher ($P \leq 0.05$) at d 120 than at d 30, 60, or 90. These concentrations follow the same slightly negative linear trend as previously reported by Ward et al. (2008), except for d 120, which increased after the feed change. Vonnahme et al. (2013) observed increased concentrations of T3 and T4, both after d 120 of gestation and with increasing intake in ewes fed to meet NRC requirements and in ewes fed to exceed NRC requirements. Therefore, the increase in T3 and T4

concentration after d 90 could have resulted from the feed change and concurrent increase in intake. However, these results could also be observed if these ewes had an early increase in T3 and T4 concentration resulting from gestation. This study was not able to differentiate between the two. The T3:T4 ratio did not differ ($P > 0.05$) from d 30 at d 60, 90, or 120. These observations contrast with Ward et al. (2008) who reported that the T3:T4 ratio decreased slightly during gestation in ewes.

IGF-I concentrations did not differ ($P > 0.05$) between d 30 and 60, or d 60 and 90, but were significantly greater on d 120. This increase in concentration of IGF-I during gestation has been reported previously in sheep (Ward et al., 2008; Verbeek et al., 2012). Cortisol and NEFA did not have d effects, but the concentrations are similar to those reported by Vonnahme et al. (2013) for ewes in gestation fed in excess of requirements.

There was no line or line by d of gestation interaction ($P > 0.05$) for concentrations of GLUC, NEFA, cortisol, T3, T4, and IGF-I, before (Table 2) gestation. There was no line or line by d of gestation interaction ($P > 0.05$) for concentrations of GLUC, NEFA, cortisol, T3, T4, T3:T4, and IGF-I, during (Table 3) gestation. These results indicate that selection for high and low reproductive rate in Rambouillet sheep, using the SI [lifetime number of lambs/(age of ewes – 1)], has not influenced systemic temporal concentrations of the metabolites and metabolic hormones assessed in the present study.

There was a line by d of gestation interaction ($P \leq 0.05$) for P4 concentrations (Fig. 2). Concentrations of P4 did not differ ($P > 0.05$) between HL and LL ewes on d 30 and 60. Concentrations of P4 increased ($P \leq 0.05$) in both HL and LL ewes from d 60 to

90. This increase in P4 concentrations in ewes of both lines at d 90 is in agreement with Sarda et al. (1973) who reported that P4 concentrations in pregnant ewes had a marked increase in P4 from d 80 to 90 of gestation. However, P4 in HL ewes increased ($P \leq 0.05$) to a greater concentration from d 60 to d 120 than P4 concentration in LL ewes.

This is the first evaluation of temporal patterns of P4 concentrations during gestation in these lines of Rambouillet ewes. The physiological mechanism(s) for this interaction in P4 concentrations from mid- to late-gestation may be related to: 1) number of corpora lutea (CL); 2) placental mass and synthesis of P4; and(or), 3) catabolism of P4. Schoenian and Burfening (1990) reported that HL ewes had more CL per ewe than LL ewes. However, it is not likely that this interaction in P4 concentrations is due to the mass of luteal tissue because concentrations did not differ between lines until d 90. Placental mass is known to be greater in ewes carrying twins than in ewes carrying singles (Vonnahme et al., 2008). Perhaps, an increase in placental mass could result in higher P4 concentrations in HL ewes late in gestation, since there was a greater proportion of HL ewes carrying twins than LL ewes. However, Sarda et al. (1973) reported that systemic P4 concentrations did not differ between ewes carrying singles and twins. Thus, it is possible that selection for high and low reproductive rate has either increased the mechanism for P4 synthesis in HL ewes or decreased this mechanism in LL ewes, resulting in divergent P4 concentrations late in gestation between ewes in these lines. However, Berardinelli et al. (1993) reported that P4 concentrations were higher in HL than LL ewes between d 4 and d 9 of the estrous cycle. This difference was not related to number of CL in the ovary because venous ovarian concentrations of P4 did not

differ between HL ewes that had 2 CL and LL ewes that had 1 CL in their ovaries (Berardinelli et al., 1995). These authors hypothesized that the clearance rate of P4 from the systemic circulation during mid-luteal phase of the estrous cycle in HL ewes was slower than the clearance rate in LL ewes. One could hypothesize that the difference in P4 concentrations that we observed late in pregnancy, between HL and LL ewes, could be a result of a change in catabolic processing of P4.

In conclusion, selection of Rambouillet ewes for high or low reproductive rate has not only caused a difference in litter size and total kg of lamb born per ewe, but it has also resulted in observable differences in late-gestation P4 concentrations and efficiency of TDN intake to kg of lamb born per ewe between these lines. Results for litter size and total kg of lamb born per ewe confirm that the use of this SI has maintained a difference in reproductive rate between these lines. These phenotypic characteristics do not appear to be driven by intake, or differences in metabolites or metabolic hormones assessed in this study. Differences in temporal P4 concentrations may reflect genetic differences caused by the SI relating to specific physiological processes affecting the reproductive rates of these sheep. The 25% increase in kg of TDN intake during gestation per kg of lamb born per ewe of LL compared to HL ewes represents a dramatic difference in reproductive efficiency, with important implications for increasing profitability of sheep production.

Table 1. Lambing rates, and least squares means for litter size, lamb birth weight (LBW), total LBW per ewe lambing, total intake, total TDN intake, and kg of TDN • total kg of lamb born⁻¹ • ewe⁻¹ of Rambouillet ewes selected for high (HL) and low (LL) reproductive rate

Item	Line			P-value
	HL (n)	LL (n)		
Reproductive measures				
Lambing rate, ¹ %	92.6 (27)	77.3 (22)	2.32 ²	0.13
Litter size ³	1.52 (25)	1.18 (17)	0.47 ⁴	0.02
LBW, kg	4.21 (32)	4.62 (17)	0.68 ⁴	0.05
Total LBW per ewe, kg	6.74 (20)	5.22 (15)	1.83 ⁴	0.02
Intake				
Total intake, ⁵ kg	322 (17)	332 (15)	67.31 ⁴	0.67
Total TDN, ⁶ kg	174.8 (17)	180.3 (15)	36.36 ⁴	0.67
kg of TDN • total kg of lamb born ⁻¹ • ewe ⁻¹ , ⁷ kg	26.7 (17)	35.0 (15)	8.04 ⁴	0.007

¹Percentages of HL and LL ewes lambing.

² $\chi^2 = 2.32$; d.f. = 1.

³Lambs per ewe lambing.

⁴Pooled SEM.

⁵Least squares means of as fed intake of standardized ewes from breeding through gestation.

⁶Least squares means of total TDN intake of standardized ewes from breeding through gestation.

⁷Least squares means of total TDN intake of standardized ewes from breeding through gestation divided by total kg of lamb per ewe lambing.

Table 2. Least squares means of metabolic hormones and metabolites before gestation for Rambouillet ewe lines selected for high (HL; n = 20) and low (LL; n = 15) reproductive rate

Item	Line		SEM	<i>P</i> value
	HL	LL		
Glucose, mg/dL	71.8	69.7	4.6	0.39
NEFA, mEq/L	0.299	0.263	0.101	0.33
Cortisol, ng/mL	16.6	19.3	4.0	0.30
T3, ng/mL	0.749	0.769	0.142	0.73
T3:T4	0.016	0.017	0.002	0.38
IGF-I, ng/mL	279	276	62	0.94

Table 3. Least squares means of metabolic hormones and metabolites during gestation for Rambouillet ewes selected for high (HL; n = 20) and low (LL; n = 15) reproductive rate

Item	Line		SEM	<i>P</i> value
	HL	LL		
Glucose, mg/dL	68.9	70.2	2.5	0.45
NEFA, mEq/L	0.317	0.317	0.089	1.00
Cortisol, ng/mL	19.8	18.5	4.2	0.30
T3, ng/mL	0.780	0.813	0.092	0.77
T4, ng/mL	51.5	52.9	6.5	0.60
T3:T4	0.015	0.015	0.001	0.77
IGF-I, ng/mL	428	431	109	0.95

Table 4. Least squares means of metabolic hormones and metabolites before gestation shown temporally for Rambouillet ewes selected for high (HL; n = 20) and low (LL; n = 15) reproductive rate.

Item	Sample day			SEM	P-value
	d -22	d -8	d 0		
Glucose, mg/dL	79.3 ^a	64.5 ^b	68.4 ^b	8.9	< 0.01
NEFA, mEq/L	0.338	0.291	0.324	0.180	0.55
Cortisol, ng/mL	28.5 ^a	13.6 ^b	15.5 ^b	9.0	< 0.01
T3, ng/mL	0.764	0.738	0.775	0.148	0.58
T3:T4	0.015 ^a	0.018 ^b	0.018 ^b	0.003	< 0.01
IGF-I, ng/mL	300	259	275	120	0.43

^{a,b} Means that have different superscript letters differ ($P \leq 0.05$).

Table 5. Least squares means of metabolic hormones and metabolites during gestation shown temporally for Rambouillet ewes selected for high (HL; n = 20) and low (LL; n = 15) reproductive rate

Item	Estimated day of gestation				SEM	P-value
	d 30	d 60	d 90	d 120		
Glucose, mg/dL	66.5 ^a	69.5 ^a	66.0 ^a	76.3 ^b	9.0	<0.01
NEFA, mEq/L	0.304	0.267	0.257	0.297	0.123	0.34
Cortisol, ng/mL	18.1	19.9	13.3	20.5	12.0	0.09
T3, ng/mL	0.741 ^a	0.773 ^a	0.652 ^a	1.058 ^b	0.175	<0.01
T4, ng/mL	49.3 ^a	48.2 ^a	46.4 ^a	64.9 ^b	7.3	<0.01
T3:T4	0.015 ^{ab}	0.016 ^a	0.014 ^b	0.016 ^a	0.003	<0.01
IGF-I, ng/mL	301 ^a	347 ^{ab}	436 ^b	633 ^c	166	<0.01

^{a,b,c} Means within a row with different superscripts differ ($P \leq 0.05$).

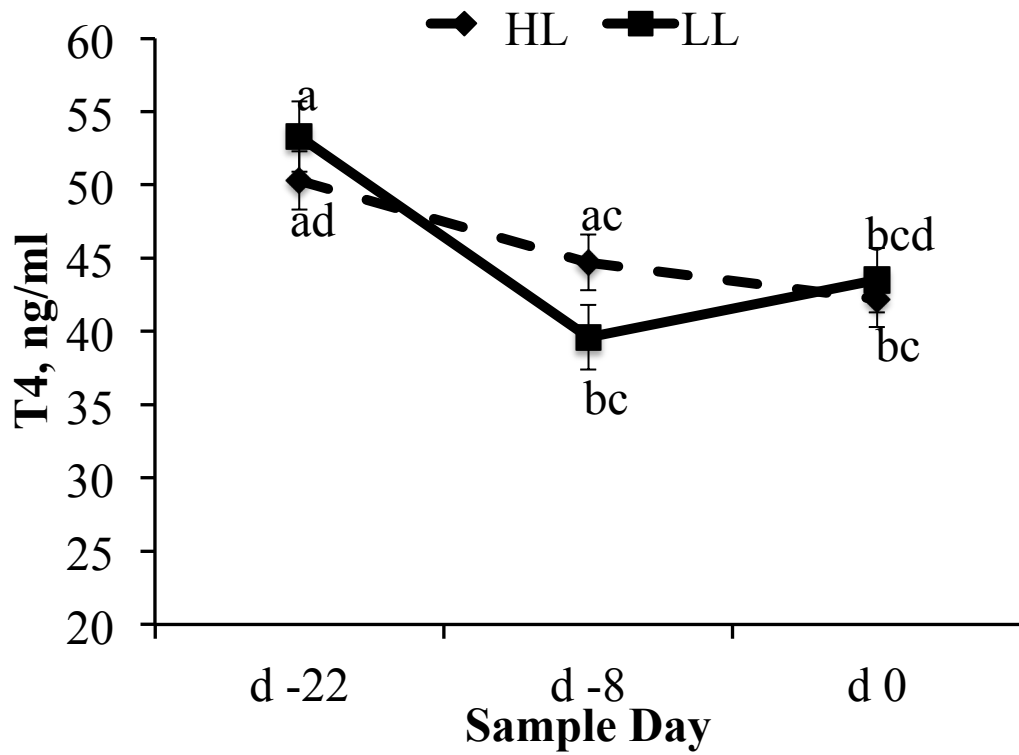


Figure 1. Least squares means of thyroxine (T4) concentrations before gestation in Rambouillet ewes selected for high (HL; $n = 20$) and low (LL; $n = 15$) reproductive rate. Error bars represent \pm SE of each mean. Line by d of gestation interaction; $P = 0.05$. Means with different superscripts differ ($P \leq 0.05$).

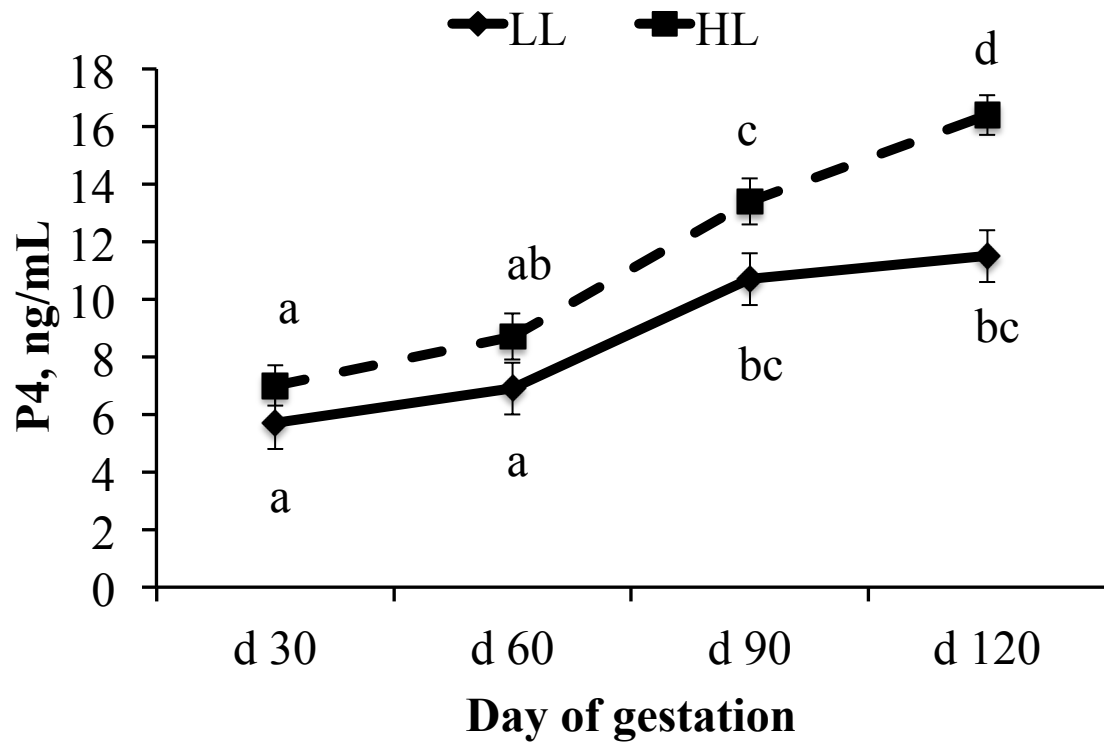


Figure 2. Least squares means of progesterone (P4) concentrations during gestation in Rambouillet ewes selected for high (HL; $n = 20$) and low (LL; $n = 15$) reproductive rate. Error bars represent \pm SE of each mean. Line by d of gestation interaction; $P = 0.04$. Means with different superscripts differ ($P \leq 0.05$).

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CHAPTER FOUR

THE VAGINAL MICROBIOTA OF EWES AND COWS ARE UNIQUE AND
DISTINCT FROM HUMANS AND NON-HUMAN PRIMATES

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PLoS One

Status of Manuscript:

Prepared for submission to a peer-reviewed journal

Officially submitted to a peer-review journal

Accepted by a peer-reviewed journal

Published in a peer-reviewed journal

Public Library of Science

The Vaginal Microbiota Of Ewes And Cows Are Unique And Distinct From Humans And Non-Human Primates

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ABSTRACT

Human vaginal microbiota affect reproductive performance and perinatal health. Although a number of common reproductive disorders in livestock involve bacterial infection, very little is known about their normal vaginal microbiota. Therefore, we sought to determine the species composition of sheep and cattle vaginal microbiota. Twenty Rambouillet ewes and twenty crossbred cows varying in age and reproductive status were sampled by ectocervicovaginal lavage. Variable regions 3 and 4 of the 16s ribosomal RNA (rRNA) were amplified by PCR and sequenced. A total of 907,667 filtered, high quality reads were obtained and subsampled to 5,000 reads per sample for analysis. Coverage estimates were identical for cattle and sheep, and indicated this approach obtained data on 98 ± 0.01 % of the total microbial genera present in each sample. Cow and ewe vaginal microbiota did not differ ($P > 0.05$), although cow

microbiota exhibited greater ($P \leq 0.05$) diversity compared to the ewe microbiota, and both differed ($P \leq 0.05$) from primates. While bacteria were numerically dominant, Archaea were detected in 95% of cow and ewe samples, mainly the order Desulfurococcales. Both ewes and cows were predominately colonized by the bacterial phyla Bacteroidetes, Fusobacteria, and Proteobacteria. The most abundant genera were *Aggregatibacter spp.*, and *Streptobacillus spp.* *Lactobacillus spp.*, while present in 80% of ewe and 90% of cow samples, were only present in low abundance. Bacteria previously described as common in the cow and ewe vaginal tract, except for *Escherichia*, were variably present, and only in low abundance. Ewe and cow pH differed ($P \leq 0.05$), with means (\pm SD) of 6.7 ± 0.38 and 7.3 ± 0.63 , respectively. In conclusion, 16s rRNA sequencing of cow and ewe vaginal ectocervicovaginal lavages revealed that cow and ewe vaginal microbiota differ from culture-led results, and reveal a microbiota distinct from previously described vaginal ecosystems.

INTRODUCTION

The typical healthy human vaginal microbiota is dominated by Lactobacilli [1]. The lactobacilli are important to vaginal homeostasis through their production of lactate and maintenance of a low vaginal pH ($\text{pH} < 4.5$) that is inhibitory to many vaginal pathogens [2]. Elevated vaginal pH is associated with vaginal irritation, odor, and discharge and is a feature always observed in bacterial vaginosis, the most common disorder among reproductive-aged women [3]. A reduction in vaginal lactobacilli in humans has been shown to negatively affect reproductive outcomes with both increases

to the risks of pre-term delivery and miscarriage [4]. The vaginal microbiota are also the first colonizers of infants [5], and the impact of these bacteria on the developing immune system could have long-term health implications [6]. Further, the widely described ability of lactobacilli to preclude common pathogens may make those vaginal lactobacilli that initially colonize neonates an important defense that bridges the period when the immune system is maturing.

Lactobacillus spp. have been reported in cows, though at a lower abundance than other genera [7-9]. *Enterococcus spp.*, *Staphylococcus spp.*, and *Streptococcus spp.* are common vaginal isolates of cows [7,8,10,11], while *Bacillus spp.*, *Corynebacterium spp.*, *Escherichia spp.*, *Staphylococcus spp.*, and *Streptococcus spp.* are commonly isolated from the ewe vagina [12-15].

However, all reported data on cow and ewe vaginal microbial communities has relied exclusively on culture techniques. The use of 16s ribosomal RNA (rRNA) gene sequencing has revealed a much greater and historically unrealized diversity in various microbial ecosystems that were obscured by traditional culturing methods [16,17]. Culture-based methods have been observed to emphasize the rarer members of communities [17] and often misses those that are more abundant. One clear example of this is the Human Microbiome Project where sequencing data collected over the past six years has identified 2,698 often abundant microbes that to this day remain to be captured by even targeted culturing approaches (http://hmpdacc.org/most_wanted/). The complete reliance on culture has left the true microbial diversity of the cow and ewe vagina hereinto undetermined. Therefore the objective of this study was to evaluate the

microbiota present in cow and ewe vagina using culture independent, 16s rRNA sequencing technology and relate their composition to other vaginal microbiota that have been defined in humans and non-human primates (Yildirim et al., unpublished data). Additionally, given the importance of pH to human vaginal health we also investigated livestock vaginal pH.

MATERIALS AND METHODS

Ethics Statement

Animal care and use protocols were approved by the Montana State University Agricultural Animal Care and Use (AACUC) committee under protocol number 2012-AA07 dated 09/20/2012.

Sampling

Vaginal lavages were collected from 20 Rambouillet ewes and 20 primarily Angus crossbred or Hereford crossbred beef cows of varied genetic composition. Ewes and cows had varied breeding method and pregnancy status (Table 1). Samples were collected by injecting 25 ml (ewes) or 50 ml (cows) of sterile, physiological saline (0.8% NaCl, w/v) into the vaginal tract via sterile catheters attached to luer-lock 60 ml syringes. Saline was injected in a continuous stream toward the cervix, aspirated 3 to 5 times, transferred to a sterile 15 ml conical centrifuge tube (Falcon®, ThermoFisher, Waltham, MA), and stored at -20 °C. A 1 ml aliquant of sample was used for measurement of pH. For DNA extraction, up to 4.5 ml of sample was centrifuged for 5 min at 20,000 x g at 4°

C.

pH Analyses

Vaginal pH was determined using an Orion 520A pH meter fitted with a ROSS Ultra Electrode (Thermo Scientific, Waltham, MA, USA). Samples for all animals were used for analysis of pH, except samples from ewes obtained before breeding (Table 1) because of the possibility that the lavage solution contained phosphate buffer (Table 1). Mean, standard deviation, boxplots, Shapiro-Wilk test of normality and two-sided Wilcoxon rank-sum test were calculated using R (<http://www.r-project.org>).

Sequencing Microbiota

Pellets obtained from up to 4.5 ml of lavage were extracted using MoBio PowerSoil DNA Isolation kits following manufacturer instructions, except a 2 min bead-beating step was used instead of a 10 min vortex. Variable regions 3 and 4 of the 16s rRNA genes were amplified using custom primers that included indexes to identify samples after sequencing. The PCR reaction ran for 30 cycles at 94 °C for 20 s, 52 °C for 30 s, and 72 °C for 45 s, using barcoded primers (SeqF(1-8) 5'-AATGATACGGCGACCACCGAGATCTACAC(adaptor)-Index2(one of eight different 8nt codes used to distinguish among samples when pooled for sequencing)-TATGGTAATT(sequencing primer pad)-AT(linker)-CCTACGGGAGGCAGCAG(341f primer)-3' and SeqR(1-12) 5'-CAAGCAGAAGACGGCATAACGAGAT(adaptor)-Index1(one of twelve different 8nt codes used to distinguish among samples when pooled

for sequencing)- AGTCAGTCAG(sequencing primer pad)-CC(linker)-GGACTACHVGGGTWTCTAAT(806r primer-3'). Amplicons were quantified using an Agilent 2200 tape station (Agilent, Santa Clara, CA, USA) and pooled at an equimolar concentration. Pooled amplicons were purified from residual PCR reagents and non-specific amplification products in an agarose gel using a QIAquick gel extraction kit (Qiagen, Valencia, CA, USA) following manufacturers instructions. Purified and pooled amplicons were subsequently quantified using a KAPA Syber quantification kit (KAPABiosystems, Wilmington, MA, USA) as per manufacturer instructions. Purified, quantified and pooled amplicons were mixed with 5 to 10% of an equimolar concentration of PhiX and sequenced at 12.5 pM. Sequencing was performed with an Illumina MiSeq using paired-ended 2 by 250 nucleotide (nt) dual-index sequencing. Custom primers (R1 5'-CCTACGGGAGGCAGCAG-3', R2 5'-AGTCAGTCAGCCGGACTAC-3', Index 5'-GTAGTCCGGCTGACTGACT-3') were used for sequencing and indexing.

Data Analyses

The resulting paired-end 16s rRNA gene reads were assembled using the 'make.contigs' command in mothur [18]. Any base call disagreement in the overlapping portions of the paired reads was ascribed to the base with a higher quality score ('deltaq = 1'). Assembled sequences were then preprocessed to remove low quality and chimeric sequence data. Briefly, sequences were removed if they were shorter than 400 nt, had a homopolymeric sequence greater than 10 base calls, had more than 2 ambiguous base

calls (1 per 200nt), or were found to be chimeric sequences by UCHIME [19]. Each of the resulting sequence data sets were subsampled to 5,000 reads to enable direct comparison. Reads were taxonomically assessed using mothur's implementation of the naïve Bayesian classifier, RDP Classifier [20]. Taxonomic assignments were considered supported if bootstrapping values were greater than 70%. Microbial 16s rRNA gene composition and diversity were compared among samples and to sequences from human and primate vaginal systems using multivariate statistical approaches provided by mothur for measures of α -diversity, and using the vegan package of R [21] for β -diversity. Richness measures included genera observed and Chao1 estimates [22] of total genera richness. These measures were derived from genus-level taxonomic classifications due to the low Bayesian support offered to the majority of sub-genus level classifications. Diversity measures were determined using Shannons diversity index. Normality of data was tested using the Shapiro-Wilk test [23], and significance determined using a two-sample t-test or Wilcoxon Mann-Whitney test for normally and non-normally distributed data, respectively. Heatmaps were constructed using gplots (<http://cran.r-project.org/web/packages/gplots/index.html>) in R with taxonomic data. Within and across microbial communities, inter-individual and inter-species similarities were determined by pairwise measurements of Bray-Curtis dissimilarity, with significance determined by analysis of similarities (ANOSIM).

RESULTS

Sequencing Overview

Twenty Rambouillet ewes and 20 crossbred cows were sampled to investigate the composition of livestock vaginal microbiota. Samples were used to generate deep V3-V4 16s rRNA gene profiles. A total of 907,667 high quality reads were obtained following processing, and samples were randomly subsampled to 5,000 reads for direct comparison. Good's nonparametric coverage estimates [24] were identical for cattle and sheep, and indicated this approach obtained data on 98 ± 0.01 % of the total microbial genera present in each sample.

α -Diversity

All α -diversity metrics were derived from genus-level taxonomic classifications due to the low bootstrap support offered to the majority of sub-genus level classifications. Greater number of genera (comparative t-test; $P \leq 0.05$) were detected in cow vaginal samples than in vaginal samples of ewes (302 ± 83 and 220 ± 102 genera, respectively). Chao1 predicted there may have been more (comparative t-test; $P \leq 0.05$) genera present vaginal samples of cows than in vaginal samples of ewes (394 ± 77 and 310 ± 103 genera predicted, respectively). Measures of diversity (richness and evenness) were normally distributed (Shapiro-Wilk $W = 0.92$; $P > 0.05$) for ewes but not for cows ($W = 0.9$; $P \leq 0.05$). Diversity was measured with Shannons diversity index, which indicated low to moderate diversity communities for both ewes and cows (2.87 ± 1.16 and $3.64 \pm$

0.96, respectively). Vaginal microbiota of cows exhibited greater diversity as measured with Shannons diversity index than ewes (Wilcoxon; $P \leq 0.05$), humans (Wilcoxon; $P \leq 0.05$), and all non-human primates (Wilcoxon; $P \leq 0.05$). However, diversity of the vaginal microbiota of ewes was not different ($P > 0.05$) from most non-human primates, but was more diverse (Wilcoxon; $P \leq 0.05$) than that found in the human vagina (Table S1 and Fig. 1).

β -Diversity

The taxonomic composition inferred from 16s rRNA gene sequence data was compared to human, and non-human primate vaginal microbiota using genus-level resolution (Yildirim et al., unpublished data). Continued use of taxonomic-inference was further necessitated by the limited overlap in the regions on the 16s rRNA gene molecule sequenced in this study and by Yildirim et al. (unpublished data). RDP Classifier genus-level taxonomy was ordinated by NMDS using Bray-Curtis dissimilarities (Fig. 2). This analysis indicated that vaginal microbiota of the ewe and cow are distinct from other equivalently interrogated primate vaginal microbiota. While ewe and cow vaginal microbiota did not differ from one another (ANOSIM $R = 0.07$; $P > 0.05$), they each differed from vaginal microbiota of humans ($R = 1$; $P \leq 0.05$) and non-human primate species ($R > 0.7$; $P \leq 0.05$).

General Taxonomic Compositional Traits

Sequencing the V3 to V4 of the 16s rRNA gene of vaginal lavages enabled the

detection of both bacterial and archaeal microbiota. Bacteria were numerically dominant, representing $98.7 \pm 0.02\%$ of 16s rRNA reads in all samples. However, Archaea were detected in 95% (19/20) of vaginal samples collected from cows and ewes. Archaeal reads were largely assigned to members of the order Desulfurococcales, occurring in 95% of cow and 85% of ewe samples. The bacterial community was most commonly dominated by members of the Proteobacteria (almost exclusively γ -proteobacteria), Fusobacteria, and Bacteroidetes phyla in both ewes and cows (Fig. 3). *Aggregatibacter spp.*, and *Streptobacillus spp.* were typically the most abundant genera in both ewes and cows; however, various other genera were also present (Figs. 4 and 5). Lactobacilli were common: detected in vaginal samples of 80% (n = 16/20) of ewes, and 90% (n = 18/20) of cows. However, lactobacilli were always found at low abundances of the total 16s rRNA gene population determined in vaginal samples of cows and ewes ($0.36 \pm 0.66\%$ and $0.53 \pm 0.65\%$, respectively). Lactobacillus species varied among individual animals and were often heterogeneous within samples (Table 2). Species often described in human vaginal microbiota, particularly those defined by Ravel et al. [1] as biotype IV, and often associated with bacterial vaginosis, were observed in ewes and cows. These include *Sneathia spp.*, found in 90% of samples from ewes and cows at $2.4 \pm 4.0\%$ and $1.9 \pm 2.3\%$ of the total microbiota, respectively; and the *Prevotella spp.*, found in 65% of ewes comprising $0.5 \pm 0.9\%$ of the total microbiota. The *Paraprevotella spp.*, a distinct but closely related genera to *Prevotella*, were also found among the vaginal microbiota of ewes and cows.

Livestock Vagina Maintains a Near-Neutral pH

Consistent with low abundance *Lactobacillus spp.* in vaginal microbiota of ewes and cows, vaginal pH was near neutral in vaginal lavages of ewes and cows. One cow was noted to have a yellow-colored sample, and possibly contained urine. Data for this cow was excluded from statistical analyses because it was determined to be an outlier using the Shapiro-Wilk test. Vaginal lavage pH of ewes and cows (6.7 ± 0.4 and 7.3 ± 0.6 , respectively) differed ($P \leq 0.05$) using Welch's and Wilcoxon two-sample tests, and ranged from 5.6 to 7.1 in ewes and from 6.5 to 8.7 in cows.

DISCUSSION

Sequencing of the microbiota in vaginal lavages of cows and ewes revealed the presence of the following phyla: Actinobacteria, Bacteroidetes, Firmicutes, Fusobacteria, Proteobacteria, and Tenericutes. Machado et al. [25] found the same phyla in the uteri of Holstein cows using 16s rRNA sequencing. The predominant bacterial genera classified in the ewe vaginal tract were *Aggregatibacter spp.*, *Cronobacter spp.*, *Phocoenobacter spp.*, *Psychrilyobacter spp.*, and *Streptobacillus spp.* This contrasts with results from culture-based studies that reported genera such as *Bacillus spp.*, *Corynebacterium spp.*, *Escherichia spp.*, *Staphylococcus spp.*, and *Streptococcus spp.* to be among the most common [12-14,26]. Of these bacteria, *Escherichia/Shigella* was detected in almost all ewes (19/20; Fig 4), whereas, the rest of the bacteria found in the present study, that were suggested to be prevalent in culture-based, studies were present at very low abundance in 3 to 11 of the 20 ewes. However, Machado et al. [25] detected many genera that were

prevalent in the vaginal samples of ewes, including *Escherichia/Shigella spp.*, *Lactobacillus spp.*, *Porphyromonas spp.*, *Prevotella spp.*, *Sneathia spp.*, *Streptobacillus spp.*, and *Ureaplasma spp.*

In vagina samples of cows in the present study we found the following genera: *Aggregatibacter spp.*, *Phocoenobacter spp.*, *Sediminicola spp.*, *Sporobacter spp.*, and *Streptobacillus spp.* were the most commonly classified. This also contrasts with culture-based studies in that the most commonly reported genera were *Bacillus spp.*, *Enterococcus spp.*, *Staphylococcus spp.*, and *Streptococcus spp.* [7,8,10,11]. These cultured-based bacteria were detected at very low abundance in 0 to 11 of the 20 cows in this study. However, Machado et al. [25] also detected many genera that were detected in the present study, including *Alistipes spp.*, *Bacteroides spp.*, *Campylobacter spp.*, *Escherichia/Shigella spp.*, *Helcococcus spp.*, *Lactobacillus spp.*, *Porphyromonas spp.*, *Sneathia spp.*, and *Streptobacillus spp.*

Results of studies that have explored microbial communities with culture-based identification methods often differ from results of studies that have used 16s rRNA gene sequences to assign taxonomy, as the latter often reveal a greater diversity in the microbial communities [16]. Shade et al. [17] studied the same soil samples using culture-based and 16s rRNA gene sequencing techniques. These authors reported that cultured organisms were often low, or even absent, when compared to that obtained from 16s rRNA community profiles. This observation is similar to the present study, which revealed many more abundant genera than was evident from previous culture-based studies, although most cultured-based genera were still detected. While one of the two

most dominant genera of our study was not reported in the study by Machado et al. [25], their study evaluated uterine bacteria, whereas, in the present study we evaluated vaginal bacteria. However, it may be reasonable to assume similar bacterial genera to be present in the vagina of cows and ewes, given the proximity of the uterus to the vagina.

In contrast to the typical human vaginal microbial community, which generally is predominately *Lactobacillus spp.* [1], the vaginal microbiota communities of cows and ewes appear to have a high prevalence, but low abundance, of *Lactobacillus spp.* This concurs with results of vaginal microbiota from culture-based studies of the cow reported previously [7-9]. *Lactobacillus delbrueckii* was one *Lactobacillus* species detected in vaginas of cows and ewes in the present study (Table. 2), which has previously been isolated from the vagina of cows [9]. The limited abundance of *Lactobacillus* led Rodriguez et al. [9] to suggest that *Lactobacillus spp.* have a restricted or limited role in the vagina of the cow.

The near neutral pH of vaginal lavages of ewes and cows is consistent with low abundance of *Lactobacillus spp.*, especially in light of the fact that this genera is known to be able to produce large quantities of lactate as a metabolic by product [27], and is generally credited with creating low vaginal pH in women. Interestingly, the vaginal microbial communities of cows and ewes share several notable genera, namely, *Sneathia spp.*, *Porphyromonas spp.*, *Prevotella spp.*, and low abundance of *Lactobacillus spp.*, with the Biotype IV described by Ravel et al. [1]. While the most acidic biotype, Biotype I, had a median pH of 4.0, Biotype IV had the highest pH at 5.3. Manes et al. [15] reported pH range between 7.0 and 7.6 in ewes before estrous synchronization with

intravaginal sponges, although they reported a pH of 6.8 at approximately 53 hours after sponge removal at breeding. Beckwith-Cohen et al. [28] reported a range of 5.52 to 8.60 for cattle vaginal pH from the literature, and found that the vaginal pH of Israeli Holstein multiparous cows was 7.35. The mean pH of 7.3 and 6.7 for cows and ewes from the present study are quite comparable to those reported in the literature.

In conclusion, 16s rRNA gene sequencing of vaginal ectocervicovaginal lavages of cows and ewes revealed that the vaginal microbiota communities of cows and ewes do not appear to differ from each other. However, the microbiota of cows exhibited greater diversity than that in the microbiota of the ewe, interestingly, and the vaginal microbiota these species differed from that found in primates. Bacteroidetes, Fusobacteria, and Proteobacteria were determined to be the dominant phyla found in cows and ewes. Archaea and lactobacilli, while prevalent, were not abundant. Previously used culture-based methods likely misidentified the most abundant species, in that organisms such as *Staphylococcus spp.* and *Streptococcus spp.* were detected at very low abundance. The two most abundant members of the vaginal microbiota of cows and ewes in the present study were *Aggregatibacter spp.* and *Streptobacillus spp.* It was confirmed that *Lactobacillus spp.*, in contrast to the human vaginal microbiota, are not an abundant genera. The near neutral pH observed in vaginal lavages of cows and ewes is consistent with the low abundance of *Lactobacillus spp.* in the microbiotic communities of these species. However, it remains to be tested whether any phyla or species are more prevalent or more abundant during the phases of the estrous cycle, or in animals who reproduce successfully. Considering the great amount of diversity and different taxa identified using

16s rRNA sequencing, it would be valuable to explore these avenues with culture-independent analyses.

ACKNOWLEDGEMENTS

The authors are grateful to Robert Brekke and Dr. Glenn Duff for providing access to the BART farm where the study was carried out. Evan Helle, Kendall Green, and Andrew Raftopoulos for their assistance in maintaining the sheep. This study was supported by the Montana Agricultural Experiment Station project (MONB00113) and the multistate project W-3177 Enhancing the competitiveness of US beef (MONB00195).

Table 1. Pregnancy status of animals used in this trial

Species	Animal ID	Pregnancy status ¹
Ewe	J0441	Not mated ²
	J1013	Not mated ²
	J0459	Not mated ²
	J9013	Not mated ²
	J8002	Not mated ²
	J1445	<48 h from first mounting
	J8030	<48 h from first mounting
	J8436	<48 h from first mounting
	J8456	<48 h from first mounting
	J8487	<48 h from first mounting
	J9444	Non-pregnant ¹
	J9017	Non-pregnant ¹
	J0025	Non-pregnant ¹
	J0037	Non-pregnant ¹
	J0447	Pregnant ¹
	J9005	Pregnant ¹
	J9014	Pregnant ¹
	J9404	Pregnant ¹
	J9445	Pregnant
	J8029	Pregnant ¹
Cow	09925	Not mated ²
	10742	Not mated ²
	09836	Not mated ²
	10896	Artificially Inseminated
	06901	Artificially Inseminated
	09808	Artificially Inseminated
	08853	Embryo transfer recipient
	09891	Embryo transfer recipient
	10840	Embryo transfer recipient
	99842	Non-pregnant ¹
	09727	Non-pregnant ¹
	10687	Non-pregnant ¹
	05E16	Non-pregnant ¹
	09703	Non-pregnant ¹
	99791	Pregnant ¹
	10E16	Pregnant ¹
	05X77	Pregnant ¹
	06981	Pregnant ¹
	06988	Pregnant ¹
	02851	Pregnant ¹

¹ Pregnancy status at time of sample acquisition. ² Not mated in the current season

Table 2. Sum of occurrence of *Lactobacillus spp.* identified in cow and ewe ectocervicovaginal lavages.

Lactobacilli	Occurance in Ewes	Occurance in Cows
<i>Lactobacillus acetotolerans</i>	1	0
<i>Lactobacillus acidophilus</i>	1	0
<i>Lactobacillus amylolyticus</i>	0	1
<i>Lactobacillus animalis</i>	1	0
<i>Lactobacillus aviarius</i>	1	1
<i>Lactobacillus capillatus</i>	0	2
<i>Lactobacillus delbrueckii</i>	1	1
<i>Lactobacillus equi</i>	6	7
<i>Lactobacillus equigenerosi</i>	3	1
<i>Lactobacillus hayakitensis</i>	10	1
<i>Lactobacillus helveticus</i>	0	1
<i>Lactobacillus intestinalis</i>	1	0
<i>Lactobacillus kalixensis</i>	0	1
<i>Lactobacillus kunkeei</i>	3	0
<i>Lactobacillus mali</i>	1	0
<i>Lactobacillus oligofermentans</i>	1	0
<i>Lactobacillus spp.</i>	84	67

Figure 1. Shannon's diversity of livestock vaginal microbiota as compared to humans and non-human primates. Boxplots showing the median, quartiles and extremities of Shannon's diversity index values calculated for individual ewes, cows, humans, and non-human primates compared in this study.

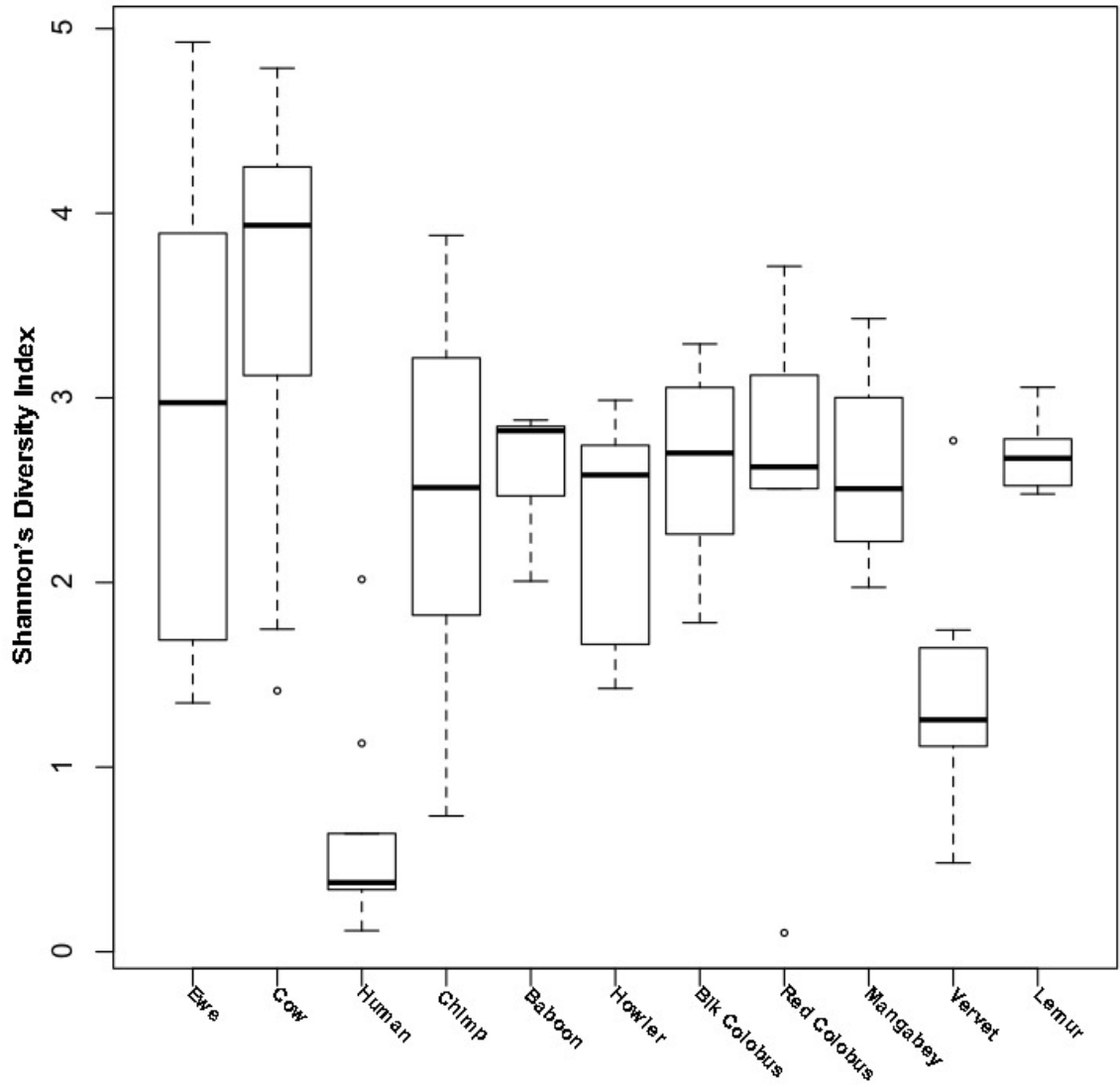


Figure 2. Bray-Curtis relationship among vaginal microbiota of livestock, humans, and non-human primates. Non-metric multi-dimensional scaling plot of Bray-Curtis dissimilarity measures of vaginal microbiota determined from individual ewes, cows, humans, and non-human primates compared in this study.

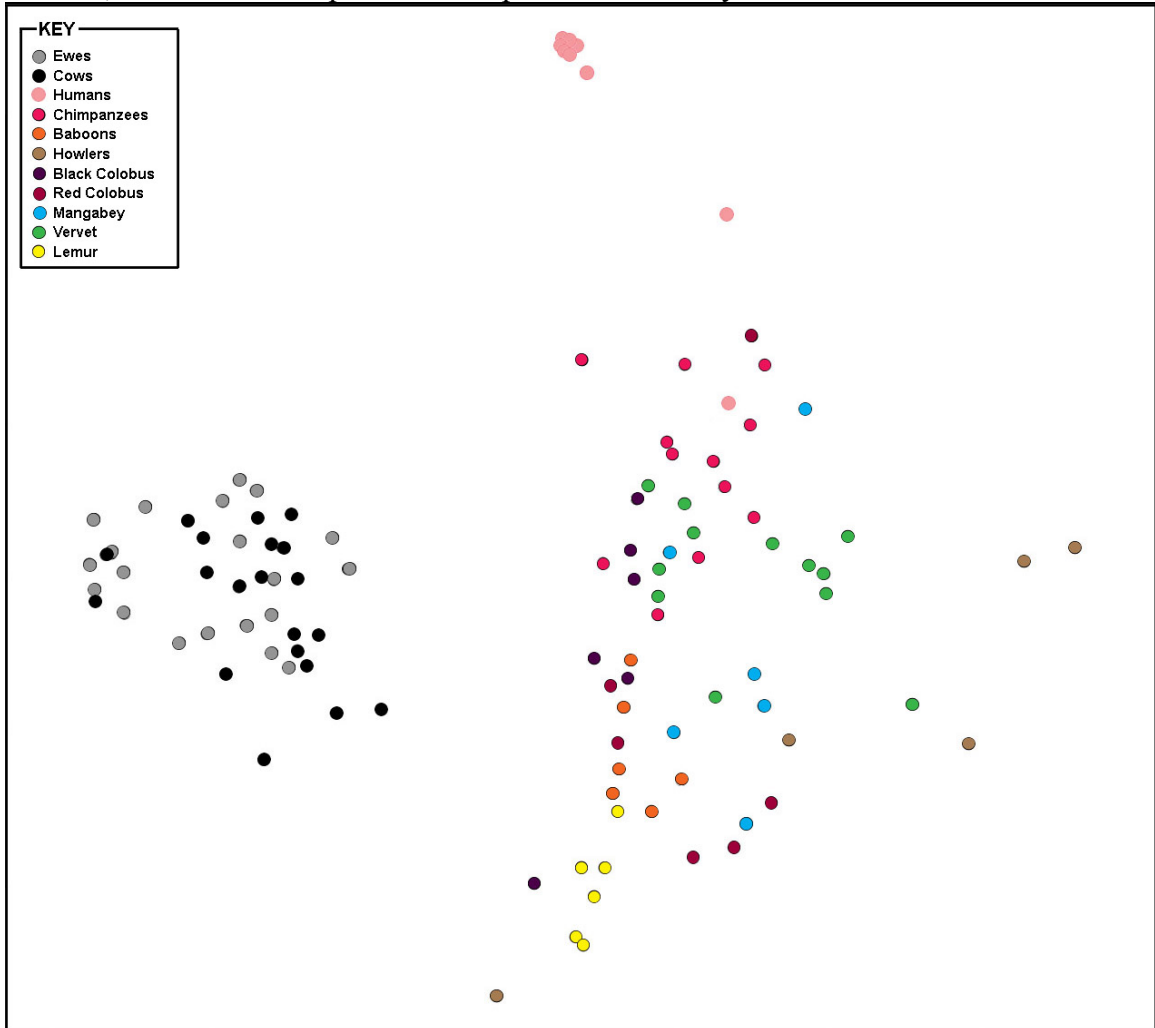


Figure 3. Phylum-level composition among livestock vaginal microbiota. Bar chart showing the proportional distribution of the six most abundant phyla.

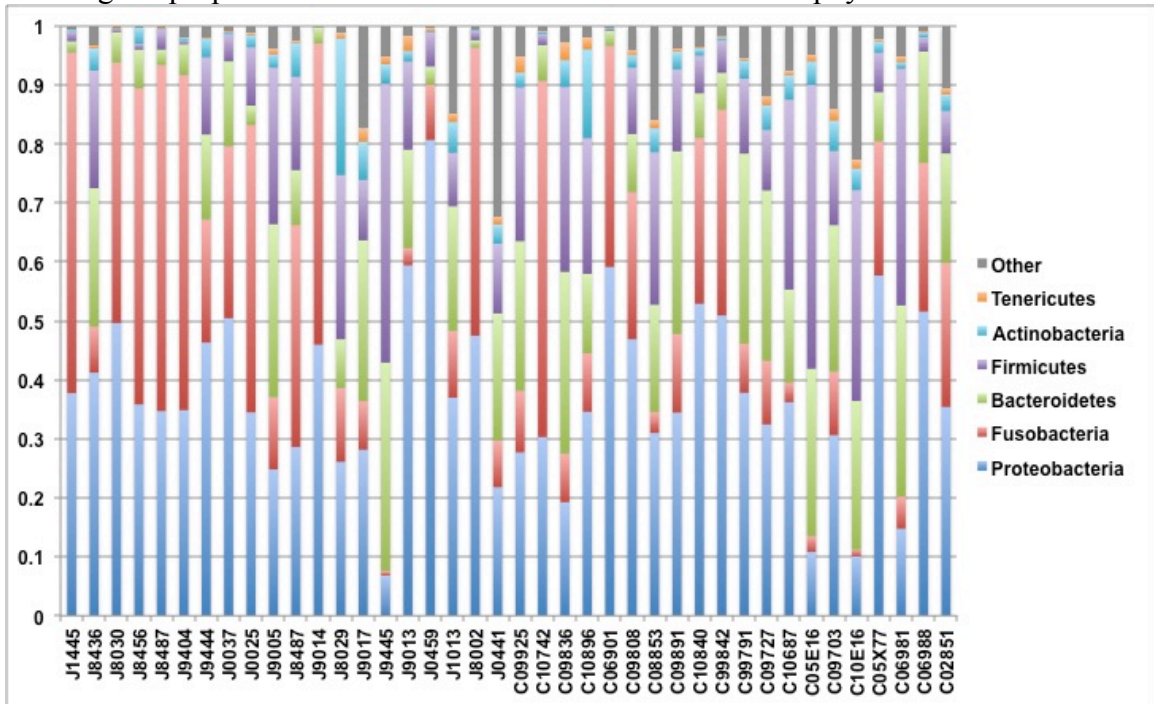
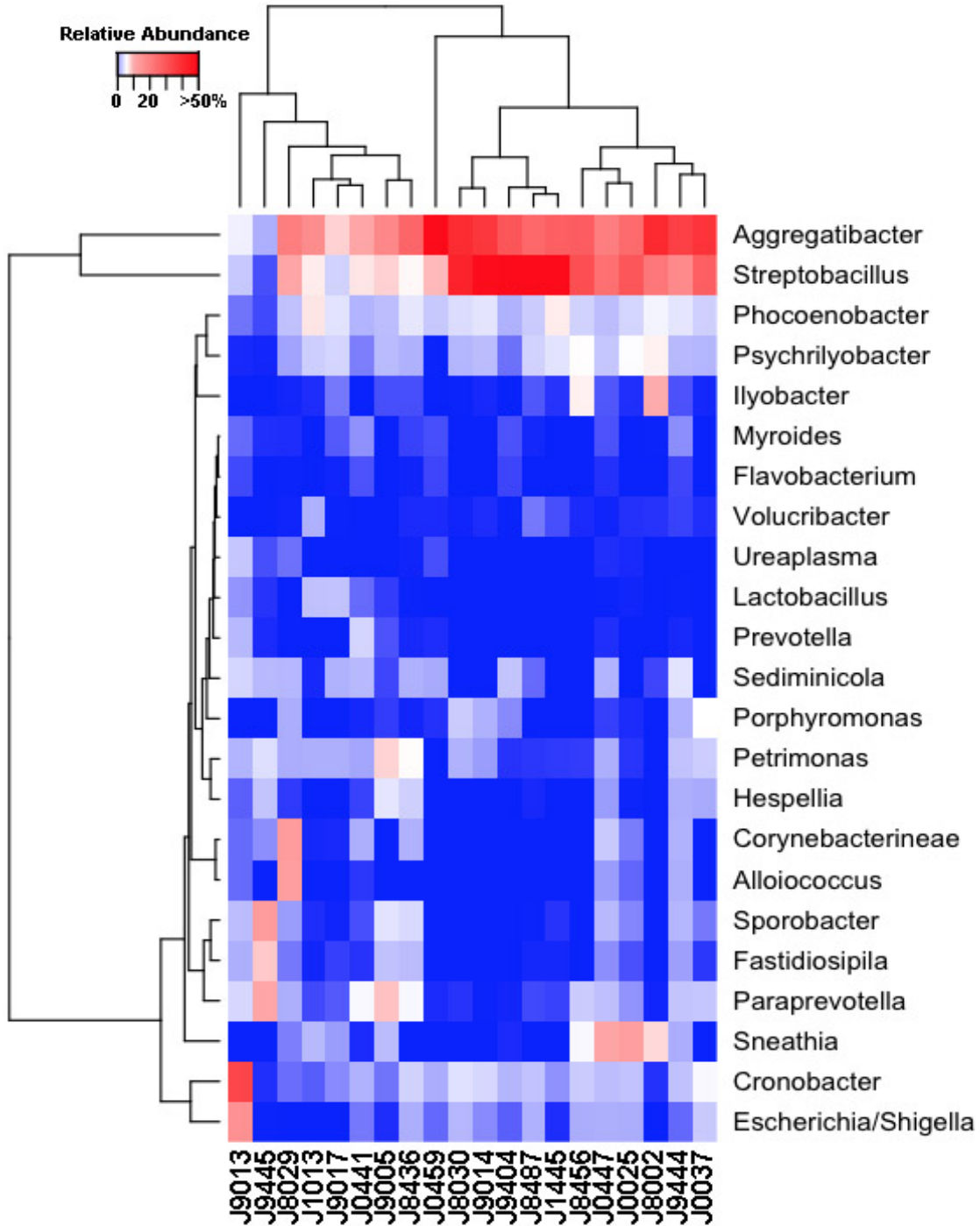


Figure 4. Genus-level composition among ewe vaginal microbiota. Heat map showing the relative abundances of the most abundant genera identified in individual ewe vaginal microbiota. Color breaks in heatmap are adjusted to show genera seen at less than 1% (blue shades), 1-10% (white shades), and greater than 10% (red shades) proportionate abundance.



SUPPLEMENTARY MATERIAL

Table S1. Mean Shannons diversity index values for cow, ewe, human, and non-human primate vaginal microbial communities.

Host	Mean	Median	Std. Dev.	Shapiro-Wilk (ρ)¹	p-value (Ewes)²	p-value (Cows)²
Ewe	2.87	2.97	1.16	0.130	N/A	0.046*
Cow	3.64	3.93	0.96	0.047*	0.046*	N/A
Human	0.64	0.37	0.59	0.011*	<0.001*	<0.001*
Chimpanzee	2.48	2.51	0.92	0.442	0.312	0.004*
Baboon	2.64	2.82	0.35	0.021*	0.790	0.013*
Howler	2.28	2.58	0.69	0.311	0.176	0.001*
Black Colobus	2.63	2.70	0.58	0.586	0.514	0.016*
Red Colobus	2.45	2.63	1.24	0.098	0.485	0.013*
Mangabey	2.61	2.51	0.53	0.875	0.454	0.013*
Vervet	1.37	1.26	0.56	0.152	<0.001*	<0.001*
Lemur	2.70	2.67	0.22	0.377	0.544	0.011*

¹ Data determined to be not normally distributed are indicated with '*'. ² Significant differences between ewe or cow diversity were assessed by two sample t-test for normally distributed data and by Wilcoxon-Mann-Whitney test for data that was not normally distributed. Significance was assessed at $p < 0.05$ and is indicated with a '*'.

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CHAPTER FIVE

SHEEP VAGINAL MICROBIOTA EXHIBIT CORRELATIVE CHANGES WITH
PROGESTERONE CONCENTRATION AND VAGINAL MUCUS
SECRETIONS, BUT NOT REPRODUCTIVE SUCCESS

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Contributions: study design, technical support, revisions.

Contribution of Authors and Co-Authors Continued

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Contributions: funding, study design, study implementation, first draft, revisions.

Manuscript Information Page

Jeffrey D. Swartz, Thomas O'Neill, Medora Lachman, Kelsey Westveer, James G. Berardinelli, Rodney W. Kott, Patrick G. Hatfield, Jennifer M. Thomson, Carl J. Yeoman
Frontiers in Veterinary Medicine

Status of Manuscript: (Put an x in one of the options below)

Prepared for submission to a peer-reviewed journal

Officially submitted to a peer-review journal

Accepted by a peer-reviewed journal

Published in a peer-reviewed journal

Frontiers

Sheep vaginal microbiota exhibit correlative changes with progesterone concentration and vaginal mucus secretions, but not reproductive success

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ABSTRACT

In livestock several microbial pathogens are known to cause spontaneous abortions. However, it remains to be determined if the vaginal microbiota can impact reproductive outcomes as has been demonstrated in humans. This study examined temporal variation in the vaginal microbiota of 15 ewes that were pregnant, non-pregnant, or aborted from before breeding through lambing. Ewes were exposed to rams marked with brisket paint for a total of 36 days. Pregnancy status was determined by the successful production of a lamb, while pregnancy was evaluated by ultrasound approximately 60 days after ewes were marked by a ram, and by temporal serum progesterone concentrations. One ewe (J1013) was found to have aborted between samples collected 90 and 120 days after being marked by a ram. Seven ewes that failed to get pregnant were matched by age, weight at breeding, and breeding ram to ewes that successfully became pregnant and lambled. Vaginal microbial samples were collected by ectocervicovaginal lavage 22 and 1 days before exposure to rams, within 48 hours of first being marked (M) by a ram (M+1), 7 days later (M+7), and approximately every 30 days

from first marking, denoted by M+30, M+60, M+90, M+120, and M+150. Variable regions 3 and 4 of the 16s ribosomal RNA genes of microbiota in lavages were amplified by PCR and sequenced using an Illumina MiSeq. Reads were filtered using mothur, and classified with RDP Classifier. All α -diversity metrics were derived from genus-level taxonomic classifications. Analysis of similarities (ANOSIM) based on composition across sampling periods did not vary ($P > 0.05$) by individual ewe, or by ewe line, or with age of ewe. The ANOSIM indicated ($R = 0.07$; $P \leq 0.05$) there were small differences between samples collected from ewes that were gestating, and samples from ewes that were pre-gestating or non-pregnant ewes. The ANOSIM also indicated ($R = 0.11$; $P \leq 0.05$) there were small differences in composition of the microbiota by sampling day. However, composition across sampling periods was not different ($P > 0.05$) for ewes who became pregnant compared to those who remained non-pregnant as a final outcome. In conclusion, we were unable to determine with genera taxonomy that community composition differed across time between non-pregnant and pregnant ewes.

INTRODUCTION

The vaginal microbiota of humans has well described impacts on reproductive performance (1). Perturbations to the vaginal microbiota can affect the menstrual cycle, fertility, and ability of women to carry a fetus to term (2-6). Bacterial vaginosis, a disorder associated with a shift to an atypical vaginal microbiota, has been determined to increase the risks of preterm-birth by 218%, and spontaneous abortion by 632% (7). Changes in the vaginal microbiota also correspond with dramatic shifts in the metabolic environment of the vagina (8) and in vaginal pH (9-11). The chemical environment of the vagina has been shown to be of critical importance to sperm motility, and sperm and embryonic survival in a variety of animals including livestock species (2,12-14). Therefore, because of the microbiota's ability to influence the chemical environment of

the vagina, the microbiota may influence reproductive outcomes by directly impacting sperm motility and survival. Further, because the biochemical effects of the vaginal microbiota have been shown to extend to the uterus they can also cause implantation failure (3). Embryo transfer experiments of twin embryos have clearly demonstrated the variable capabilities of the livestock uterine environment to support pregnancy (15); though it remains to be explored if this is affected by differences in the microbial-influenced vaginal and uterine environments. Despite the potentially critical role of the vaginal microbiome in reproductive performance, and the importance of reproductive performance to the economic sustainability of livestock agriculture, little is known about the vaginal microbiota of livestock. Recently, Gatti et al. (16) used a culture-based approach and reported $3.5 \log$ aerobic colony forming units ml^{-1} associated with vaginal mucous in the ewe vagina before insertion of intravaginal sponges for estrous synchronization. Also, it is known that opportunistic pathogens can be present in the vaginal microbiota of the cow and ewe that limit reproductive success (17,18), and nonspecific bacterial infections are known cause infertility in cattle (19). However, knowledge of the community structure present in livestock vaginal tracts related to reproduction has been severely limited by a reliance on culture-based techniques, as it is well documented that culture-dependent techniques often only partially reveal the diversity present in microbial communities (20-22). Our compositional understanding has also been limited by research focusing on a discrete number of pathogens. Swartz et al. (unpublished data) provided the first culture-independent characterization of the vaginal microbiota of livestock, specifically sheep and beef cattle. Herein, we provide the first

complete, deep, temporal exploration of the vaginal microbiota of a major livestock species across a breeding season and attempt to unravel questions about the factors that influence the vaginal microbiota composition of the ewe, and test the hypothesis that variation in vaginal microbiota of the ewe can influence reproductive outcomes.

MATERIALS AND METHODS

Ethics Statement

Animal care and use protocols were approved by the Montana State University Agricultural Animal Care and Use (AACUC) committee under protocol number 2012-AA07 dated 09/20/2012.

Sampling

Fifty nulli-, primi-, and multiparous Rambouillet ewes, ages 1 to 4 years (Table 1) were maintained from one month before breeding through lambing at the Bozeman Agricultural Research and Teaching Facility. Ewes were single-sire mated to Rambouillet rams for 18 days, followed immediately by exposure to Suffolk rams for another 18 days. Mating attempts were monitored by brisket paint, which was applied to rams prior to introduction to ewes and every 3 days thereafter. All ewes showed signs of paint (mounting activity) in the first 18 days of the breeding season. Vaginal microbial samples were collected by ectocervicovaginal lavage of sterile physiological saline (0.8% NaCl, w/v) twice before introduction of rams (days -22 and -1), within 48 hours of first being marked (M) by a ram (M+1), 7 days later (M+7), and approximately every 30 days from

first marking, denoted by M+30, M+60, M+90, M+120, and M+150. The contents of the uterus of each ewe was examined by ultrasound per rectum between 52 to 66 days after being marked with brisket paint by a fertile ram. Pregnancy status was also assessed by progesterone concentrations in serum samples collected by jugular venipuncture on days M+30, M+60, M+90, and M+120 of gestation. Progesterone concentrations were determined in duplicate using solid-phase RIA kits (Siemens Healthcare Diagnostics, Los Angeles, CA). Failure to lamb and a concurrent absence of continually elevated progesterone concentrations over the sampling days were used to determine failure to maintain a pregnancy, and to identify ewes that aborted embryos or fetuses. Evaluation of progesterone concentrations over the sampling period indicated that 1 ewe (J1013) aborted a fetus between sampling days M+90 and M+120. Seven ewes failed to become pregnant, and these ewes were matched by age, weight at breeding, and breeding ram to ewes that successfully lambed (Table 1). Vaginal samples were collected by ectocervicovaginal lavage described by Yeoman et al. (2013). Samples were collected by injecting 25 ml of sterile saline into the vaginal tract using sterile catheters attached to luer-lock 60ml syringes. Saline was injected in a continuous stream towards the cervix, aspirated 3 to 5 times, then transferred to a sterile 15 ml conical centrifuge tube (Falcon®, ThermoFisher, Waltham, MA), and stored at -20 °C. The presence or absence of mucus was evaluated by visual appraisal. Vaginal pH was determined using an Orion 520A pH meter fitted with a ROSS Ultra Electrode (Thermo Scientific, Waltham, MA, USA). Samples for all animals were used for analysis of pH, except samples obtained at M-22 because of the possibility that the lavage solution contained phosphate buffer.

Lavages were centrifuged for 5 min at 20,000 x g and 4 °C and the supernatant analyzed for pH.

Sequencing Microbiota

For DNA extraction, up to 4.5 ml of sample was centrifuged for 5 min at 20,000 x g and 4 °C. Pellets were extracted using MoBio PowerSoil DNA Isolation kits following manufacturer instructions. The PCR reaction was run for 25 cycles to amplify variable regions 3 and 4 of the 16s ribosomal RNA (rRNA) genes. The reaction ran at 94 °C for 20 s, 52 °C for 30 s, and 72 °C for 45 s using custom primers. These included eight nucleotide (nt) indexes for sample identification after sequencing (SeqF(1-8) 5'-AATGATACGGCGACCACCGAGATCTACAC(adaptor)-Index2(one of eight different 8nt codes used to distinguish among samples when pooled for sequencing)-TATGGTAATT(sequencing primer pad)-AT(linker)-CCTACGGGAGGCAGCAG(341f primer)-3' and SeqR(1-12) 5'-CAAGCAGAAGACGGCATAACGAGAT(adaptor)-Index1 (one of twelve different 8nt codes used to distinguish among samples when pooled for sequencing)-AGTCAGTCAG(sequencing primer pad)-CC(linker)-GGACTACHVGGGTWTCTAAT(806r primer-3'). Amplicons were quantified using an Agilent 2200 TapeStation (Agilent, Santa Clara, CA, USA) and pooled at an equimolar concentration. Pooled amplicons were purified from PCR reagents and non-specific amplification products using an agarose gel and QIAquick gel extraction kit (Qiagen, Valencia, CA, USA) following the manufacturer's instructions. Purified and pooled amplicons were subsequently quantified using a KAPA Syber quantification kit

(KAPABiosystems, Wilmington, MA, USA) as per manufacturer instructions. Purified, quantified and pooled amplicons were mixed with 5 to 10% equimolar concentration PhiX and sequenced at 12.5 pM. Sequencing was performed with an Illumina MiSeq using paired-end 2 by 250 nt dual-index sequencing. Custom primers (R1 5'-CCTACGGGAGGCAGCAG-3', R2 5'-AGTCAGTCAGCCGGACTAC-3', Index 5'-GTAGTCCGGCTGACTGACT-3') were used for sequencing and indexing.

Data Analyses

Resulting paired-end 16s rRNA gene reads were assembled using the 'make.contigs' command in mothur (23). Any base call disagreement in paired read overlap was ascribed to the base with a higher quality score ('deltaq = 1'). Assembled sequences were then filtered to remove low quality and chimeric sequence data. Briefly, sequences were removed if they were shorter than 400 nt, had a homopolymeric sequence greater than 10 base calls, had more than 2 ambiguous base calls (1 per 200nt), or were found to be chimeric by UCHIME (24). To enable direct comparison, each resulting sequence data set was subsampled to 5,000 reads. Reads were taxonomically assessed using mothur's implementation of the naïve Bayesian classifier, RDP Classifier (25). Taxonomic assignments were considered supported if bootstrapping values were greater than 70%.

Microbial composition and diversity were compared among samples using multivariate statistical approaches provided in the vegan package of R (26). Sequencing coverage was estimated by Good's non-parametric coverage estimator (27). Because the

majority of sub-genus level classifications had bootstrap values under 70%, analysis was performed using genus-level taxonomic classifications. Temporal and between group similarities of ewe vaginal microbiota were determined by pairwise measurements of Bray-Curtis dissimilarity. Significance was examined using analysis of similarities (ANOSIM) to non-parametrically test variation between and within groups by rank order similarity relationships ($R = 0$ when within and between relationships equal; 28). Total richness estimates were obtained by Chao1 analyses (29) on rarefied data, and diversity was determined using Shannons diversity index. Taxa defining between class differences were obtained by similarity percentage (SIMPER; 28). Tests of significance for differences in relative abundances of individual genera among groups (grouped by age, line, day of sampling, pregnancy status, pregnancy outcome, or observations of mucus) were performed using false discover rate corrected p-values obtained from Welch's t-tests. Pearsons and Spearmans correlation coefficients were generated to evaluate associations between progesterone concentrations and genera, presence or absence of vaginal mucus and genera, and vaginal pH and progesterone concentrations. Observations of mucus were tested against vaginal pH and progesterone concentrations using Welch's Welch's t-tests.

RESULTS

Sequencing Overview

Samples were sequenced, and 917,544 high quality reads were obtained. Individual samples were randomly subsampled to 5,000 reads. Good's Coverage estimates were 98.22 ± 1.10 % (range 93.5 – 99.7 %) for all samples after rarefaction.

Temporal Changes in α -Diversity of Ewe Vaginal Microbiota

All α -diversity metrics were derived from genus-level taxonomic classifications. Measures of diversity were similar to those determined in our preceding study (Swartz et al., unpublished data), and did not change ($P > 0.05$) between successive sample points (Table 2). However, vaginal microbial diversity was greater ($P \leq 0.05$) at M+120 in pregnant ewes than in non-pregnant ewes. However, diversity did not differ ($P > 0.05$) between pregnant and non-pregnant ewes on other sampling days (Table 2). Greater microbial diversity in pregnant than non-pregnant ewes was not correlated ($P > 0.05$) with serum progesterone concentrations using either a linear (Pearsons) or rank-sum (Spearman's) test of correlative relationship. Closer examination of these data indicated this was probably due to substantial inter-individual variation in vaginal microbial diversity at low progesterone concentrations.

Compositional Changes

Consistent with our previous observations (Swartz et al., unpublished data), *Aggregatibacter spp.* and *Streptobacillus spp.* were typically the most abundant genera, with an average abundance of $36.6 \pm 21.3\%$ and $19.6 \pm 14.6\%$, respectively. Both taxa were present in samples from all ewes, and when abundance was rank-ordered, these genera were present within the top 2 in 78% and 56% of samples from all ewes, respectively. When the rank-order was expanded to the top 5 genera, *Aggregatibacter spp.* and *Streptobacillus spp.* were present in 89% and 82% of samples, respectively. Samples in which *Aggregatibacter spp.* and *Streptobacillus spp.* were not in the top 5 most abundant were from non-pregnant ewes in 71% and 48 %, or in samples from pregnant ewes following parturition in 29% and 36%, respectively. By separate analysis of similarities (ANOSIM), the composition of ewe vaginal communities over time did not differ by individual ewe (ANOSIM $R < 0$, $P > 0.05$), by ewe line ($R = 0.01$, $P > 0.05$), or with age of the ewe ($R < 0$, $P > 0.05$), suggesting a common ewe vaginal microbiota. The ANOSIM indicated ($R = 0.07$; $P \leq 0.05$) there were small differences between microbiota of vaginal samples collected during gestation and microbiota of vaginal samples collected from non-pregnant and pre- or post-gestation samples of ewes that lambed. The ANOSIM indicated ($R = 0.11$; $P \leq 0.05$) there were small differences in composition of the microbiota on sample day. However, composition across sampling periods was not different ($P > 0.05$) for ewes who became pregnant compared to those who remained non-pregnant as final outcome. ANOSIM also indicated vaginal microbiota co-varied with presence or absence of mucus secretion in the vaginal lavage samples (ANOSIM R

= 0.16, $P \leq 0.05$). Similarity percentage (SIMPER) analyses indicated that the relative abundances of *Aggreratibacter spp.* (19% of total explanatory power), *Streptobacillus spp.* (13%), and *Escherichia/Shigella spp.* (7%), were most dissimilar between non-pregnant and pregnant ewes in samples collected during exposure of ewes to rams, and when all samples for each ewe were analyzed together. However, pregnant and non-pregnant ewe microbiota did not have genera that differed ($P > 0.05$) in abundance in samples collected during exposure of ewes to rams, or when samples for each ewe were analyzed together.

Correlative relationships between vaginal microbial genera and mucus, pH, and systemic progesterone concentrations were investigated using Spearman's and Pearson's correlations. The ANOSIM indicated that when vaginal mucus was observed in lavage samples, compositional differences were detected in the vaginal microbiota. The relative abundances of several taxa were found to exhibit consistent decreases after ewes were marked by rams (from M1 – M120), including *Xenophilus spp.* (Spearman's $\rho = -0.41$), *Olsenella spp.* ($\rho = -0.33$) and *Pantoea spp.* ($\rho = -0.30$). Progesterone concentrations had positive rank or linear relationships with several microbial taxa (Table 3). *Brevundimonas spp.* showed the strongest Spearman's correlation to progesterone at $\rho = 0.54$ ($P < 0.05$). *Lactobacillus spp.* and *Sediminicola spp.*, two genera previously detected in ewe vaginal lavages (Swartz et al., unpublished data), exhibited correlative relationships ($P < 0.05$) by Spearman's correlation to progesterone concentration ($\rho = 0.32$ and $\rho = 0.41$, respectively). *Lactobacillus spp.* (Pearson's $R = -0.30$), and *Stentrophomonas spp.* ($R = -0.33$), each showed a negative correlation ($P < 0.05$) with increasing vaginal pH. No

difference ($P > 0.05$) was found between progesterone concentrations or pH from samples with and without reported mucus. However, a significant (Pearsons $R=-0.39$, $P < 0.05$) negative correlation was found between vaginal pH and progesterone concentrations for all sampling times.

DISCUSSION

The vaginal microbiota of non-pregnant and pregnant ewes was investigated temporally from before breeding to after lambing. Our previous finding (Swartz et al., unpublished data) that *Aggregatibacter spp.* and *Streptobacillus spp.* are among the most abundant genera was confirmed. The vaginal microbiota of ewes appears to be shared across age, individuals, and between lines. No bacterial genera were determined to exhibit differences in relative abundance ($P > 0.05$) between ewes that did not breed successfully and those that produced lambs. It has been reported in a culture-based study that frequency of occurrence of four families of bacteria and the genera *Corynebacterium* did not differ between repeat breeder and fertile cows, although in contrast to the present study, they found that total counts of *Corynebacterium* and Enterobacteriaceae were higher in repeat breeder cows than in fertile cows (19).

Differences were seen in microbial diversity between pregnant and non-pregnant ewes at M+120, but diversity did not differ between these classes at the other sampling times. One hypothesis that may explain this result could be that diversity was related to progesterone concentrations, since samples were obtained in March when ewes in Montana are usually anestrus (30). However, progesterone concentrations were not

correlated with differences in vaginal microbial diversity using Pearsons and Spearmans correlations.

The microbiota of pregnant and non-pregnant ewes did not differ using ANOSIM. Microbial composition was found to differ with sample day, as well as in samples taken during gestation compared to all non-gestating samples of both non-pregnant and pregnant ewes. However, low ANOSIM R-values associated with these differences indicate that the microbiota had limited compositional changes. In addition, microbiota co-varied with observations of mucus secretion. Various lab species, bitches, and cows have been reported to have increased microbial populations during estrus (31-35), although not every study was able to link microbial populations to stages of the estrous cycle (18). As a hormone of pregnancy and estrous cyclicity (36), it could be hypothesized that progesterone was causing or co-varying with these effects. Consistently, we observed correlation between progesterone concentration and relative abundance of several genera, including *Brevundimonas spp.*, *Myroides spp.*, and *Lactobacillus spp.* (Table 3). The genus with greatest correlation was *Brevundimonas spp.* Interestingly, this genera has been shown to convert estradiol-17 β to estrone in isolation, without the benefit of metabolites from other bacteria (21). We did not find a difference in pH or progesterone concentrations with observations of vaginal mucus. There was a negative correlation between vaginal pH and progesterone concentrations. *Lactobacillus spp.* were positively correlated with progesterone concentrations, while also negatively correlated with pH. *Lactobacillus spp.* are credited with developing and maintaining the typically low vaginal pH environment of the human vagina (37). It could

be hypothesized that *Lactobacillus spp.* increased in response to changes in systemic progesterone and through their homolactic metabolism caused a decrease in vaginal pH. However, given their low abundance in ewes relative to that reported for women (Swartz et al., unpublished data), that *Lactobacillus spp.* are causally related to this drop in pH seems doubtful.

In conclusion, progesterone concentration and the observation of mucus in vaginal lavages correlated with changes in relative abundance of several microbial genera. However, Rambouillet ewes that became pregnant and ewes that remained non-pregnant did not exhibit significant compositional differences in their microbial communities. These analyses were performed at the genera level, and future research is needed to determine the variety of species or strains found in the vaginal tract. A more globally oriented study of the diversity of microbiota may provide a more robust test of important differences between pregnant and non-pregnant ewes not found at the genera level that could impact reproductive success and the prevention of disease in sheep.

Table 1. Ewe ear tag, line, age, weight at breeding, rams exposed to for breeding (Rambouillet/Suffolk ram), and number of lambs born from each ewe sampled by ectocervicovaginal lavage for vaginal microbiota.

Ewe	Line	Age	Weight (lb)	Breeding Rams	Lambs
OPEN EWES					
J0025	Low	2	119.6	J0005 / O1187	0
J0033	Low	2	141.1	J0005 / O1187	0
J0037	Low	2	99.1	J0005 / O1187	0
J1026	Low	1	107.2	J0005 / O1187	0
J9017	Low	3	140.6	J009 / O251	0
J1469	High	1	98.0	J0437 / O251	0
J9444	High	3	112.5	J0437 / O251	0
Avg. +/- Std. Dev.			116.9 +/- 18.0	N/A	0 +/- 0
MATCHED EWES					
J0004	Low	2	100.3	J0005 / O1187	1
J0017	Low	2	141.3	J009 / O251	1
J0032	Low	2	133.6	J009 / O251	2
J1006	Low	1	100.1	J0005 / O1187	1
J9003	Low	3	137.1	J0005 / O1187	2
J9013	Low	3	117.8	J0005 / O1187	1
J1435	High	1	123.3	J0437 / O251	2
J9407	High	3	115.3	J0437 / O251	2
Avg. +/- Std. Dev.			121.1 +/- 15.8	N/A	1.6 +/- 0.5
OTHERS					
J1013	Low	1	109.1	J009 / O251	0 (aborted)

Table 2. Shannon's Diversity Index values, and relationships between open and pregnant ewes and current and preceding time points within pregnancy status.

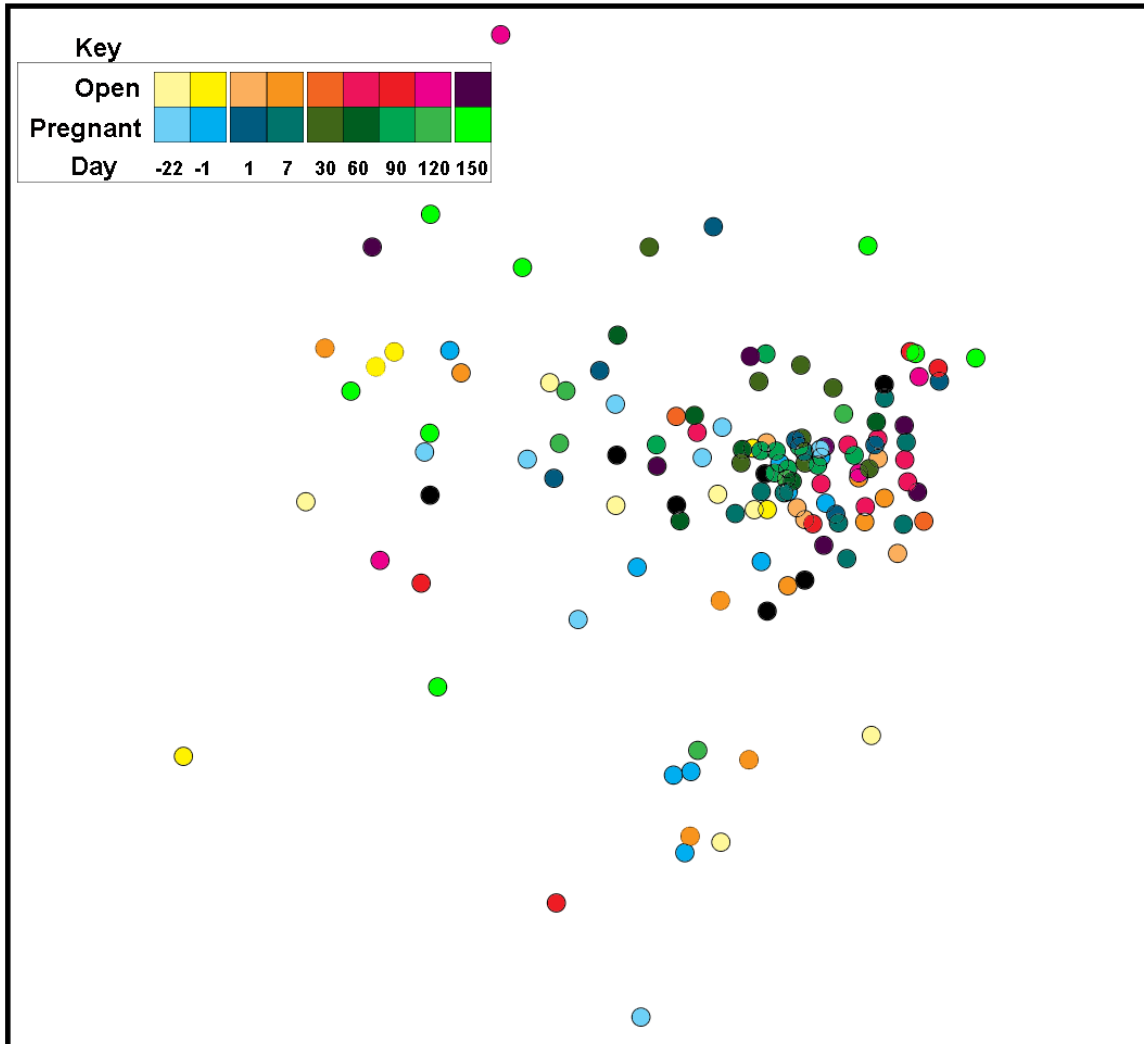
Pregnancy Result ¹	Period	Mean	Median	Std. Dev.	Shapiro-Wilk (p) ²	Temporal p ³	Preg_Result p ⁴
Open	M-22	2.80	3.02	1.16	0.44	0.776	0.613
Pregnant	M-22	3.11	3.42	1.06	<0.05*	0.235	
Open	M-1	3.00	2.81	1.24	0.55	N/A	0.434
Pregnant	M-1	2.50	2.32	0.97	0.64	N/A	
Open	M+1	2.19	2.28	0.61	0.89	0.267	0.699
Pregnant	M+1	2.42	2.30	1.41	0.67	0.279	
Open	M+7	2.01	1.60	0.99	<0.05*	0.432	0.999
Pregnant	M+7	1.91	1.80	0.79	0.58	0.397	
Open	M+30	2.87	2.72	1.32	0.86	0.268	0.632
Pregnant	M+30	2.56	2.68	0.43	0.16	0.083	
Open	M+60	1.93	1.71	0.89	0.31	0.217	0.099
Pregnant	M+60	2.80	2.83	0.89	0.80	0.518	
Open	M+90	1.76	1.66	1.05	0.70	0.781	0.058
Pregnant	M+90	2.99	2.93	0.57	0.28	0.625	
Open	M+120	1.81	1.74	0.78	0.36	0.928	0.050*
Pregnant	M+120	2.88	2.82	0.85	0.57	0.791	
Open	M+150	2.40	2.37	0.98	0.87	0.278	0.330
Pregnant	M+150	3.05	3.47	1.48	0.49	0.791	

¹ Pregnancy result indicates success or failure to become pregnant across the complete breeding season. ² Data determined to not be normally distributed are indicated with an '*'. Subsequent tests for significance temporally (3) or by pregnancy result (4) assessed by two sample t-test for normally distributed data and by Wilcoxon-Mann-Whitney test for data that was not normally distributed. Significance was assessed at $p \leq 0.05$ and is indicated with an '*'. ³ Examines significance within pregnancy result (1; Open or Pregnant) between the preceding and current measures of diversity. ⁴ Examines significance between groups of ewes classified by pregnancy results (1; Open vs. Pregnant) at the current sample time point.

Table 3. Vaginal microbial genera whose relative abundance correlates with systemic progesterone concentration. All reported correlative relationships were found to be significant ($p \leq 0.05$).

Genera	Spearman's ρ	Pearson's R
<i>Brevundimonas spp.</i>	0.54	0.40
<i>Myroides spp.</i>	0.47	N/A
<i>Flavobacteria spp.</i>	0.47	N/A
<i>Flavobacterium spp.</i>	0.45	N/A
<i>Sediminicola spp.</i>	0.41	N/A
<i>Stentrophomonas spp.</i>	0.39	0.42
<i>Fusobacterium spp.</i>	0.38	N/A
<i>Phenylobacterium spp.</i>	0.37	0.30
<i>Sphingobacterium spp.</i>	0.36	N/A
<i>Ralstonia spp.</i>	0.33	N/A
<i>Azobacter spp.</i>	0.33	N/A
<i>Kaistella spp.</i>	0.33	0.31
<i>Caenimonas spp.</i>	0.33	0.35
<i>Lactobacillus spp.</i>	0.32	0.33
<i>Streptophyta spp.</i>	0.32	N/A

Figure 1. A NMDS plot of open and pregnant ewe microbial communities using Bray-Curtis dissimilarity matrix. The ANOSIM provided weak support for clustering by ewe pregnancy status at time of sampling (pregnant or not pregnant; $R = 0.07$; $P \leq 0.05$) and day of trial ($R = 0.11$, $P \leq 0.05$), but not for final pregnancy outcome (pregnant, open, or aborted; $R < 0$, $P > 0.05$) of ewes.



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CONCLUSION

Variations exist in the reproductive performance of agricultural animals that impact the sustainability and financial success of livestock operations. We employed a multifaceted approach to determine measurable factors that could account for this variation. As a primary model we used the Montana Agricultural Experiment Station's Rambouillet sheep flock that had been selected for high or low reproductive rates for 44 years. In the present study, there was no statistical difference in lambing rate between ewes of these lines. Nevertheless, high line ewes continue to produce more lambs, yielding a greater total birth weight of lamb than that of low line ewes. This is consistent with the results reported by Schoenian and Burfening (1990) more than 20 years ago. Average intake of feed per ewe from breeding through gestation did not differ between lines, and as a result high line ewes were more efficient at converting total digestible nutrients during this period into kg of lambs at birth. One could hypothesize that different systemic concentrations of energy-related metabolites and metabolic hormones might play a role in these determining these differences between ewes of these line; allowing high line and low line ewes to produce more or less lambs, respectively. However, the results of the present study did not support that hypothesis. Surprisingly, systemic concentrations of progesterone increased to greater concentrations after d 90 of gestation in high line ewes than in low line ewes. This is the first report of a line difference in progesterone concentrations during pregnancy between ewes of these lines. Whether differences in systemic progesterone concentrations were caused by differences in the

synthesis or catabolism of progesterone between ewes of these lines could not be determined in the present study.

Independent of effect of nutrition, we explored the microbiota that colonize the vaginal tracts of livestock for their potential role in reproductive success in livestock species. Results in this area from human studies have firmly established that vaginal microbiota can impact reproductive success. Unfortunately, at the outset of this project there were no reports describing the composition of livestock vaginal microbiota using culture-independent analyses techniques, such as sequencing of the 16s ribosomal RNA gene content present in vaginal samples. Therefore, one of the aims of the present study was to determine the composition of, and individual variation in, vaginal microbiota of Rambouillet ewes and cross-bred cows. The results of the present study indicated that the predominant microbial taxa reported from culture-based studies available in the literature were only found at low abundance. While cows and ewes exhibited inter- and intra-specific variation, they did not differ significantly from each other. *Aggregatibacter spp.* and *Streptobacillus spp.* were found to dominate both cow and ewe vaginal microbiota, whereas, *Lactobacillus spp.*, which predominate in the healthy human vagina, were in very low abundance. An important finding from the present study was that the vaginal microbiota of these two livestock species differs significantly from other vaginal systems described in both humans and non-human primates.

To determine if the vaginal microbiota of sheep influence reproduction, 15 ewes that had aborted, failed to become pregnant, or successfully produced a lamb were analyzed. The Rambouillet ewes that became pregnant and those that were not pregnant

did not exhibit significant differences in their vaginal microbial communities. However, we noted the presence or absence of visible mucus in samples, and found mucus to be positively correlated with changes in vaginal microbiota. Additionally, when all samples, taken from ewes during gestation, were compared to samples from all pregnant and non-pregnant ewes at non-gestating time points, there was a small but significant difference in their vaginal microbiota compositions. Likewise, vaginal microbiota composition of ewes was affected by sampling day: at 120 days after being marked by rams we observed significant differences in microbial diversity between non-pregnant and pregnant ewes. Through monitoring progesterone in our first study, we knew progesterone concentrations peaked around this time, leading us to hypothesize that hormonal changes associated with pregnancy, such as progesterone concentrations, may be responsible for these differences in vaginal microbiota between pregnant and non-pregnant ewes. Testing Pearson's and Spearman's correlative relationships of individual microbial taxa with serum progesterone concentrations revealed positive relationships with several microbial genera. However, as the lines did not have different compositions, and non-pregnant and pregnant ewes did not differ, it would appear that at this level of taxonomic-resolution, failure of ewes to get pregnant cannot be ascribed to the vaginal microbiota, nor can they explain differences in reproductive rate between the high and low line ewes.

In conclusion, results for litter size and total kg of lamb born per ewe confirmed that the use of the selection index has maintained a difference in reproductive rate between these lines of Rambouillet sheep. The ~25% increase in kg of TDN intake during gestation per kg of lamb born per ewe of the low line relative to that of ewes in the

high line, represents a dramatic difference in reproductive efficiency; with important implications for increasing profitability of sheep production. In addition, Rambouillet ewes that became pregnant and those that remained open did not exhibit significant differences in their microbial communities. These analyses were performed with genus-level resolution of taxonomic composition and may not have detected important features occurring at the species, strain or molecular levels. Future research should explore species or strain-level variation, changes in microbial gene content (metagenomics), or gene-regulation (metatranscriptomics), or their metabolic impact (metabolomics) before the vaginal microbiota can be considered to not influence reproductive performance, or suppress disease associated with reproductive performance, in livestock species.

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