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Impact of the Estrous Cycle on the Vaginal Microbiota and Its Association to Pregnancy Rates in Dairy Cows

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The Impact of the Estrous Cycle on the Vaginal Microbiota and Its Association to Pregnancy Rates in Dairy Cows

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Résumé

Plusieurs recherches ont été conduites pour déterminer les phases du cycle œstrus chez les vaches. Toutefois, peu est connue de leur microbiote vaginal. Cette étude a pour but de déterminer la composition du microbiote vaginal de vaches laitières durant les quatre phases du cycle œstrus, pour détecter son impact sur la fertilité de ces vaches. Cette information nous permettra d'un jour manipuler le microbiote vaginal d'autres vaches afin d'améliorer leur taux de fécondité. Vint-et-une vaches Holstein multipares de la même ferme ont subi l'insémination artificielle (IA) suite à la détection du début des chaleurs. Quatre frottis vaginaux ont été faits aux jours 1,3,15 et 19 du cycle. Au jour 31, une échographie a confirmé que neuf vaches étaient enceintes. Les données ont ensuite été analysées avec avec le séquençage rRNA 16S de la région hypervariable V4. Les résultats ont montré une prédominance nette de certains embranchements bactériens. Les firmicutes, bacteroidetes et protéobacteéries composait plus de 80% de la population microbiotique vaginale avec certaines fluctuations importantes. Une analyse statistique a déterminé qu'il y a eu des changements significatifs entre l'œstrus et le proestrus chez les vaches qui ne sont pas devenues enceintes de (P=0.028), et entre l'œstrus et le dioestrus chez les vaches enceintes de (P=0.043). Cette recherche est un premier pas important dans l'identification du microbiote vaginal et son impact possible sur la santé vaginale et la fertilité de vaches laitières. Cette recherche pourrait contribuer aux études futures tentant d'améliorer la fécondité bovine avec la manipulation du microbiote vaginal.

Mot clés: **Holstein, vaches laitières, 16S, rRNA, séquençage, V4, microbiote, vaginal, œstrus**

Abstract

A great deal of research has been done on the four phases of the estrous cycle in cows, yet very few studies exist regarding their vaginal microbiota. This study explored the various differences in the vaginal microbiota in pregnant and non-pregnant multiparous Holstein dairy cows. This information will allow us to see what a healthy vaginal microbiota looks like in pregnant cows and to someday try to achieve similar microbiomes in other cows to aid in fertility issues on farms. The objective of this study is to investigate variations in microbiota populations during the estrous cycle and their possible association with pregnancy rates using next generation sequencing of the V4 hypervariable region of the 16S rRNA gene. Twenty-one multiparous Holstein cows on the same farm underwent artificial insemination (AI) after estrous detection. Vaginal swabs were collected four times, on days: 1 (before AI), 3, 15, and 19. Ultrasonography performed at day 31 confirmed that 9 cows became pregnant. A clear predominance of certain phyla was found, with Firmicutes, Bacteroidetes and Proteobacteria making up over 80% of the vaginal microbiota composition throughout, with notable variations between individuals. Differences in beta-diversity (community composition) that were proven statistically significant were between the estrus-proestrus phases in non-pregnant cows (P=0.028) and between the estrus and diestrus phase in pregnant cows at (P=0.043). These findings are a clear first step in identifying possible beneficial vaginal microbiota in pregnant cows that may help to determine how to proceed in manipulating other cows' vaginal microbiota that have suffered from reproductive failure in the past.

Keywords: **Holstein, dairy cows, 16S, rRNA, V4, AI, vaginal, microbiota, estrous**

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List of Acronyms and Abbreviations

AI	Artificial insemination
AMOVA	Analysis of Molecular Variance
ANOVA	Analysis of Variance
DFETC	Development Finance Corporation
E2	Estradioll-17β
ET	Endometrial thickness
FSH	Follicular stimulating hormone
GDP	Gross Domestic Product
GnRH	Gonadotropin Releasing Hormone
hCG	Human chorionic gonadotropin
LAB	Lactic acid bacteria
LEfSe	Linear discriminant analysis effect size
LH	Luteinising hormone
NGS	Next generation sequencing
OTU	Operational Taxonomic Unit
P4	Progesterone
PCoA	Principal coordinates analysis
PG	Prostaglandin
PGF-2α	Prostaglandin F2 Alpha
PPA	Postpartum amenorrhea
SARA	Subacute ruminal acidosis

Dedication

This thesis is lovingly dedicated to my mom, without whom I wouldn't have made it this far…

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I'd like to acknowledge the wonderful efforts of all the managers, owners and workers at the farm in Castro, Brazil who cared for the cows involved in this study, as well as the detailed work of my collaborators in Brazil who took all the samples, especially Dr. Marcello Seneda and Amanda Zangirolamo.

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Chapter 1 - Introduction

Dairy is a multibillion-dollar industry in Canada, with net farm receipts totaling nearly 7 billion dollars and manufacturing shipments more than double that amount, for a GDP of nearly 20 billion dollars in 2019, according to statistics Canada. Of the over 10 000 dairy farms in Canada, more than 4/5 are in Québec and Ontario, with 4766 and 3367 active dairy farms in 2020 respectively (Canadian Dairy Information Centre, 2020). Yet, fertility rates of dairy cows remain relatively low, with pregnancy rates averaging between 20 and 24%, according to Lactanet Canada, 2020. In addition, reproductive issues are one of the leading economic losses in the dairy industry across Canada, (Tiwari et al., 2008) as they are responsible for over 30% of culling. Repeat implantation failures lead to culling rates of 67% (Bonneville et al., 2011).

1. Rationale behind this research

Given the importance of this sector to the Canadian economy, along with the financial losses incurred by farmers through poor fertility rates, it is imperative to determine what combination of factors create an optimal environment for fertilisation and pregnancy. In order to sustain or even surpass a 30% reproductive rate in Canadian dairy cows, we must therefore examine the vaginal microbiota at length in an attempt to discern any patterns or differences between cows that become pregnant and those that fail. The purpose of this study is to examine the fluctuations in vaginal microbiota during the four phases of the estrous cycle. Though other studies have done similar research, to the best of our knowledge, none have yet looked at the fluctuations of microbiota over all four phases of the cycle, before and after insemination, to compare the microbiota of pregnant and non-pregnant cows over this 21-day period. Furthermore, though there is a growing body of research that has attempted to quantify the vaginal microbiota in dairy heifers and cows, most of these studies were done to examine the impact of synchronization protocols and assorted hormone or drug therapies on microbiota, and few if any, allowed natural estrous to occur.

1.1 Methodology

Conversely, no synchronization protocol was performed on cows for this study. This allowed us to see the natural microbial presence within the vaginal canal. In addition, all cows were healthy

and multiparous, and were let out to pasture regularly; again, to optimize the natural evolution of microbiota over the 21-day cycle. Moreover, to the best of our knowledge, no other studies actually took samples that coincided with all four phases of the Estrous cycle. These differences are key, since it is crucial to clearly identify naturally occurring microbiota changes throughout the cycle to further our understanding of the impact of fluctuating hormones during that time, and also, to see if there are any significant differences between cows that succeed to become pregnant and those that fail.

To further minimize the possible impacts of human interference, estrus detection was done through the use of Allflex necklace technology, and 21 cows were artificially inseminated according to the optimal timing recommended by the data collected through these devices. Four samples were taken, on day 0 before AI, day 3, at the Metestrus phase, day 15, at Diestrus, and day 19, at Pro-estrus, after which ultrasound determined that 9 of the 21 cows were pregnant (a 43% pregnancy rate!) All of these cows carried to term and had successful births. The collected data was then sequenced and analyzed using Next generation sequencing (NGS) by amplifying the V4 region of the 16S rRNA gene.

1.2 Hypotheses and objectives

We hypothesize that bovine vaginal microbiota will fluctuate during the four phases of the estrous cycle, and that these changes will correlate to hormonal changes. Second, we expect that vaginal microbiota will differ between cows that succeed to become pregnant and those that fail. We also expect to see changes at Estrus, just before AI, and at Metestrus, just after AI, since these moments are key to successful implantation.

We also hypothesize that these microbiota fluctuations may impact pregnancy outcomes, and that non-pregnant cows may show differences in richness or biodiversity, with the possibility of higher levels of bacteria that may destabilize vaginal pH levels or pathogens that are known to cause dysbiosis.

Therefore, the objectives of this study are twofold:

1. To investigate the vaginal microbiota during the different phases of the estrous cycle following artificial insemination (AI) in dairy cows

2. To compare the vaginal microbiota of cows that succeed or fail to become pregnant after artificial insemination (AI).

In so doing, our ultimate goal would be to determine the possible reasons for these differences, and to propose pathways of further exploration to eventually encourage the optimal microbiota to ensure and maintain higher pregnancy rates in the dairy industry. Indeed, learning all we can of the vast interactions between microbiota and host, is the only way to steward the health of each.

Chapter 2 - Literature Review

Introduction

Pinpointing probable reasons for fertility issues and poor pregnancy rates in cattle has been an ongoing challenge for researchers. The unimaginable complexity of microbiota communities certainly adds to that hurdle. Nonetheless, the advancement and vulgarisation of revolutionary DNA sequencing technologies have permitted much more precise study of the microbiota makeup of the cow. A great deal of research has examined microbiota in the digestive tract especially and has helped link dysbiosis to health issues. Diet supplementation with assorted probiotics is now commonplace and is at least partly based on such studies. Yet research into the makeup of vaginal microbiota is still relatively limited and improving pregnancy rates through any type of microbiota manipulation is still in its infancy. Many variables affect microbiota, thus complicating attempts at finding any clear patterns. Outside influences like diet and environment along with physiological changes due to hormone fluctuations, pH, stress or disease, all impact and modify microbiota membership. Bacterial communities are in a constant state of flux since many generations of one particular genus may evolve over the course of a few days, or even a few hours. Therefore, more frequent study at different key points in time is crucial to understanding microbiota fluctuations. That was the intent of this study: To look at vaginal microbiota communities in cows over the course of a full Estrus cycle, in order to identify and classify any possible changes in microbiota during the four phases of the cycle. These observations may then be linked to other known factors, such as hormonal changes and pH fluctuations, in the attempt to better understand the reasons for

these fluctuations. Ultimately, finding any variances in microbiota between cows that fail to become pregnant and those that succeed could open new pathways for research that may eventually greatly increase pregnancy rates in the dairy and beef industries.

In order to accomplish this analysis, the first step is to gather and sort through the existent literature on microbiota, and on vaginal microbiota specifically. Another important factor is to look at the analysis and collection methods used, in order to confirm their efficacy. Finally, comparing studies done with other species also helps to validate our findings. Therefore, this literature review is divided into eight sections: the first looks at the evolution of microbiota research and culturing techniques up to 16s rRNA sequencing, which was the method used to analyse our samples, along with a quick look at the progress in estrous detection. The second section looks at microbiota and their association to reproductive issues, the third gives an overview of physiological factors that may have an important impact on vaginal microbiota, such as hormonal and pH changes. The fourth examines research of the vaginal microbiota in other species, while the fifth examines the links between uterine and vaginal microbiota. The sixth section examines studies relating illness and poor fertility rates to possible pathogenic bacteria, while the seventh section looks at possible links between digestive and vaginal microbiota populations. Finally, the eighth section examines certain microbiota manipulation techniques commonly used in the cattle industry, and the ongoing controversy surrounding their efficacy, in order to determine possible future perspectives for this study.

Section 1 - Evolution of microbiota research and methods and the progress in estrous detection

1.1 The evolution of microbiota research

Initial evidence of microorganisms in the body was discovered by Leeuwenhoek in 1676, when he described oral microbes that were living in the mouth. Later, in the mid-1880s, Theodor Escherich, an Austrian paediatrician, found evidence that microorganisms live symbiotically in humans. He compared the bacteria present in the intestines of healthy and sick children with diarrhoea. These bacteria were later named Escherichia coli (Rogers, 2019). However, the term *'microbiome'* was coined in 2001 by Dr Joshua Lederberg, while working on microbial genetics and artificial intelligence (Goins, 2019). This term is now used to describe the genetic material of microorganisms inhabiting a specific environment, while microbiota describes the microorganisms living in a particular environment. Works published before 2001 have used both terms interchangeably.

Though microbiota research has progressed rapidly in the past decade, its evolution took much longer, and a large range of varying strategies of cultivation are still being used, from basic culture-based methods to a more precise differentiating media, to DNA sequencing using sophisticated technology

1.1.1 Culture Based Methods

Years of experimentation occurred before scientists could establish a reliable culturing method. The first culture media used were heart and brain cells. These were nonselective, thus permitting the excessive growth of undesired microorganisms. Through further experimentation, scientists discovered that yeast is a much more reliable and effective media, and it is still used today (Bonnet et al., 2019).

The advent of the Petri dish, with its transparent lid that allowed researchers to observe the formation of colonies while limiting contamination, was revolutionary. Then, solid culture media, with the use of gelatine and agar, allowed researchers to observe the growth of pure cultures

(Guthertz, 2017). Next, scientists enriched this growth media through the addition of animal blood. This encouraged the growth of varied fastidious microorganisms. (Russell et al., 2006).

Finally, researchers determined that select cultures could be developed by regulating four primary environmental parameters. These included: the choice of nutrients, atmosphere and humidity, optimal temperature, and incubation time (Bonnet et al., 2019). This led to the creation of selective or differential media, that could encourage the growth of certain microorganisms while inhibiting the growth of others. This media is created through the addition of substances like organic and inorganic components and minerals. For example, crystal violet is a dye that helps to classify bacteria, since it inhibits the growth of Gram-positive bacteria. Antibiotics and antiseptics are also used to prevent or correct contamination of samples and decrease the rapid overgrowth of commensal bacteria (Bonnet et al., 2019).

Other forms of control in selective media are just as important. For instance, regulating environmental parameters, such as temperature and atmospheric conditions, provides aerophilic or anaerobic conditions based on varying oxygen levels (Stieglmeier et al., 2009). Meanwhile, temperature is key when developing cell cultures to grow and isolate intracellular bacteria: Mammalian cells must be kept at 36^0C, while other cell lines, usually amphibian, tick, mosquito and fish, prefer 28^0C. (Stewart, 2012; Penzo-Méndez, et al., 2012). A third method of control is to regulate incubation time, since most pathogens grow over 24 to 48 hours, while bacteria require a much longer time, up to five days (Dunn et al., 1997). All these factors must be considered in order to provide optimal growth conditions for the desired microorganism being cultured.

1.1.3 The Polymerase Chain Reaction (PCR)

The Polymerase Chain Reaction (PCR) was invented in 1985 by Kary B. Mullis, and this process revolutionised genetic research. It allowed scientists to make millions of copies of a tiny piece of DNA, thus opening up related avenues of study, such as the diagnosis of genetic defects, or comparative DNA testing in criminology. PCR is also very useful in the study of evolution, since it allows comparative study of live samples with DNA from fossils that can date back millions of years (Butler, 2015).

PCR works through temperature regulation. A thermocycler is programmed to change the temperature of the reaction every few minutes. First, the sample is heated to amplify a segment of DNA through denaturation, which will separate it into two pieces of single stranded DNA. Next, the enzyme Taq polymerase is added to build a new strand of DNA using these single strands as templates, to create an exact duplicate of the original. These steps will be repeated multiple times, through a cycle of denaturing and annealing, until billions of exact copies are replicated, in only a few hours. (Garibyan & Avashia, 2014).

1.1.4 Next Generation Sequencing (NGS)

DNA sequencing is the process by which the sequence of nucleotides in a section of DNA can be determined. The first method of DNA sequencing was invented by Frederick Sanger in 1977, and it remained the preferred method for the next forty years (Heather & Chain, 2016). Next Generation Sequencing (NGS), also known as high-throughput sequencing, is a term that covers a number of different modern sequencing technologies. These technologies can sequence DNA and RNA at a very fast rate and are much cheaper than the Sanger sequencing technique. The Illumina next-generation sequencing (NGS) method is based on sequencing-by-synthesis (SBS), and reversible dye-terminators that enable the identification of single bases as they are introduced into DNA strands. This method has become the most prevalent, since it is extremely reliable. Another method, known as the Roche 454 sequencing method, is based on detecting pyrophosphate release, also through fluorescence, similar to Illumina sequencing. The advantage of this method is its capacity to sequence much longer reads than the Illumina method. A third method is Ion Torrent or Proton/ PGM sequencing. This method does not rely on optical signals (or fluorescence), since it detects the direct release of protons when DNA polymerase individually incorporates bases into the chain. (Heather & Chain, 2016).

NGS can analyse large-scale genomic and transcriptomic sequencing because of its high-throughput production and outputs at a gigabase level. NGS platforms can sequence millions, even billions of small fragments of DNA in parallel. These sequences are then analysed with the help of bioinformatics, computer programs based on a vast digital library customised with precise search parameters. (Kulski, 2016). Clinicians are exploring the use of NGS to help determine minute mutations in DNA such as: substitutions, insertions and deletions.

The greatest hurdle in the widespread use of NGS sequencing is the cost of the technology capable of the fast data processing and large storage capacity required to run a sophisticated bioinformatics system, along with the specialised training of staff able to analyse the resulting data. (Kulski, 2016)

1.1.5 16S rRNA sequencing

Another NGS function is in metagenomics, the study of genetic material derived directly from the environment. This is readily applied to the study of microbiota through the sequencing of the 16S rRNA gene. This gene is unique to bacteria, since it is a component of the 30S small subunit of a prokaryotic ribosome. Its analysis has been going on for decades. However, high-throughput sequencing of the full gene is uncommon, since it is about 1500 base pairs long, and therefore too expensive a program. Therefore, only some regions are sequenced at a time, dependant on the researcher's specific search parameters. (Behjati & Tarpey, 2013). This gene in particular is used in reconstructing phylogenies, because it is highly conserved. It contains 9 hypervariable regions (V1 to V9) that all code for different things. The degree of conservation varies significantly between these regions: the less conserved regions tend to show genus and species, while the more conserved regions tend to show higher-level taxonomy (Johnson et al., 2019). Illumina platforms are widely used when sequencing for bacteria. However, Illumina can only read 75-250 base pairs at a time (Burk & Darling, 2016).

Other microorganisms are also easily identified through 16s sequencing, including Archaea and Eukaryotes like fungi. These microorganisms are all commonly associated with a variety of health conditions in animals, including dairy cows, and they also tend to vary during pregnancy. Many studies, including my own, focus on the V4 region of the gene, since its semi-conserved state makes it ideal to identify bacterial genera (Johnson et al., 2019). Other lesser conserved regions have also proven informative: For example, the V3 region has been useful in identifying various pathogens while the V6 region has proven most accurate in the differentiation of species (Chakravorty et al., 2007). Though some research in microbiota is still being done through culture methods, a great deal more information is now available through NGS, which is quickly opening the doors to a better understanding of the role of various microbiota in their symbiotic relationship with their host.

2.1 Technological advancements in Estrus detection

The first phase of the Estrus cycle in cows lasts for only one day. Thus, early detection of heat is essential for timely insemination. Any delay may lead to a failed conception. Traditionally, farmers relied heavily on behavioural cues from a cow heading into estrus. But with the advances in detection software and hardware, there are many options that are proving much more effective and reliable.

2.1.1 Behavioural cues

Many behavioural cues have been observed to pinpoint the start of estrus in cows. The primary sign is a cow's standing heat. That is when a cow will allow another cow to mount her, and will move forward slightly under its weight. This period of standing heat is when oestrogen levels are at their highest. A standing heat typically lasts for 15 to 18 hours. Secondary signs are numerous, and they vary in intensity and duration. Such signs may also occur before, after or during standing heat. These include mounting other cows; mucous discharge from the vulva which is usually clear and elastic; swelling and reddening of the vulva; bellowing, restlessness and trailing; rubbing tailhead hair and dirtying flanks; chin resting and back rubbing (usually right before a mount); head raising and lip curling; and decreased feed intake and milk yield. (O'Connor, 2016).

2.1.2 Chemical means

Other ways to detect the onset of estrus is by human intervention and chemical and hormonal means. It is common to have an Estrus synchronization protocol in place with larger herds. Estrus synchronization usually requires hormonal therapy with a combination of different hormones. The main hormones used in Estrus synchronization programs are: Progestins, Prostaglandins, and Gonadotropin releasing hormone (GnRH). Progestins work by keeping the animals out of heat. They mimic the functions of natural progesterone produced by the CL after ovulation. This will trick the body into thinking it is pregnant, thus preventing the ovulation that would start another cycle. Other progestins commonly used on heifers and cows are Melengestrol Acetate, given orally through food, and Controlled Intervaginal Drug Release device (CIDR), a plastic device that is inserted into the vagina that will release small amounts of progesterone periodically. This device can be left in the vagina for 7 to 14 days. Prostaglandins (PGF2α) on the other hand, will cause luteolysis of the CL, thus causing the animal to return into heat. Products that contain PGF2α used

in synchronization programs are: Lutalyse®, In-Synch®, and ProstaMate®. Cloprostenol is a synthetic analog to $PGF_{2\alpha}$ and it is available as Estrumate® and estroPLAN® (Hall, 2009). Finally, GnRH is a 10-amino-acid polypeptide secreted by the hypothalamus that will trigger the release of LH. This will in turn cause ovulation of the oocyte from the follicle as well as follicular growth. Products available with this hormone include: Factrel®, Fertagyl®, Cystorelin®, and OvaCyst® (Hall, 2009).

There are still other methods that have also proven effective in determining and encouraging Estrus. First, placing a heifer that has been treated with testosterone in the herd will stimulate more mounting in the cows. Also, treating a few cows with prostaglandin will increase the overall estrous behaviour in the herd. Finally, sexually active cows in their proestrus phase and cows nearing their estrus phase tend to congregate together (O'Connor, 2016).

2.1.3 Manual examination

During a herd health-check it is common practice for the veterinarian to synchronize the cows. A vet will also check the overall condition of the cow's uterus and where she is in her cycle. This is done by transrectal ultrasonography and can help in determining if the cow is pregnant or not. Following the size of the follicles and seeing if there is a presence of a small or large CL can determine if the cow is ready to be inseminated, or if the farmer should wait before synchronizing her again.

2.1.4 Technological detection

More and more farmers are now moving away from traditional methods and looking to the future, by using precision dairy monitoring technologies, such as ear tags or collars. Using these technologies in combination with traditional methods can allow for better estrus prediction for more successful insemination leading to better overall fertility rates in cattle. These detection collars follow the changes in rumination and activity levels of the cow. A sharp decrease in rumination along with an increase panting are two key indicators of heat. This information will be detected through the collars and sent wirelessly to a computer database which can graph changes in activity. An alert will be sent to warn the farmer of any changes. The program will even determine how many hours are left for best AI results.

One brand of detection collars is the SCR necklace by Allflex. These collars have been shown to be 90-95% accurate in successfully detecting the onset of heat in dairy cows and heifers, as well as beef cattle (Allflex, 2020). These collars were used to detect the start of Estrous for this study. With this system, the cow's activity is continuously monitored and compared to a baseline to detect and flag any changes that may occur. The system can also detect weak heat signs, help to avoid false heats and provide precise insemination timing guidance, which will improve the cost effectiveness of AI. Not only do these systems help to increase the efficiency and profitability of a farm by reducing the calving interval, improving milk production and reducing involuntary calving, they also have a positive impact on cow welfare, by eliminating the need for hormonal therapy. With these systems in place, there is no need to synchronize the animals with hormones, instead the cow can express her natural cycle with no external disturbances (Allflex, 2020).

A study done by MacMillan et al., 2020, used a very similar system like that of Allflex, through the use of ear tags by eSense to detect the onset of estrus in dairy heifers. They had promising results with a 91% sensitivity rating, and a positive predictive value to detect true estrus at 83.5% (MacMillan et al., 2020).

Section 2 - Microbiota linked to health and reproductive issues

2.1 Microbiota communities and their link to health in humans

Many studies now exist that describe microbiota communities and their interactions with humans, animals, plants and soil (Wang et al., 2018, Li et al., 2018, Lev-Sagie et al., 2019). Much attention has been given to gut microbiota and its impact on human health. It has been determined that intestinal microbiota play an important role in an individual's immunity, metabolism and neurobehavioral traits (Kim & Shin, 2018). A study done with twins showed that there is a genetic component to the gut microbiota, but it is mainly influenced by environmental factors like drugs, diet and stress (Wen and Duffy, 2017). The health of this microbiome has also been linked to obesity, since overweight individuals show a dysbiosis in their gut microbiota with a clear lack of biodiversity (Valdes et al., 2018). In addition, lower bacterial diversity has been observed in people

with inflammatory bowel disease, psoriatic arthritis, diabetes, atopic eczema, coeliac disease, and arterial stiffness (Scher et al., 2015).

The clear relation between intestinal microbiota and health encouraged subsequent research focusing on the health of vaginal microbiota in women and its possible link to infertility and neonatal issues (Barrientos-Durán et al., 2020). Healthy vaginal microbiota in human females is composed primarily of *Lactobacilli*. *Lactobacilli* create lactic acid to maintain vaginal homeostasis by keeping pH levels between 4.2 and 5 (Ravel et al., 2011). Lactic acid acts as an important microbicide that prevents the growth of other bacteria, thus avoiding any dysbiosis (O'Hanlon et al., 2013). A lower abundance of *Lactobacilli* in pregnant women seems to lead to late abortions or premature births (Freitas et al., 2018). Bacterial vaginosis, which affects a third of all women, is associated with a decrease in *Lactobacilli* with an inversely related increase in pH, which allows growth of harmful bacteria. This may in turn lead to a possible failure of reproduction. (Eastment & McClelland, 2019; Kitaya et al., 2019).

2.2 Reproductive failure in cows

Unfortunately, infertility and reproductive issues are major problems in the cattle industry. In a study done in 2019, Dr Ryon Walker, a livestock consultant with the Nobel Research Institute in Oklahoma, identified 5 reasons of reproductive failure that deserve closer attention:

The first cause: early embryonic mortality. Survival rates drop significantly during the progression of pregnancy in cows. Within the first 7 days there is a 95% survival rate, but by day 28 the survival rate drops to 70%, and by day 42 it drops to 62%. The reason for this dramatic drop is still not understood.

The second cause of infertility is related to the sire. Many bulls are opted out of the breeding soundness exam before breeding, since one bull can be used to sire up to 30 calves per season, as compared to one calf per cow. Statistics show that 1 in 5 bulls are sub-fertile, through tests done across a random population (Nani et al., 2019). Another study showed that major reasons for bull sub-fertility were poor libido and inferior semen quality (Khatun et al., 2013).

The third cause is poor nutrition. Dairy cows, especially, have high energy demands related to milk production, which may compromise its ability to support a calf through pregnancy. A cow's poor

physical condition may also inhibit her ability to recover after pregnancy in order to resume cycling.

The fourth cause of reproductive failure is infection. These are relatively rare, but may still have serious consequences, like stillbirths and abortions. Some of these infections include: Bovine viral diarrhoea (BVD), Infectious Bovine Rhinotracheitis (IBR), Brucellosis, Leptospirosis and many others.

Finally, the fifth cause of infertility is lack of planning or poor timing. Bulls left with cows for too long will impact the calving season. Consequently, part of the herd may calve later in the season, which may also impact the viability of the calf.

Pinpointing the reasons behind these reproductive issues is key to ensuring the continued success of the cattle industry. Therefore, research into the cow's vaginal microbiota is critical, in order to understand both the circumstances affecting it as well as their effect on the reproductive health of their host.

Section 3 - Physiological aspects of the estrous cycle

3.1 Hormones Related to The Four Phases of the Estrous Cycle

3.1.1 Estrus (Day 1)

The estrous cycle in cattle lasts for about 21 days. During that time, various hormones play an important role as both triggers and regulators of physiological changes that promote an ideal environment for pregnancy. The major hormones involved in the estrous cycle are: Progesterone (P4), Estrogen (E2), and Prostaglandin (PG). The estrous cycle is comprised of four different phases. The first, the estrus phase, will start on day 0 and will last for only one day. This is why it is vitally important for farmers and veterinarians to be aware of the exact moment when the cow's cycle begins, in order to inseminate on the appropriate day to ensure better chances of pregnancy. At this phase, the E2 concentrations will be very high so that the ovum will be released from the follicle.

3.1.2 Metestrus (Days 2 to 3)

The metestrus phase follows the estrus. This phase will last for 2 days and is considered one of the transitionary phases, part of the luteal phase of the cycle. The ovulation of the follicle will occur, led on by an increase in gonadotropin hormones with a peak in both luteinising hormone (LH) and follicular stimulating hormone (FSH). This generates a second cohort of follicles to be released so that one may become dominant and be ovulated. A physical indicator that the cow is in her metestrus phase is the appearance of bloody discharge protruding from her vagina. This is pseudomentruation caused by the seepage from uterine vessels into the vagina, known as diapedesis.

3.1.3 Diestrus (Days 4 to 18)

Following the metestrus phase is the second and final transitionary phase, the diestrus. This phase is part of the luteal stage and lasts for 15 days. During this phase, large amounts of progesterone are released in the bloodstream to maintain pregnancy. This will follow the increasing growth of the CL from days 1-16. The larger the CL, the higher the concentrations of progesterone. However, if the cow is not pregnant, prostaglandin will be secreted between days 17 to 20 to complete and restart the cycle. In addition, there is often another spike in LH around Day 15 in some cows to generate a third cohort of follicles to be released so that one may become dominant and be ovulated.

3.1.4 Proestrus (Days 19 to 21)

Finally, the proestrus phase lasts for the final 3 days of the 21-day cycle. This phase makes up the follicular phase, and is composed of various and significant transformations, depending if the cow became pregnant or not. In non-pregnant cows: the uterus will release prostaglandin F2α on day 17 to lyse the CL. P4 levels will start to decrease over the next 5 days, the preovulatory follicle will undergo its final growth phase, and estradiol concentrations increase. At day 21, P4 levels will have decreased considerably, and E2 concentrations will be high again, to restart the cycle.

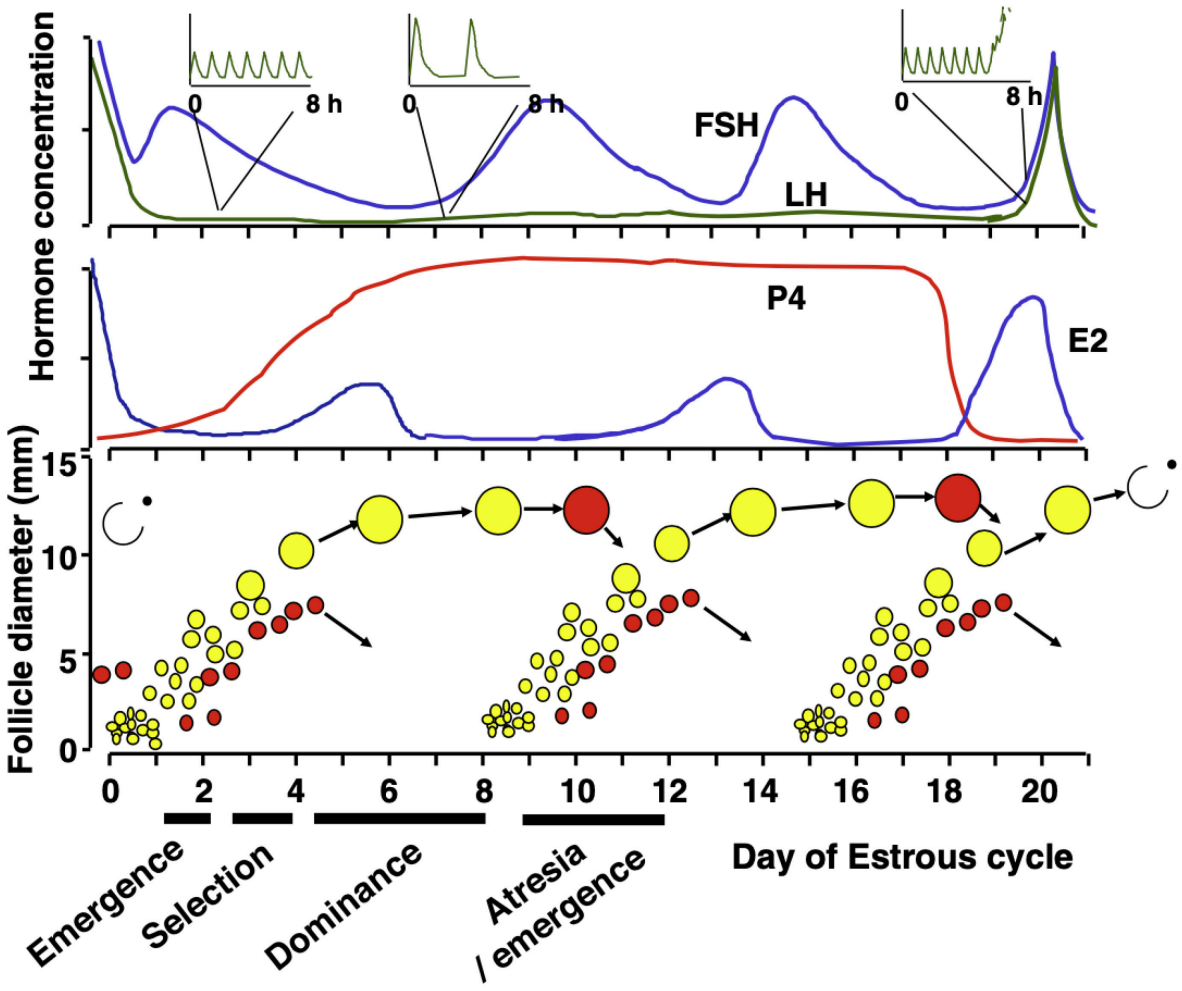


Figure 1 -Schematic depiction of hormonal patterns of secretion during the estrous cycle in cattle, From: Crowe, M. (2016). Reproduction, Events and Management: Estrous Cycles: Characteristics. 10.1016/B978-0-08-100596-5.01039-8. Reprinted with permission from the author (Mark A. Crowe)

However, in pregnant cows, P4 levels will remain high throughout gestation to ensure the survival of the embryo and foetus. Other hormones take over during the later stages of gestation. The conjugate form of Oestrone, Oestrone sulphate, will be present at elevated levels during mid-pregnancy until the third stage of parturition or until the cow has expelled the foetal membranes

(Kindahl et al., 2002). 10 days prior to parturition, glycoproteins originating from the trophoblastic binucleate cells will increase. The increasing levels of oestrone are related to the increased synthesis of prostaglandin F2α, which are in turn related to elevated levels of 15-ketoduhydro-PGF2α. This increase will cause prepartal luteolysis that will trigger hormones such as cortisol, prostaglandin F2α, and oxytocin to also escalate during labour (Kindahl et al., 2002).

3.1.5 Follicle growth throughout the estrous cycle

Follicle growth in cattle is dependent on the elevated concentrations of the follicle-stimulating hormone (FSH). Once FSH begins to rise at the start of the estrous cycle, it will be followed by a cohort of 24 small (3-5mm) follicles where they will start to grow pass 4mm in diameter. When FSH will start to decline in the next 2-3 days, follicles from the original cohort will stop growing. This will allow the selection of one dominant follicle form the cohort while the others undergo atresia (Crowe et al., 2014). The newly selected dominant follicle will have advanced growth and steroidogenesis with very high levels of estrogen secretion as compared to the other follicles. The dominant follicle will grow to about 12-20mm in diameter by the time it is selected. Its large size allows the secretion of estrogen which will inhibit the secretion of FSH, preventing a second cohort from starting. Continuation of growth and estrogen secretion from the dominant follicle does not continue for more than 3-4 days after, as the developing CL with its progesterone secretion will negatively impact the LH pulse pattern. Without the LH pulse frequency, the dominant follicle will become atretic. Come days 7 and 9 once the first dominant follicle loses dominance, FSH will start to rise again. Selecting a new dominant follicle from a second wave. If luteolysis happens with the new dominant follicle then it will ovulate. However, if the CL is still active suppressing LH then a third wave will take over, selecting a 3rd dominant follicle for ovulation. A strong and frequent LH pulse during the follicular phase will support the dominant follicle and promote ovulation (Crowe et al., 2014).

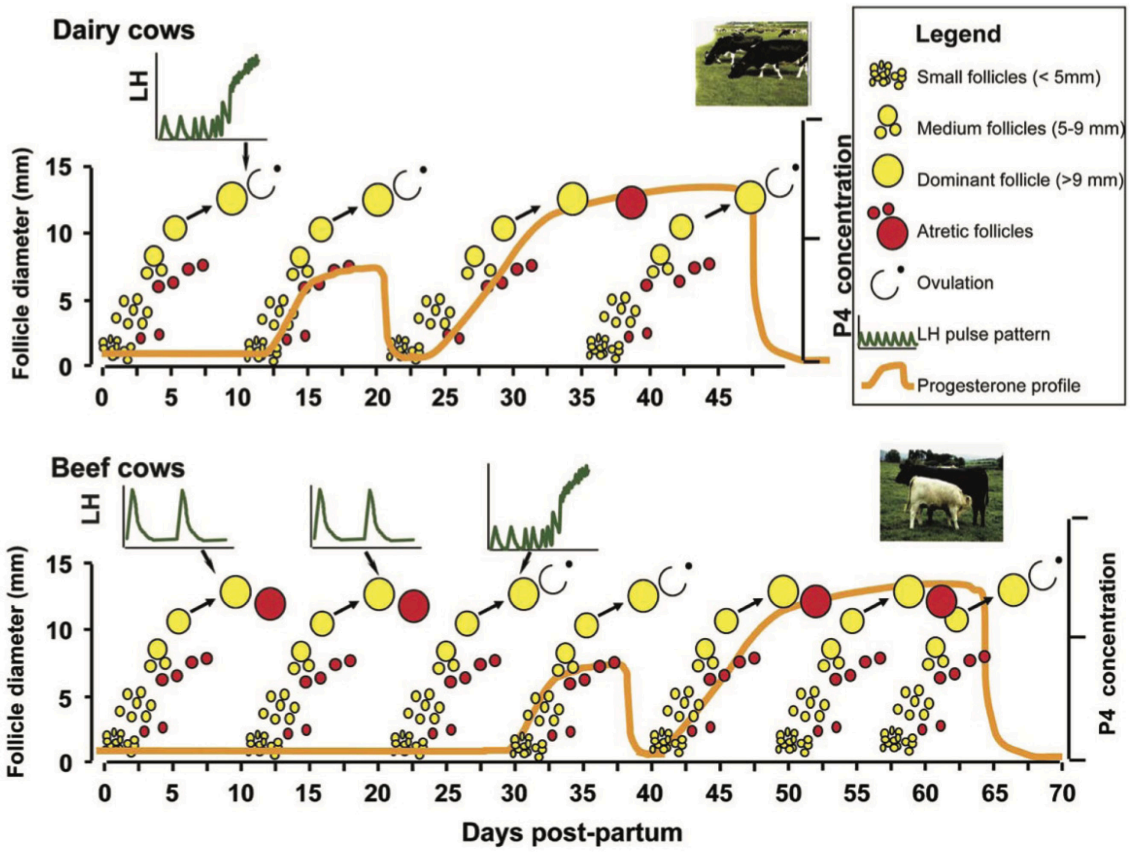


Figure 2 – Diagrammatic scheme of resumption of dominant follicles and ovarian cycles during the postpartum period in dairy and beef suckler cows. From: Crowe, M. A., Diskin, M. G., & Williams, E. J. (2014). Parturition to resumption of ovarian cyclicity: comparative aspects of beef and dairy cows. *Animal : an international journal of animal bioscience*, *8 Suppl 1*, 40–53. https://doi.org/10.1017/S1751731114000251. Reprinted with permission from the author (Mark A. Crowe)

The association between vaginal microbiota and reproductive health in humans, and the lack of reproductive efficiency in cattle, have led researchers to study the microbiome in the female reproductive tract of cows. A recent study by Ault et al., 2019 showed that there is a decrease in bacterial diversity within the uterus during the synchronisation protocol before artificial insemination (AI), while research by Deng et al., 2019, shows a shift in the vaginal microbiota of beef cows in later gestational stages. That study found a continuous increase in vaginal microbiota

from the pre-breeding stage to the second trimester; while there was a marked decrease from the second to the third trimester (Deng et al., 2019). However, changes in the vaginal microbiome during the four phases of the estrous cycle have not yet been clearly investigated, while the manipulation of vaginal microbiota to improve reproductive rates is still in its infancy.

3.2 Studies measuring direct effects of hormones on cow reproductive physiology

A study by Šuluburic et al., 2017, looked into how a lack in progesterone (P4) might affect early embryonic development in dairy cows. This study was done on 110 healthy Simmental dairy cows. Cows were given an injection of $PGF_{2\underline{\alpha}}$ to start estrus synchronisation after the presence of a CL was confirmed by rectal palpation. Cows who showed signs of estrus 2 to 5 days after this treatment were then randomly assigned into the following groups:

- 26 control cows were allowed to go through a spontaneous estrus and were artificially inseminated soon after.
- 26 cows were treated with Gonadotropin-releasing hormone (GnRH) followed by AI.
- 30 cows went through AI and given an injection of Human chorionic gonadotropin hormone (hCG) 7 days later.
- 28 cows were given GnRH treatment followed by AI with an injection of hCG 7 days later.

Blood serum and milk samples were collected at estrus and then again on days 14, 21 and 28 after AI to measure P4 levels. The results showed that out of the four treated groups, the cows that responded most were those treated with hCG on days nine through twelve following the onset of estrous. There was a significantly higher tendency for increased milk P4 concentrations in hCG-treated cows on days 21 and 28. It was also noted that there was a significant increase in pregnancy rates in all treated groups compared to the controls at 54% (GnRH), 62% (GnRh/hCG), 63% (hCG) vs. 31% (Controls), with $P<0.05$. Calving rates in all treated groups were also higher, with the hCG treated group being the most significant. However, calving rates, although still relatively high, decreased and did not follow through with the higher pregnancy rates. The highest level of pregnancy loss was in the GnRh/hCG treated group at 19%, while the lowest pregnancy loss was

observed in the GnRH group at 7%, while pregnancy loss in the hCG treated group was between the two, at 11%.

3.3 Endometrial thickness at estrus related to pregnancy rates

Other studies focused on the impact of hormones on physiological factors at the start of estrous. One such study measured endometrial thickness (ET) and its relationship to fertility in Holstein dairy cows (Souza et al., 2010). They found that an ET thicker than 8 mm resulted in a significantly higher rate of ovulation as well as increased pregnancy rates. Another study done by Sugiura et al., 2018, related hormonal profiles to endometrial wall thickness. They used 15 lactating cows, 8 primiparous and 7 multiparous. The cows were all between 35-86 days postpartum. Each cow was induced to start cycling with a controlled internal drug release (CIDR) containing 1.9 g of progesterone, then given 25 mg of prostaglandin (PGF2α) about a week later, once the CIDR was removed. A control group of 9 cows was allowed to cycle naturally. They found that in the induced estrus group, ET was significantly greater between 6 and 12 hours before ovulation compared to the natural estrus group. However, 6 hours before ovulation ET in both groups were in a similar state. Subsequently, in the natural estrus group, P4 concentrations decreased and E2 concentrations rose prior to an increase in ET. While in the induced estrus group, an increase in ET started at the same time as a decrease in P4 and an increase in E2. Yet, this research did not relate microbiota fluctuations to hormone levels.

3.4 Impact of hormone manipulation on the vaginal microbiota in cows

A study by Quadros et al., 2020, looked into the effects an intra-vaginal progesterone device would have on the vaginal microbiota in Holstein dairy cows. Twenty cows were used with a mix of different parturition states, some being primiparous and other multiparous. Each cow went through an estrus synchronisation protocol with use of a CIDR implant containing 1.9g of progesterone. Vaginal swabs were collected from days 0 (before implantation) and day 10 (after the implant was taken out). The cows were randomly allocated to two different groups where ten animals received an intramuscular injection of ceftiofur hydrochloride on day 9 while the control group received a saline injection the same day. On day 11 all cows were artificially inseminated. The V3 and V4 region of the 16S rRNA gene was sequenced from the samples. The four major bacterial phyla

found were: Firmicutes at approximately 37.61%, Tenericutes (29.45%) Bacteroidetes (13.73%) and Proteobacteria (17.47%), totalling 98.26% relative abundance.

Major genera found were as follows:

- Firmicutes: Over 101 000 OTU reads, composed of 31 different families, Ruminococcaceae (48.23%), Lachnospiraceae (26.88%) and Rikenellaceae (10.03%).
- Tenericutes: Over 79000 OTU reads, with 5 families: more than 95% of the reads mapped to the Mycoplasmataceae family (95.44%), and by the family Anaeroplasmataceae with 1.58% of the reads.
- Proteobacteria: 47 families sequenced, with a dominance in Succinivibrionaceae (67.76%) followed by Pasteurellaceae (11.24%).
- Bacterioidetes: 15 families sequenced, Bacteroidaceae (26.89%), Prevotellaceae (16.09%), Rikenellaceae (10.03%) and Bacteroidales (9.66%).

The rest of the microbiota consisted of Actinobacteria with 19 families sequenced, especially Bifidobacteriaceae (37.32%) and Corynebacteriaceae (26.57%) and Spirochaetae, with all the reads mapped to the Spirochaetaceae family.

Although there was no significant difference between the vaginal microbiota of multiparous and primiparous cows, multiparous cows did show a greater microbial colonisation of the vaginal tract. Also, in multiparous cows, the implants increased the number of unclassified bacteria, and significantly affected certain bacterial strains, including an increase in the proportion of the *Family XIII AD 3011* in the Firmicutes phyla ($p = 0.027$), and a reduction in the number of the Clostridiales in BB60 group genus ($p = 1.55\times10^{-3}$). Finally, the cows who were injected with ceftiofur hydrochloride on day 9 did show a decrease in vaginal bacterial proliferation of some bacteria.

Though most of this research is based on induced cycling and hormonal manipulation, these studies seem to link certain hormones to variances in the abundance and richness of vaginal microbiota. However, scarce literature can be found on natural cycling patterns and the effect hormonal changes during estrous may have on microbiota membership. Unfortunately, it is still very challenging to find the precise composition of vaginal microbiota, in part because of the effects of these hormonal changes and other factors, which lead to a continual flux in its structure and

membership. Nonetheless, ongoing research to classify the vaginal microbiota of cows is now giving us a clearer picture of its particularly volatile makeup.

In a study done by Messman et al., 2020, researchers wanted to characterize the vaginal microbiota along with estradiol concentrations at the time of AI. The study looked at 70 Brangus heifers which all went through a controlled estrus synchronization protocol before sampling. At AI, swabs were taken within the anterior vagina of the heifers and blood samples were taken immediately after to measure hormone levels. The V4 region of the 16S rRNA gene was later sequenced. The four most abundant phyla found were Tenericutes, Proteobacteria, Fusobacteria and Firmicutes. 29 heifers later become pregnant while 49 did not. Comparing the differences between the pregnant and non-pregnant heifers, they found that three OTUs were significantly different, including *Pasteurealla multocida, Pasteurellaceae unclassified, and Fusobacterium unclassified.*

Comparing estradiol concentrations with microbiota did prove beneficial, since they did show significant differences. The heifers were placed into 3 different groups based on whether they had high, medium or low concentrations of estradiol. When examined in this fashion, eight statistically significant OTUs at the species level were observed: heifers with high concentrations compared to heifers with medium and low concentrations had a higher abundance of: *Leptotrichiaceae unclassified* ($P < 0.0001$), *P. multocida* ($P < 0.001$), and *Pasteurellaceae unclassified* ($P < 0.001$). Heifers with medium and low concentrations of estradiol had a higher abundance of: *Histophilus somni* ($P < 0.001$), *Actinobacillus seminis* ($P < 0.001$), and *Fusobacterium unclassified* ($P = 0.001$) compared to heifers with a high concentration of estradiol. Finally, *Bacteroidetes unclassified* ($P < 0.001$) were more abundant in the vaginal microbiome of heifers with low estradiol concentrations compared to the rest. The paper goes on to conclude that there were various species-level differences between pregnant and non-pregnant cows which could possibly alter the vaginal environment that could influence fertility. Although more research is needed, this study shows promising results worth further investigation.

Section 4 - Vaginal microbiota during Estrous cycle in different species

4.1 Ruminants - buffalo and heifers

A study done on the vaginal microbiota of buffalo showed many similarities with the vaginal microbiota of cows. Mahalingam et al., 2019, quantified vaginal microbiota during 3 phases of the estrous cycle in nine sexually mature buffalo from 4 to 5 years of age. The 16S rRNA gene was sequenced through NGS, amplifying the V3 and V4 regions. The results were, from most abundant to least: in the pre-estrus phase: Proteobacteria, Actinobacteria, Firmicutes and Bacteroidetes. In the estrus phase: Proteobacteria, Firmicutes, Actinobacteria, Bacteroidetes, and Tenericutes. In the diestrus and final phase they found Proteobacteria, Actinobacteria, Firmicutes, Bacteroidetes and Tenericutes. Each phylum of bacteria presented was given different percentages of abundance and it was evident that during the estrus phase, there was a sharp decrease in the bacteria present. However, the percentages of certain bacteria in the estrus phase did increase. Firmicutes, Actinobacteria and Bacteroidetes were most abundant in the estrus phase only. The pre-estrus and diestrus phases had higher similarities in phylogenetic composition. They noticed that the estrus phase had more anaerobic and facultative anaerobic bacteria than the other phases. These same bacterial phyla have also been found to dominate the vaginal microbiome of cows.

A recent study by Quereda et al., 2020, looked into the changes in vaginal microbiota during the estrous cycle in dairy heifers. In their study, twenty 13-to-16-month-old heifers were used, and a single vaginal swab was taken during the follicular (estrus phase, 6-8 hours before AI) and luteal (diestrus phase, 14 days after AI) of the estrous cycle. All heifers were submitted to estrus synchronization with the use of a cloprostenol injection. Blood samples were taken during the same time points as the swabs to run a plasma progesterone immunoassay. The onset of estrus was also followed by a monitoring system. 16S rRNA sequencing was performed on the samples focusing on the V3 and V4 hypervariable regions. Their results showed that richness variance was significantly higher in microbial communities during the follicular phase as compared to the luteal phase samples at ($P<0.016$). They showed taxonomic results by comparing individual mean relative abundances from both phases.

They found 27 bacterial phyla in the follicular (estrus) phase and 23 bacterial phyla in the luteal (diestrus) phase. The most abundant phyla were: Tenericutes (35.6%), Firmicutes (25.2%) and Bacteroidetes (14.9%) Genera: 17 genera and 4 families showed relative abundance over1% *Ureaplasma, Histophilus, Corynebacteriaceae, Porphyromonas, Mycoplasma, Ruminococcaceae UCG-005, Leptotrichiaceae, Bacteroides, Leptotrichia, Helcococcus, Campylobacter, Rikenellaceae RC9* gut group, *Alistipes, Streptococcus, Lachnospiraceae,* (Eubacterium) the coprostanoligenes group, and *Facklamia* were the most abundant genera (Quereda et al., 2020).

They concluded that researching beneficial bacteria in healthy heifers can help to develop a probiotic based treatment to correct and improve fertility rates in cattle. This may in turn aid in finding certain biomarkers in reproduction as well (Quereda et al., 2020)

4.1.1 Microbiota in horses

A study by Barba et al., 2020, investigated the vaginal microbiota composition in eight adult Arabian mares during the estrus and the diestrus phases of the estrous cycle. They found that the vaginal microbiota remained stable throughout these two phases and that there were no significant differences in their microbial community composition and structure. There were also no significant differences between the most abundant taxa between the two phases. The V3 and V4 regions of the 16S rRNA gene were sequenced and in all the samples they found a substantial number of bacteria as well as archaea. The most abundant phyla found were Firmicutes, Bacteroidetes, Proteobacteria and Actinobacteria. At the genus level, the most abundant were *Porphyromonas, Campylobacter, Arcanobacterium and Corynebacterium.* The researchers also remarked that *Lactobacillus,* unlike the elevated levels found in human studies, was below 2% in the mares sampled, with only 0.18% during estrus and 0.37% during diestrus.

4.2 Other species

4.2.1 Baboons

A study published in 2017 by Miller et al., described the vaginal microbiota of 48 wild baboons during various reproductive states. Pregnancy, postpartum amenorrhea and ovarian cycling were the dominant predictors of all baboon vaginal microbiota. The baboons lived in five different social

groups and had been heavily monitored since 1971 by the Amboseli Baboon Research Project. This allowed the caretakers to be very familiar with their mating routines, sexual behaviours and the overall history of each animal. NGS was used to sequence the 16S rRNA gene amplifying the V4 region. Vaginal pH was also measured but from a separate group of 20 female baboons. They found 29 bacterial and archaeal phyla. 11 phyla were found in 100% of the samples. *Firmicutes, Fusobacteria, Proteobacteria, Bacteroidetes*, *Actinobacteria* and *Tenericutes* all had mean relative abundances greater than 1%. This is very similar to what was found in the cow vaginal microbiota, however in different orders of abundance. Still, the median relative abundance of *Lactobacilli* was only 0.00063%, which is vastly different when compared to human studies where *Lactobacilli* represents 96%.

Social group size influenced microbial alpha diversity. Females who were members of larger social groups had lower Shannon's diversity compared to those in smaller groups. This discovery suggests that this tendency may also be a factor for dairy cows. For instance, those cows who remain in tie-stall barns may show a lower diversity than those in larger groups that spend most of their time out to pasture.

Cycling individuals had higher levels of common BV associated genera like *Sneathia*, *Prevotella* and *Mobiluncus* when compared to both pregnant and those in postpartum amenorrhea (PPA). Cycling females also had low levels of all other relative taxa. Surprisingly, there was an inverse relation between *Firmicutes* and *Fusobacteria* across females in different reproductive states. Cycling females exhibited high levels of *Fusobacteria* and low levels of *Firmicutes* while PPA females had the opposite.

Two phases that were of particular interest were the periovulatory and the anestrus phase. The first is comparable to the estrus phase in dairy cows while the anestrus refers to the prolonged period of sexual rest where the reproductive system is quiescent. These two phases represent the periods of maximum and minimum estrogen and vaginal glycogen availability. During the preovulatory phase in the baboons, there was a higher presence of *Bacilli* and very low relative abundance of the family *Fusobacteriaceae*. Around ovulation, there were high levels of Firmicute*s* and low levels of Fusobacteria. The opposite was observed during anestrus. PH was also observed to change significantly during the different phases. This paper also speculated that major shifts in the

bacterial composition during ovulation and AI may increase the risk of disease and subsequently affect conception.

4.2.2 Microbiota of rodents under electrical stimulation

A recent study published in March 2020 by Levy et al., describes the rodent vaginal microbiome across the estrous cycle and the effects genital nerve electrical stimulation has on the residing microbiota (Levy et al., 2020). The goal was to find a possible treatment avenue for postmenopausal women who suffer from female sexual dysfunction (FSD). Ten nulliparous female Sprague-Dawley rats were used in this study. Vaginal lavages were performed in order to determine which phase of the estrous cycle the rats were in as well as to gather samples for quantifying their microbiota. A total of 1591 OTUs were used in the dataset. The study showed that the most abundant phyla found belonged to *Proteobacteria, Firmicutes*, and *Actinobacteria*. It was noted that the vaginal microbiota did fluctuate during the different phases of the estrous cycle. There was a significant difference between estrus and diestrus samples at P=0.002 as well as a near significant difference in metestrus and diestrus samples at P=0.059. PCoA graphs showed a degree of clustering during estrus and diestrus phases. More specifically, the samples in estrus clustered around OTU3 or the *Streptococcus* bacteria. While during diestrus there was evident clustering around OTUs 1 and 2, or the *Proteus* and *Escherichia/Shigella* bacteria respectfully. LEfSe analysis revealed that *Streptococcus* bacteria was significantly more abundant in the estrus phase than any other phases (P=0.0001). Likewise, *Corynebacterium* was significantly more abundant during the diestrus phase compared to any other phase (P=0.0003).

4.2.3 Microbiota in pigs

Another study done by Lorenzen et al., 2015, quantified the vaginal microbiota during estrous in Göttingen Minipigs for the first time. The aim was to determine the composition of the vaginal bacterial microbiota by 16S rRNA sequencing to see if this breed of pig was a good model for future genital tract research in women. Ten prepubertal pigs and ten sexually mature, unbred pigs were used. Each pig was regularly tested for various pathogens following federation guidelines. The estrous cycle of the sexually mature group where synchronised orally for a period of 18 days. Each pig was then sampled on days 0, 5, 9, 13 and 21 during a full estrous cycle. The pigs were sampled deep in the vagina close to the vagino-cervical transition. The V3 and V4 regions of the

16S rRNA gene were amplified. The resulting dataset consisted of 7 516 123 high-quality sequences. Five major phyla were present in the sexually mature pigs during estrus, including: Firmicutes (49.3%), Proteobacteria (35.2%), Tenericutes (6.3%), Actinobacteria (5.7%) and Bacteroidetes (3.5%). PCoA plots showed no significant differences in the taxonomic composition of vaginal microbiota. Firmicutes showed the greatest diversity in membership compared to the other bacteria present.

4.2.4 Microbiota in dogs

A study done by Lynman et al., 2019, looked into the vaginal and uterine microbiota in in 25 bitches and quantified the microbiota at the different stages of estrous before an elective ovariohysterectomy. Blood samples were taken from each dog before swabbing to run hormonal analyses, using an ELISA progesterone assay. Vaginal smears were also conducted to classify the stages of estrous. Five dogs were placed into 5 different groups based on which phase of the estrous cycle they were in. This included: the pre-pubertal stage, anestrus phase, proestrus stage, estrus stage and diestrus stage. The V4 region of the 16S rRNA gene was amplified using primers 515F and 806R. Results generated 3 527 169 reads after ambiguities were removed. Overall, Proteobacteria, Bacteroidetes and Firmicutes were the three most abundant phyla in both the uterus and vagina. Bacteroidetes (34.3%), Proteobacteria (26.2%), Tenericutes (15%) and Firmicutes (12.9%) were the most prevalent phyla in the vagina of the bitches while Proteobacteria (38.8%), Firmicutes (26.2%), Actinobacteria (18.2%) and Bacteroidetes (9.4%) were the most prevalent phyla in the uterus. At the genus level, the bacterial community in the vagina was higher in richness, while the uterus was more diverse. There were also no significant differences in diversity or richness between endometrial and vaginal microbiota during the different phases of the estrous cycle. Although running a Tukey's HSD analysis did show a significant difference in diversity among vaginal microbiomes of animals in estrus and those who were pre-pubertal ($P<0.05$).

There seems to be a pattern between non-human mammals and their vaginal microbiota. Thus far, baboons, rats, cows and dogs all have similar phyla of bacteria. Baboons actually seem to share many similarities with the vaginal microbiota in cows. Yet very few papers mention the presence of estrogen and glycogen in cows, though this hormone and related glucose molecules have been studied frequently in humans, primates and other animals (Amabebe & Anumba, 2018, and Yildirim et al., 2014).

Section 5 - Uterine and vaginal microbiota comparisons

5.1 Uterine microbiota in humans

A study by Moreno et al., 2016 was conducted on women and revealed that the endometrium microbiota has an effect on the implantation success and failure. Three pilot studies were done on different groups of women. These groups consisted of 13 fertile women, 22 fertile women during different phases of their menstrual cycle and 35 infertile patients that were undergoing IVF treatments. Samples were taken from the uterus and the vagina of each patient and were later sequenced using 16S rRNA sequencing amplifying the V3 and V5 regions of the gene. They found that there were different communities present between the uterine cavity and vagina of some subjects. In other patients the microbiota between the uterus and the vagina were completely different. In some asymptomatic patients, in 6 of the paired samples taken, the bacterial communities in the uterus had a high proportion of potential pathogenic bacteria which included: *Atopobium, Clostridium, Gardnerella, Megasphaera, Parvimonas, Prevotella, Sphingomonas,* or *Sneathia* genera (Moreno et al., 2016).

18 of the 22 fertile patients showed a stable uterine microbiota profile during the transition from pre-receptive to the receptive phase. These results indicated that there was no hormonal influence on the acquisition during the endometrial receptivity. Based on the samples taken from the uterus of the fertile women in the study, 28 were quantified as being *Lactobacillus*-dominated and 16 were classified as non-*Lactobacillus*-dominated. Concerning the infertile women, it was found that there was a significant difference in the bacterial diversity with the non-*Lactobacillus*-dominated group that showed a higher diversity then those in the *Lactobacillus*-dominated group. The non-*Lactobacillus*-dominated group had significantly lower implantation (60.7% vs 23.1%, P=.02), pregnancy (70.6% vs 33.3%, P = .03), ongoing pregnancy (58.8% vs 13.3%, P=.02), and live birth (58.8% vs 6.7%, P=.002) rates, as well as higher miscarriage rates, although this was not statistically significant (16.7% vs 60%, P=.07) (Moreno et al., 2016). The study concludes that any pathological modification of the microbiota profile is associated with poor reproductive outcomes for IVF patients.

Although humans have a very different vaginal and uterine microbiota profile compared to animals, this study does give us insight to future research that should be taken into consideration when dealing with infertility in dairy cows and other animals. In particular the increase in diversity in the uterus could lead to potential reproductive failure during AI or even later in gestation.

5.2 Vaginal and uterine microbiota during pregnancy in ewes, cows and heifers

A study done by Swartz et al., 2014, compared the vaginal microbiota of ewes and cows. They determined that these two ruminants share the greatest diversity in microbiota, effectively demonstrated in a box plot (See Figure 3). This comparison clearly shows the vast difference between the microbiota of cows and humans, with humans showing the least abundance of all species.

Researchers found that vaginal microbiota in both animals included significantly lower levels of *Lactobacilli* as compared to humans, resulting in a near neutral pH, while most prominent bacterial phyla included Bacteroidetes, Fusobacteria and Proteobacteria. Cows however, had a greater number of genera compared to ewes. Archaea were also detected in 95% of vaginal samples in both cows and ewes. However, the effect of the estrous cycle on microbiota structure and membership was not assessed.

This information is crucial to developing any possible microbiota manipulation strategies in the future, since there is mounting evidence that maintaining a high diversity is vital to maintaining a health microbiota in these animals. This is contrary to strategies already used to correct dysbiosis in humans, whose microbiome is very much dominated by *Lactobacilli*. Such strategies usually include supplementation with more *Lactobacilli*, which have an acidifying effect on the microbiome, thus acting as an effective microbicide.

5.2.1 Determining the presence of microbiota in utero during pregnancy

The association between vaginal microbiota and reproductive health in humans, and the lack of reproductive efficiency in cattle, have led researchers to study the microbiome in the female reproductive tract of cows. A recent study by Ault et al., 2019 showed that there is a decrease in bacterial diversity within the uterus during the synchronisation protocol before artificial insemination (AI), while research by Deng et al., 2019, shows a shift in the vaginal microbiota of

beef cows in later gestational stages. That study found a continuous increase in vaginal microbiota from the pre-breeding stage to the second trimester; while there was a marked decrease from the second to the third trimester (Deng et al., 2019). However, changes in the vaginal microbiome during the four phases of the estrous cycle have not yet been clearly investigated, while the manipulation of vaginal microbiota to improve reproductive rates is still in its infancy.

A study by Moore et al., 2019 revealed the uterine microbiome of virgin heifers and pregnant cows. 10 virgin heifers and 5 pregnant cows were used in this study. All 5 pregnant cows were slaughtered, and samples were collected post culling. Amniotic fluid, placentome, intercotyledonary placenta, cervical lumen and external cervix surface (as the control tissue) were sampled. The three most abundant phyla found in both heifers and cows were Firmicutes, Bacteroidetes, and Proteobacteria.

This study confirms that the uterus is not a sterile environment as previously thought (Moore et al., 2019). Researchers also noticed the presence of many bacterial species usually associated with postpartum uterine disease in both heifers and cows. These species included: *Trueperella* spp., *Acinetobacter* spp., *Fusobacteria* spp., *Proteus* spp., *Prevotella* spp., and *Peptostreptococcus* spp.

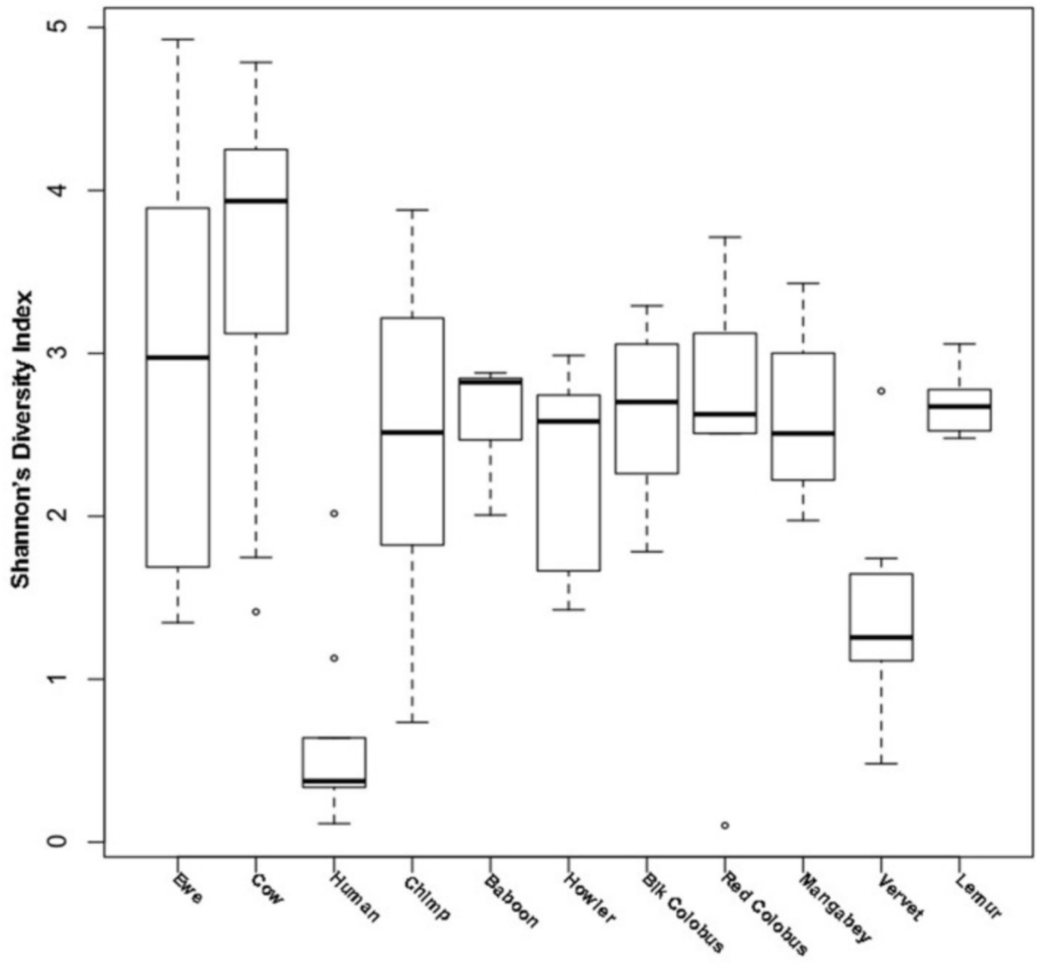


Figure 3 - Shannon's diversity of livestock vaginal microbiota as compared to humans and non-human primates. Boxplots showing the median, quartiles, and extremities of Shannon's diversity index values calculate from individual ewes, cows, humans, and non-human primates compared in this study, from © 2014 Swartz, Lachman, Westveer, O'Neill, Geary, Kott, Berardinelli, Hatfield, Thomson, Roberts and Yeoman. Open access paper available at: 10.3389/fvets.2014.00019

5.2.2 Comparison of microbiota in heifers and cows

Laguardia-Nascimenta et al., 2015, attempted to classify the vaginal microbiota of Nellore cattle using NGS sequencing. They studied four different groups of cows: Non-pregnant heifers and pregnant heifers as well as non-pregnant cows and pregnant cows. The samples were collected by vaginal lavage, and sequencing was done using universal primers to measure the presence of

archaea, bacteria and fungi. There was a noticeable lack of diversity in pregnant cows compared to non-pregnant ones, while Archaea populations were significantly more abundant in pregnant than in non-pregnant cows. The most abundant phyla of bacteria found included Firmicutes, Bacteroidetes, and Proteobacteria, which are also those found predominantly in the gastrointestinal tract (GIT).

Pregnant cows also tended to have a higher proportion of the Archaea, *Methanobrevibacter*. Two phyla of fungi detected were Ascomycota and Basidiomycota. Ascomycota seemed to dominate all four groups, with a reduction in pregnant animals. Fungi of the Mycosphaerella genus was also found, which is known to make compounds that inhibit protozoa. Researchers considered this significant, since this fungus may protect the vaginal canal against these parasites. This study confirmed that microbiota presence did not vary significantly between heifers and cows, though there were many individual variations.

5.3 Vaginal and uterine microbiota linked to disease in pregnant and postpartum cows

Inflammation or infection present in the uterus can compromise the reproductive state of an animal, as shown in multiple studies with dairy and beef cattle (Wang et al., 2017; CasLuengo et al., 2019). A study done by Jeon et al., 2018, revealed that there is a shift in uterine microbiota with different antibiotic treatments on mastitis in postpartum dairy cows. The study was done on 44 Holstein dairy cows diagnosed with mastitis and later randomly assigned to three different groups. The first group was treated with ceftiofur for 5 days, the second group received ampicillin trihydrate for 5 days and the 3rd group was untreated. The results showed that the Chao1 index was significantly decreased ($P \leq .05$) in all groups from day 1 to day 6, while the evenness increased in all groups from day 1 to day 6, although the change was only significant in the ceftiofur and untreated groups.

The study demonstrated how different antibiotics had equally diverse effects on uterine microbiota. 99.2% of bacterial phyla found were, from most abundant to least, Bacteroidetes, Fusobacteria, Firmicutes, Tenericutes, and Proteobacteria. Researchers found a significant increase ($P < .01$) in the relative abundance of Bacteroidetes and a significant decrease ($P < .01$) in the relative abundance of Tenericutes from days 1 through 6 (Jeon et al., 2018).

They noticed a similar pattern in genera populations: *Bacteroides, Porphyromonas*, and *Prevotella* within the phylum Bacteroidetes, *Helcococcus, Sporanaerobacter*, and *Peptoniphilus* within the phylum Firmicutes, and the genus *Campylobacter*, a Proteobacteria were significantly increased ($P \leq .05$), while the genus *Mycoplasma*, a Tenericutes, and *Sneathia*, a Fusobacteria, were significantly decreased ($P \leq .05$) from days 1 through 6. They concluded that the group treated with ceftiofur led to significant changes in most categories while the group treated with ampicillin, following a similar pattern to the untreated group, did not prove to have significant impact on microbiota changes (Jeon et al., 2018).

A study done by Moore et al., 2019 associated the uterine microbiota to the uterine transcriptome from cycling and non-cycling postpartum dairy cows. Their hypothesis was that the transcriptome in postpartum cows would be associated with the cyclicity status of the cows as well as the microbiota during the uterine involution (Moore et al., 2019). The study used 35 first lactation Holstein x Jersey breeds of dairy cows. Three endometrial samples were collected on 1, 5 and 9 weeks postpartum. These samples were then analysed by amplifying the V4 region of the 16S rRNA gene. They found that there were greater reads found during week 1 in the non-cycling cows compared to the cycling cows when compared to weeks 5 and 9. Four of the most significant OTUs found included: *Bacteroidales* S24–7, *Lachnospiraceae* NK4A136, *Clostridium sensu stricto* 1, and *Ruminococcaceae* UCG-005. It was noted that their combined relative abundance was greater on week 5 comapred to the rest (Moore et al., 2019). 809 genes were differentially expressed when comparing the samples from week 1 to the samples from non-cycling week 5 cows ans cycling week 5 cows. Cycling week 5 cows had a downregulation of protein synthesis genes as well as possibly harmful biological functions as compared to the non-cycling genes. In response to this, cycling week 5 cows therefore had an upregulation in other pathways as well as an increase in certain target molecules as well. There were 1489 genes that were expressed from the non-cycling week 5 cows when they were compared to the now cycling week 9 cows. Come week 9, since all cows were now cycling there was a lot of downregulated pathways in comparison to week 5 non-cycling cows. The paper goes on to conclude that the microbiota may have an indirect role in restoring cyclicity in cows, although more research is needed.

Another study took a different approach and compared the uterine and vaginal microbiota in dairy cows that were in the stages of developing postpartum endometritis. The study hypothesized that

during and after parturition the uterine and vaginal microbiota will mix which will be a possible cause to infection and once the vagina and uterus have a chance to go back to normal, they will be different in healthy compared to cows that go on to development postpartum endometritis (Miranda-CasoLuengo et al., 2019). 97 cows from 3 different Irish farms were used in this study. Duplicate samples were taken from both the uteri and the vaginas of each cow on days 7, 21 and 50 postpartum. The samples were later sequenced by amplifying the V1 and V3 hypervariable regions of the 16S rRNA gene. 26 out of the 97 cows were diagnosed healthy, and it seemed that even with the individual variation, the uteri and vaginas of the healthy cows shared core microbiota. However, the remainder of the cows all suffered from various forms of post-partum endometritis. They found that the higher the relative abundance of shared OTUs in the vagina, the higher the frequency of detection in the uterus. In correlation to this, they found that at day 7 postpartum, the vaginal and uterine microbiomes that later developed endometritis were more similar than in healthy cows. It was noted that a delay in the differentiation of the uterine and vaginal microbiota increased the chances of dysbiosis which resulted in the cow to become ill after parturition. The 6 most abundant phyla found were Firmicutes, Bacteroidetes, Fusobacteria, Proteobacteria, Tenericutes and Actinobacteria. In sick cows, there was a significant reduction in overall OTUs, bacterial diversity and species evenness as compared to the healthy cows. This study also showed that the healthy cows had a higher amount of Firmicutes while the unhealthy cows showed a high amount of Bacteroidetes. Finally, it was remarked that with the loss of diversity in the vaginal microbiome of sick cows, there was a higher presence of dominating OTUs from the genus, *Streptococcus, Bacteroides*, *Porphyromonas* and *Helcococcus* (Miranda-CasoLuengo et al., 2019).

This study showed the impact uterine and vaginal microbiota have on each other and how their differentiated microbiota is crucial for a healthy reproductive tract in cows. Unhealthy cows seem to show a decrease in bacteria with emerging dominant bacteria taking over the vaginal tract. Researchers surmised that a healthy and diverse vaginal microbiota can be restored. In turn, this may help the uterine microbiota to differentiate as well. This may avoid obstacles during breeding and may help prevent possible diseases postpartum.

Section 6 - Pathogenic Bacteria

An increasing abundance of literature points to dysbiosis as a crucial factor that may lead to reproductive failure. Delayed differentiation of microbiota in utero also seems to be related to postpartum disease. Therefore, many researchers have looked at the possibility of pathogenic bacterial strains that may ultimately lead to pregnancy failures.

6.1 Pathogens most likely associated with metritis and reproductive failure

A study of potentially pathogenic bacteria was done by Wang et al., 2013 using both a culture-based method and DNA sequencing. Acidfying bacteria like *Lactobacillus*, *Pediococcus, Staphylococci* and *Enterococcus* were in greater abundance in cows that developed metritis postpartum. Toxins created by other bacteria, for example, a Shiga-like toxin, SLT from *E. Coli*, and bacteriocin pediocin AcH/Pa-1, created by *Pediococcus acidilactici*, also seemed to negatively impact the reproductive tract. They concluded that overgrowth of pathogenic bacteria after parturition, especially *E. coli*, likely contributes to the development of metritis in dairy cows.

In addition, Rodrigues et al., 2015, compared the vaginal microbiota of five healthy and five sick cows with signs of reproductive disease. They found that sick cows presented many more potentially pathogenic and acidifying bacteria, such as: *Histophilus, Lactobacillus, Victivalles, Fibrobacter and Bacteroides.* These acidifying bacteria may be harmful to the vaginal canal by lowering its pH, thus inhibiting the growth of other beneficial bacteria. Other studies of the GI tract in humans and animals (Rodrigues et al., 2015; Runci et al., 2019) determined that some bacterial genera would compete for key nutritional elements, like iron. Such bacteria may therefore be especially harmful to the vaginal tract. These were: Proteobacteria like *Histophilus* and *Acinetobacter*, and *Corynebacterium* (Actinobacteria). Headley et al., 2014, also found that *Histophilus-somni* may be associated with spontaneous abortions in dairy cattle from Brazil.

Finally, a study by Díaz et al., 2019, confirmed that *Ureaplasma diversum* is especially toxic, with a clear link to reproductive diseases in cattle. This extensive study tested for *Ureaplasma diversum* in various locations on farms and slaughterhouses. They found that 64% of cows with

GGV-lesions tested positive for these bacteria as compared to those without lesions, 57% with poor reproductive efficacy also tested positive as compared to only 18% that tested negative.1/4 of farms with high abortion rates also tested positive. *Ureaplasma diversum* was also prevalent in the liver of aborted foetuses. All these studies seem to indicate links between certain levels of potentially harmful bacteria and their acidyfying effects on the vaginal microbiome as causes reproductive issues such as infertility, metritis or early embryonic loss.

Section 7 - Interplay of gut, faecal and vaginal microbiota

7.1 Intestinal microbiota during the estrous cycle in mice

One of the first hormonal studies of microbiota in mice during their estrous cycle found a decrease in progesterone during diestrus, demonstrating that there is a close and important relationship between intestinal bacteria and steroid hormone levels related to the estrous cycle (Syukuda, 1978).

Another study with mice showed a significant shift in the intestinal microbiota during the onset of pregnancy and throughout the remaining trimesters. This study also showed that there was a significant increase in the relative abundance of four genera: *Akkermansia*, *Bifidobacteria*, *Clostridium*, and *Bacteroides* (Gohir et al., 2015). Therefore, researchers assumed that there would be a shift in microbiota during estrous, as bacteria are highly involved in the development and metabolism hormones. Yet another study concluded that fecal microbiota is not influenced by the varying hormones during estrous (Wallace et al., 2018).

7.2 Influence of pregnancy on the vaginal and fecal microbiota in cattle

A study done by Deng et al., 2019, looked at how the vaginal and fecal microbiota are related to pregnancy in beef heifers. They used 72 heifers and synchronized their estrous cycles to prepare them to be bred. Once signs of estrus were detected, the heifers were left in the field with bulls for a period of 50 days. 56 out of the 72 heifers became pregnant. Samples were taken from the vaginas at the pre-breeding stage, during the first and second trimesters and for those that did become pregnant they were sampled at the third trimester. Fecal samples were taken at the pre-breeding

stage and at the first trimester. All samples were sequenced using 16S rRNA sequencing amplifying the V4 region of the gene. Interestingly, they found that the vaginal microbiota was dominated by an unclassified Enterobacteriaceae (21.05%), followed by *Ureaplasma* (4.37%) and an unclassified Bacteroidaceae (2.49%).

At the phylum level, Firmicutes was the most dominant at (31.57%) followed by Proteobactera (24.08%), Bacteroidetes (12.96%0 and Tenericutes (4.95%) (Deng et al., 2019). With the fecal microbiome, the top 15 features included several features associated with Ruminocaccaceae and Bacteroidaceae. At the phylum level, Firmicutes (45.93%), Bacteroidetes (18.83%), Euryarchaeota (6.14%) and Actinobacteria (2.57%) were 4 most abundant (Deng et al., 2019). To determine if the vaginal and fecal microbiota can be used to determine the success of of pregnancy, the researchers developed a Random Forest model to identify the bacterial features that were most likely to be biomarkers to pregnancy.

The top three most abundant bacterial features found in non-pregnant cows from the vaginal samples were *Histophilus somni,* Clotridiaceae 02d06, and *Campylobacter.* The fecal samples had a higher accuracy rating than the vaginal samples at 93%. Bacteroidales and Lachnospiraceae were the most abundant in the feces of cows that established pregnancy after breeding. Thus, the paper concludes that vaginal and fecal microbiota could be used as biomarkers of bovine reproductive success.

7.2.1 Correlation between blood, fecal and uterine microbiota in cattle postpartum

Finally, a study by Jeon et al., 2017, compared the blood, faecal and uterine microbiota in postpartum Holstein dairy cows. They wanted to investigate whether blood in the uterus after calving, would be an invitation to disease such as postpartum metritis. Blood, faecal and uterine samples were collected from 12 Holstein dairy cows on day 0 (day of calving) and on day 2 (2 days postpartum). Later, the samples were sequenced by 16S rRNA sequencing and the V4 region of the gene was amplified.

The 5 most abundant phyla found in the blood, feces and uterus were: Tenericutes, Bacteroidetes, Firmicutes, Proteobacteria, and Fusobacteria which accounted for 99.6% in blood microbiota, 91.4% in fecal microbiota, and 98.5% in uterine microbiota (Jeon et al., 2017). The microbiota in the blood was relatively stable from day 0 to day 2, with a high abundance of Tenericutes. In feces,

Firmicutes and Bacteroidetes were the most abundant phyla and also remained stable; however, rare phyla such as Tenericutes (2.3 vs. 0.9%, $P < 0.01$) and Fusobacteria (0.24 vs. 0.18%, $P = 0.02$) significantly decreased. The uterine microbiota was the only environment that was very dynamic throughout days 0 to 2. Tenericutes (1.9 vs. 21.9%, $P = 0.02$) and Fusobacteria (7.2 vs. 37.3%, $P < 0.01$) increased while Proteobacteria (44.1% vs. 6.2%, $P = 0.04$) decreased (Jeon et al., 2017).

Many statistical tests confirmed that the uterine microbiota was more similar to the fecal microbiota than blood microbiota. The fecal samples on day 0 tended to separate from fecal samples on day 2 ($P=0.067$), indicating a shift in microbiota in the gut after calving which resulted in a decline in diversity.

The blood core microbiota contained many major uterine pathogens such as *Bacteroides, Fusobacterium* and *Porphyromonas. Prevotella* and *Peptoniphilus* were also found and have been shown to contribute to metritis. Another core microbiota found in the blood was *Helcococcus,* another emerging uterine pathogen. *Mycoplasma* was also quite abundant in the blood as well. These findings show that pathogens can survive being transported from the blood to the uterus during and after parturition. The previously mentioned genus was not found as core microbiota in the fecal samples.

Another result supporting the hypothesis of bacterial transport from the blood to the uterus was an observed tendency of an inverse relation between the blood and uterus. Indeed, bacterial levels decreased in the blood as uterine bacterial levels increased. This paper concluded that the unique microbiota found in the blood that harboured various major pathogenic uterine bacteria could possibly transfer over to the uterus after calving (Jeon et al., 2017).

Section 8 - Supplemental: Microbiota manipulation strategies and their impact on vaginal microbiota

8.1 Intervention and supplementation

Given mounting literature tracing dysbiosis and pathogens to poor pregnancy rates and failed pregnancies, many companies and researchers advocate for the use of supplementation as a type

of microbiota manipulation for many uses. Adding probiotics and prebiotics to cattle feed to increase milk yield and aid digestion is now commonplace. Research is ongoing to expand such supplementation regimens to target vaginal microbiota, in the hope of preventing dysbiosis and known diseases, such as metritis, that affect pregnancy rates.

Most microbiota manipulation strategies involve a form of enrichment of the native microbiome through supplementation with beneficial bacteria. This is done with the goal to correct any dysbiosis in microbiota. Two forms of this manipulation are through the use of probiotics and prebiotics. Another aspect of manipulation is through targeted inhibition of pathogenic bacteria through the use of antibiotics. Most manipulation strategies have focused on gut microbiota, and many probiotic and prebiotic supplement regimens are readily available. Some researchers have taken these findings and applied them to vaginal microbiota to observe whether such supplementation may improve the reproductive health of cows. (Otero at al., 2006) The development of a reliable strategy may be key to improving fertility rates in cows by maintaining a healthy microbiome.

8.1.1 Probiotics

Probiotics are live microbes that can have many health benefits when consumed or applied to the body. They can be found naturally in fermented foods such as yogurt and kimchi, or can be taken in a supplement form. They are also used in beauty products, since they are added to many moisturisers. Probiotics can aid in digesting food or destroy disease causing cells, and can even produce vitamins. They interact with the gut microbiome by producing specific metabolites or cell components, including SCFAs (Short-chain fatty acids). They can improve the gut barrier and reduce inflammation through their interactions and secretions (Wieërs et al., 2019). Probiotics contain a variety of different microorganisms. The most common for human consumption are *Lactobacilli* and Bifidobacteria, used to relieve IBS symptoms and promote gut health. The benefits of *Lactobacilli* are far-reaching, as they may also help synthesise vitamins like B2, B11 and K (Gu and Li, 2015). Yeast is also a major probiotic, like *Saccharomyces boulardii*, often used to relieve diarrhoea (Rezac et al., 2018). Probiotics may also improve immunity to act as a defence against illnesses and pathogens.

Given the success of *Lactobacilli* and other acidifying bacteria in maintaining a healthy gut and vaginal microbiome in humans, researchers have attempted to create a similar probiotic for use in cattle. Otero et al., a culture-based study published in 1999, had the objective of designing a probiotic for veterinary use with cows to re-establish an optimal vaginal microbiome. They collected samples during the proestrus, estrus, metestrus and diestrus phases during two cycles in 15 Nellore Hereford heifers. They found that a total number of aerobes and facultative anaerobes was maintained at 10^2 and 10^5 CFU per sample during the estrous cycle. *Lactobacilli* was present in low numbers in the 3 phases while increasing slightly in the estrus phase. With 10^0 and 10^2 CFU/sample across the entire cycle. Of the 34 families of *Lactobacilli*, most were heterofermentative. (Otero et al., 1999) Similarly, Enterococci was seen to be at its highest levels during the estrus phase and slightly lower in the three remaining phases. However, it was much higher in value compared to *Lactobacilli* at 10^2 and 10^4 CFU/sample along the cycle. (Otero et al., 1999).

Elevated levels of these bacteria during the estrus phase would coincide with the increase in estrogen at estrus as seen in the hormonal study by Parish et al., 2016, since increased levels of estrogen will inhibit the growth of various bacteria while allowing a select few to remain. Given their results, researchers suggested that the development of a probiotic containing precise strands of *Lactobacilli* and enterococci could contribute to the therapeutic treatment of genital tract infections in postpartum dairy cows (Otero, et al., 1999).

The Otero group has continued research to develop a strain of probiotics for cows containing strains of *Lactobacilli*. Their findings confirm that the creation of lactic acid by *Lactobacilli* is an effective antimicrobial agent against pathogenic bacteria associated with “acute (bovine *E. coli*) and chronic (*Act. Pyogenes*) endometritis in cows.” (Otero et al., 2006) All their tests were started in vitro, and some lab experiments on mice were promising. Live experiments on cows have not yet been attempted. They are still searching for the most effective and resilient strain that can adhere to bovine vaginal epithelial cells while still being easily neutralised once the desired inhibitive effects are achieved.

Another study by Adjei-Fremah et al., 2018, noticed that probiotics increased milk production and weight gain in cows and calves, and had similar beneficial effects on other ruminants. Use of *Lactobacillus* and other acid producing bacteria provide protection against enteric infection. They also promote the use of supplements containing both bacteria and yeast "as multi-strain probiotics or a broad-spectrum effect against infections." (Adjej-Fremah et al., 2018)

Yet, A study by Zawistowska-Rojek et al., 2018, states that further research must be done on the long-term effects of probiotic use. Researchers noticed that microorganisms used as probiotics may actually cause infection and disturb the metabolism. In addition, a study done by Uyeno et al., 2015, recommends caution in the use of these probiotics and prebiotics, especially in healthy cows and calves. Though they have been traced to increased milk-production and weight gain, secondary effects of long-term use have not been fully assessed, nor have the precise strains of effective microbiota for probiotic use been determined. Probiotics may benefit unhealthy calves by moderating diarrhoea and re-establishing a certain balance in the GI tract, but their effects on healthy calves is minimal. Also, overuse of probiotic supplements in the early stages of life may disrupt the establishment of a healthy gut microbiome in young calves. Probiotic yeast strains are especially unreliable, and their effects inconsistent. The long-term effect of probiotics on the whole gastrointestinal microbiome is also relatively unknown, and their use may prove disruptive to a healthy microbiome (Uyeno et al., 2015). In addition, no research on their long-term impact on other microbiomes, like the vagina, has been done.

Another caution is the application of probiotic bacteria effective in the health of the human gut health to ruminants, since these species have vastly different physiology and diets, and therefore sharply contrasting microbiomes. Specifically, the use of *Lactobacilli* as a probiotic in cows is questionable, since the nature of this bacteria is to claim dominance over residing microbiota. In addition, ruminants like cows, have very trace amounts of *Lactobacilli;* in fact, these bacteria, found in greater abundance in calves, decrease with maturity (Uyeno, et al., 2010). Therefore, one can question why the agricultural industry is avidly promoting adding these bacteria to a probiotic regimen in cattle feed.

8.1.2 Prebiotics

Prebiotics are a group of nutrients within plant fibres that are degraded by gut microbiota. Though these fibres are indigestible to humans, they are a ready source of nutrition for probiotics and gut microbiota. A recent study by Davani-Davari et al., 2019, found that prebiotics feed the intestinal microbiota and that their degradation results in short-chain fatty acids (SCFAs). In turn, these fatty acids are the main source of energy for colon cells and therefore promote gut health and may reduce obesity, diabetes, fatty liver disease and other diet-related diseases. (Devani-Davari et al., 2019).

All high-fibre plants, including leafy greens, some fruit, legumes and grains contain prebiotics. Examples of these include: asparagus, sugar beet, garlic, chicory, onion, Jerusalem artichoke, wheat, honey, banana, barley, tomato, rye, soybeans, peas, beans, etc., along with seaweeds and micro-algae (Carlson et al., 2018).

Prebiotics leave by-products in the bloodstream including SCFAs and other elements like antioxidants, saccharides and proteins, that may provide the entire body with multiple benefits: Prebiotics may increase general cognition, decrease the process of dementia, increase recall and memory, and help with learning and overall mood. Prebiotics can also aid in calcium uptake, increase water retention, keratin retention and collagen formation as well as lower low-density lipoproteins (LDL) (Devani-Davari et al., 2019)

Most prebiotics used in cattle are the addition of high-fibre diets and grains to encourage rapid weight gain. These must also be supplemented with yeast, a probiotic, to improve digestion. Though yeast supplementation seems effective (Uyeno et al., 2015), adding grains and other high calorie supplements to feed (like corn), also has a disruptive effect on the gut microbiome.

8.1.3 Antibiotics

Antibiotics, although very important when dealing with illnesses and infections, have been linked to very negative effects on the gut microbiota (Francino, 2016). Antibiotics are, for the most part, non-selective, and therefore can destroy large numbers of both beneficial and pathogenic bacteria. Overuse of antibiotics causes dysbiosis through a reduction in biodiversity of (gut) microbiota.

This in turn negatively impacts the host's overall health and immune system. A recent study done by Zhang et al., 2019, found that these effects may cause or aggravate various diseases, such as: diabetes, obesity, inflammatory bowel disease, asthma, rheumatoid arthritis, depression, autism and superinfection in critically ill patients (Zhang et al., 2019).

That study also found that antibiotics impact the host's immunity by altering bacterial metabolites and the signals transmitted from gut microbiota to the host. These signals have a particular effect on intestinal epithelial and immunity cells. Metabonomic analysis shows that lipids, bile acids, amino acids and amino acid-related substances in the gut all suffer from antibiotic use. Antibiotics also affect the function of the bacteria to make short-chain fatty acids (SCFAs), a very important energy source for colon cells and gut microbiota. This can lead to inflammatory issues in the gut. Meanwhile, their effect on immunity cells can render the host more susceptible to invading pathogens (Zhang et al., 2019).

A study done by Holman et al., 2019, looked into the effect of 2 antibiotics on the fecal and nasopharyngeal (NP) microbiota in feedlot cattle. 36 Angus × Hereford cattle were used, divided by sex: 18 steers and 18 heifers. All cattle were randomly assigned to one of three treatment groups, with 12 in each: control, oxytetracycline and tulathromycin. Prior to this experiment, none of the animals were subject to any antibiotics, vaccines or hormone implants. Fresh fecal and NP swabs were taken on day 0 (before the injections) and later on days: 2, 5, 12, 19, and 34. Afterwards, the V4 region of the 16S rRNA gene was sequenced. Researchers discovered that both residing fecal and NP microbiota were significantly affected. To this, NP microbial community structure was very sensitive to the oxytetracycline treatment group. Oxytetracycline and tulathromycin also increased the amount of a lot of antibiotic resistant determinants in both fecal and NP animals. Typically, these antibiotics are meant to be used on a long-term basis, however, NP microbiota were affected on a large scale on days 2 and 5 of treatment, after the first initial doses of antibiotics were administered. Given the very dynamic nature of microbiomes, it is of no surprise that any manipulation can have far-reaching consequences.

Such studies accumulate compelling evidence of the potentially negative effects of long-term use of antibiotics, and also of the indiscriminate use of probiotics and prebiotics. In our attempts to inhibit the growth of one pathogen through the addition of acidifying bacteria, we also affect the

delicate balance of a healthy microbiota. Similarly, adding prebiotics to a ruminant's diet to affect weight-gain and milk production, also carries harmful side-effects. Therefore, it is imperative that research continues, not only to find a precise strategy of manipulation, but better yet, in order to find more effective ways to achieve and maintain an optimal balance in this living microbiome.

Conclusion

Microbiota research is a vast and dynamic field of study. Sampling strategies and cultivation methods have evolved greatly over the past century, to the point that cutting-edge technologies and DNA sequencing are now providing researchers with an ever-widening bank of precise data. What we have learned about interactions and impacts of microbiota in humans can be transferred, in part, to animals, and more precisely, to cattle. Scientists have mapped the microbiota in the GI tract of cattle, and some have also begun the study of the vaginal microbiome in cows. This is a crucial step to maintaining a profitable cattle industry, since reproductive inefficiency is a great source of revenue loss.

Researchers are now successfully mapping the great variety of bacteria populating the vaginal tract, and some have expanded their research to uterine microbiota. Some commonalities of phyla can be seen, as can some differences in populations between pregnant and non-pregnant cows. Yet this research is often disparate: Some studies focus on hormones, others on microbiota, others on pathogenic bacteria. Most are actually experimenting with hormone additives or replacements to induce Estrus, while others are making crude attempts at microbiota manipulation with other drugs and interventions. Most research is now based on a synchronization protocols which cloud or destroy the natural microbiota makeup of cows. Few, if any, have taken the time to step back to theorise on the links between all of these factors. The same holds true with studies on nutrition and supplementation.

Probiotic and prebiotic supplements are quickly replacing the use of antibiotics in the cattle industry. Acidifying bacteria and fermenting yeast are now common additives to feed, as are calorie-rich grains. Yet this practice comes with a word of caution: the effect of the long-term use of these additives is still unknown. Some compelling data shows that these supplements may indeed have adverse effects, especially in young calves and in healthy cattle. Of greatest concern

are those researchers who seem to be attempting to replace antibiotics by probiotics, with little regard as to the natural composition of bovine microbiota. Too many studies seem to be focusing on lactic acid creating bacteria, which are prominent in a healthy human microbiome, but are found in very trace amounts in the vaginal microbiota of cows.

All these studies confirm that greater time and care must be taken to observe the natural interactions between vaginal microbiota and their host. Such analyses will help to better understand the links between microbiota fluctuations, the estrous cycle, hormones, pH and other factors, that all work in symbiosis to ensure a healthy host and reliable pregnancy rates.

Chapter 3 - Scientific Manuscript

Impact of the Estrous Cycle on the Vaginal Microbiota and Its Association to Pregnancy in Dairy Cows

To be submitted to the Canadian Journal of Veterinary Research

Abstract

A great deal of research has been done on the four phases of the estrous cycle in cows, yet very few studies exist regarding their vaginal microbiota. This study compared the fluctuations in vaginal microbiota in pregnant and during the estrous cycle in non-pregnant multiparous Holstein dairy cows. The objective of this study was to investigate variations in microbiota populations during the estrous cycle and its possible association with pregnancy. Twenty-one multiparous Holstein cows on the same farm underwent artificial insemination (AI) after estrous detection. Vaginal swabs were collected four times, on days: 1 (before AI), 3, 15, and 19. Ultrasonography was performed at day 31 to confirm pregnant. The vaginal microbiota was assessed using Illumina sequencing of the V4 region of the 16S rRNA gene. A clear predominance of certain phyla was found, with Firmicutes, Bacteroidetes and Proteobacteria making up over 80% of the vaginal microbiota composition throughout, with notable fluctuations between individuals. Changes that were proven statistically significant were between the estrus-proestrus phases in non-pregnant cows (P=0.028) and between the estrus and diestrus phase in pregnant cows (P=0.043). These findings are a clear first step in identifying vaginal microbiota in Holstein dairy cows and may help determine the potential impact of microbiota on fertility rates and overall vaginal health of cows.

Section 1 - Introduction

In Canada, dairy farming alone accounts for one of the largest agricultural sectors and contributes close to 20 billion dollars to the economy annually and employs nearly a quarter million people (Canadian Dairy Sector Overview, 2018). Unfortunately, infertility is one of the leading causes of high economic losses in the dairy and beef industries. Many infections in cattle may cause infertility, stillbirths and abortions, and these seem to be on the rise (Walker, 2019). In addition, lower pregnancy rates following artificial insemination (AI) in dairy cattle are also of concern. Unfortunately, the reasons for these lower rates are not yet clearly understood. Possible causes include improper timing, improper technique and the overall condition of the uterine environment during AI.

A great deal of research has focused on the health of vaginal microbiota in women and its possible link to infertility and neonatal issues (Barrientos-Durán et al., 2020). Typically, healthy vaginal microbiota in women is composed primarily of *Lactobacilli*. These bacteria create lactic acid that not only maintains vaginal homeostasis by establishing a lower pH, (Ravel et al., 2011) but also acts as an important microbicide, thus avoiding possible dysbiosis (O'Hanlon et al., 2013). Lower abundance of this bacteria may lead to shorter gestational lengths and even bacterial vaginosis, which affects one-third of women around the world (Freitas et al., 2018) (Eastment & McClelland, 2019). Vaginosis may also be linked to infertility and possible repeated implantation failure (Kitaya et al., 2019). Given the magnitude of data from multiple studies clearly relating women's vaginal health and fertility with their microbiota, especially the importance of *Lactobacilli*, it would therefore be the next logical step to focus on vaginal microbiota in cows to determine their possible link to their vaginal health and fertility rates.

In dairy cattle, issues with fertilisation of the oocyte or problems with embryo implantation were linked to early embryonic death (Parmar et al., 2017). Many recent studies have attempted to trace possible links and patterns in microbiota from conception to birth. A recent study by Ault et al., 2019, showed a decrease in bacterial diversity within the uterus during the synchronization protocol before AI. Another study showed a shift in the vaginal microbiota in beef cattle in the later gestational stages (Deng et al., 2019). This recent study indicated a possible continuous

increase in vaginal microbiota from the pre-breeding stage to the second trimester of pregnancy; with a marked decrease from the second to the third trimester. There have also been certain studies (Otero et al., 1999 & 2006) that made acidifying bacterial probiotics and have given them to cows through their diet, but not in the goal to improve fertility.

Quereda et al., 2020, stated that future research should be focused on discriminating beneficial bacterial groups so that biomarkers for reproductive health can be established. This information may allow researchers to design a probiotic-based treatment for cows that fail to become pregnant. This current study falls into this philosophy: by comparing the microbiota of cows that succeeded to become pregnant to the microbiota of those that failed. With biomarkers that ensure a healthy pregnancy, more informed research may be done on how best to provide an optimal microbiota to improve overall pregnancy rates for the cattle industry.

Therefore, the objectives of this study were to investigate the vaginal microbiota present at the different phases of the estrous cycle preceding artificial insemination (AI) in dairy cows. It also compared the vaginal microbiota of cows that succeeded to become pregnant to the microbiota of those that did not. Since hormonal profiles vary widely during the estrous cycle, the study attempted to relate these changes to microbiota fluctuations. Data collected tested the hypotheses: i) Bovine vaginal microbiota change during the different phases of estrous. ii) Bovine vaginal microbiota composition impacts pregnancy rates.

Section 2 - Materials and Methods

2.1 Ethics Statement

Animal care and use protocols were approved by the University of Londrina's Animal Care and Use Committee under protocol number 10878.2019.88, dated the 6th of August 2019.

2.2 Subject and Sample Collection

Vaginal swabs were collected from 21 multiparous Holstein dairy cows housed in a farm located in the city of Castro, state of Paraná, Brazil. The selected cows were all submitted through a screening process: no history of reproductive disease or any use of antibiotics before the study. All cows had a high milk production with an average of 42.4L with varying stages of life and ages. The cows were milked twice a day, followed by feeding of a commercial concentrate diet consisting of 16% crude protein and corn silage. The cows spent the remainder of the day in the field where they were allowed to graze on pasture that consisted of Azavém (*Lolium multiflorum*). For the entire study the cows remained with the rest of the herd with no changes in reproductive, sanitary, food or zootechnical management already carried out on the farm.

The cows were not submitted to any estrous induction protocol. Instead, estrous detection was performed using SCR necklaces by Allflex. These collars were attached around the subjects' necks to monitor and capture signals of the cow's metabolic, rumination and panting activities. Data from each cow was automatically sent to a management software. An observed descending peak in rumination or a sudden increase in activity (panting) would indicate that the animal was in heat and was ready to be inseminated.

The ovarian activity was verified by transrectal ultrasound after each sample collection. The first sample was taken on the first day of estrus. Samples from the metestrus phase were taken on day 3, diestrus phase samples on day 15, and proestrus phase samples on day 19. Finally, on day 31, an ultrasound was done to confirm pregnancy.

Collection protocol: First, the perineum area was cleansed and disinfected with 70% ethanol, next a sterile cotton swab was inserted into the vaginal canal and was circled around the vagina five

times. The swabs were removed from their plastic rods with sterile forceps and immediately transferred into a liquid nitrogen container at a temperature of -80°C to await analysis.

2.3 DNA Extraction Protocol

The PowerSoil® DNA isolation kit (Qiagen 2019, Hilden, Germany) was used to extract the DNA from these samples. All steps conducted on the samples were followed exactly from the manual provided in the kit.

2.4 16S rRNA Gene (V4) Amplification and Sequencing

The V4 region of the 16S rRNA bacterial gene was amplified by PCR using the two primers: 515F (GTGCCAGCMGCCGCGGTAA) and 806R (GGACTACHVGGGTWTCTAAT). Sequencing was done using an Illumina MiSeq platform, using the V2 reagent kit (2 x 250 cycles) at the McGill University and Genome Québec Innovation Centre. Sequence data was processed using the software Mothur v.1.41.3, following a Standard Operating Procedure (Kozich et al., 2013). Reads were clustered at 97% similarity and classified based on the Ribosomal Database Project (RDP). Operational taxonomic units (OTUs) were considered by merging reads classified within the same genus.

Three alpha diversity indices: Chao 1, Inverse-Shannon and Shannon-Weiner, were calculated. The similarity of bacterial communities found in each sample (beta diversity) was addressed by the Jaccard and Yue and Clayton indices to compare membership and structure respectively and were visualized by the principal coordinates analysis (PCoA).

2.4.1 Statistical Analysis

Further analysis of molecular variance (AMOVA) was conducted to determine statistical significance of results for both membership and structure of microbiota during all four phases of the estrous cycle, with a p value of $p<\alpha$, $\alpha = 0.05$.

Two-way repeated measures ANOVA testing was performed to compare alpha diversity indices, considering pregnancy outcome and sampling time as variables. P values were determined as significant to $P<0.05$. LEfSe analysis was also performed to determine statistical differences in

genera between pregnant and non-pregnant cows at estrous and variations during the four phases of the cycle.

2.4.2 Descriptive Statistics

The differences between individual groups of bacterial phyla during the different phases of the estrous cycle of non-pregnant cows and comparing these to the bacterial phyla at similar time points in pregnant cows were determined using a 2 tailed t-test. Significance was set at $P<0.05$.

Section 3 - Results

A total of 84 samples (21 cows at 4 different time points) were collected from the vaginal swabs. Out of the 21 cows, 9 became pregnant while the remaining 12 failed to become pregnant after AI. The total amount of births ranges from 2-7 calves per cow. The youngest cow used in this study was 37 months in age while the oldest was 108 months of age. The intensities of heat varied between each cow and the total milk production average at 42.2L.

3.1 Alpha Diversity Indices

Of the three alpha diversity indices calculated, the Chao 1 Index showed the greatest standard deviations, while the Shannon-Weiner Index showed very low SD. Nonetheless, there was no significant statistical differences between alpha diversity indices of vaginal microbiota in cows that tested positive for pregnancy and those that were negative throughout the different phases of the cycle as compared by ANOVA t-tests (all $P>0.05$). (See Appendix 3)

3.2 Beta Diversity Membership and Structure

3.2.1 Membership: Jaccard Index

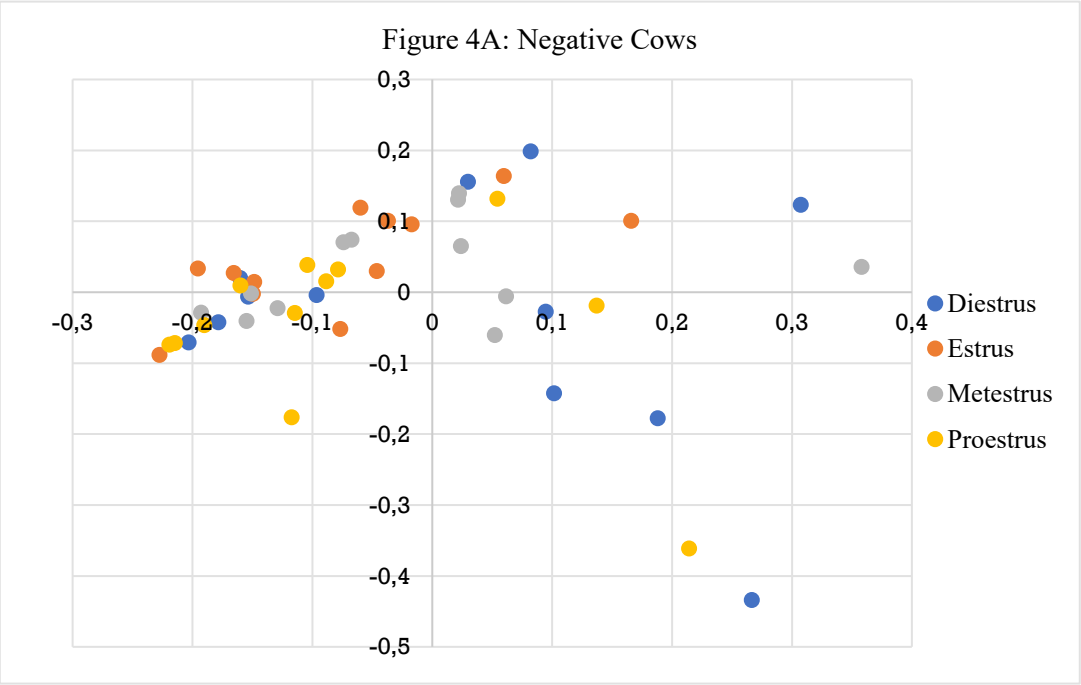
There was no statistically significant clustering during the different phases of the estrous cycle in pregnant and non-pregnant cows as seen in PCoA graphs A and B in Figure 4. There were no statistical differences addressed by the AMOVA test.

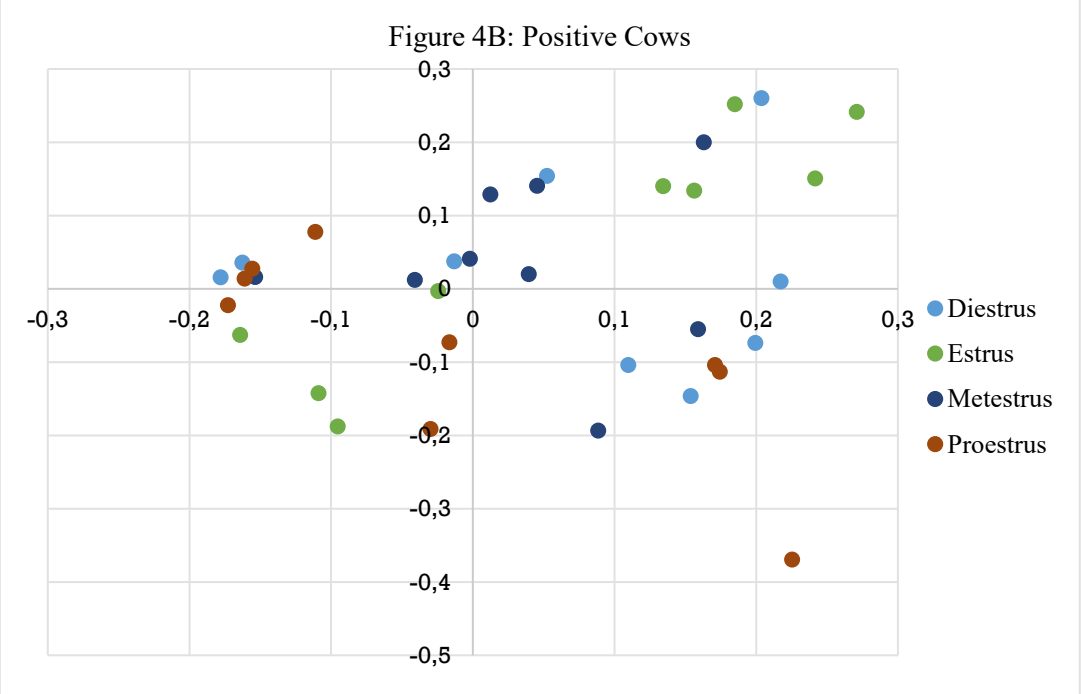
Likewise, there was no obvious clustering in membership in the comparison between pregnant and non-pregnant cows during three of the four phases of the cycle, at the metestrus, diestrus and proestrus, with a small grouping of cows that failed to become pregnant at the estrus phase. (See Appendix 1: PCoA Graphs A to D.) There was a difference in membership in negative cows, between Day 1 and Day 19 of sampling ($P=0.028$).

3.2.2 Structure: Yue and Clayton Index

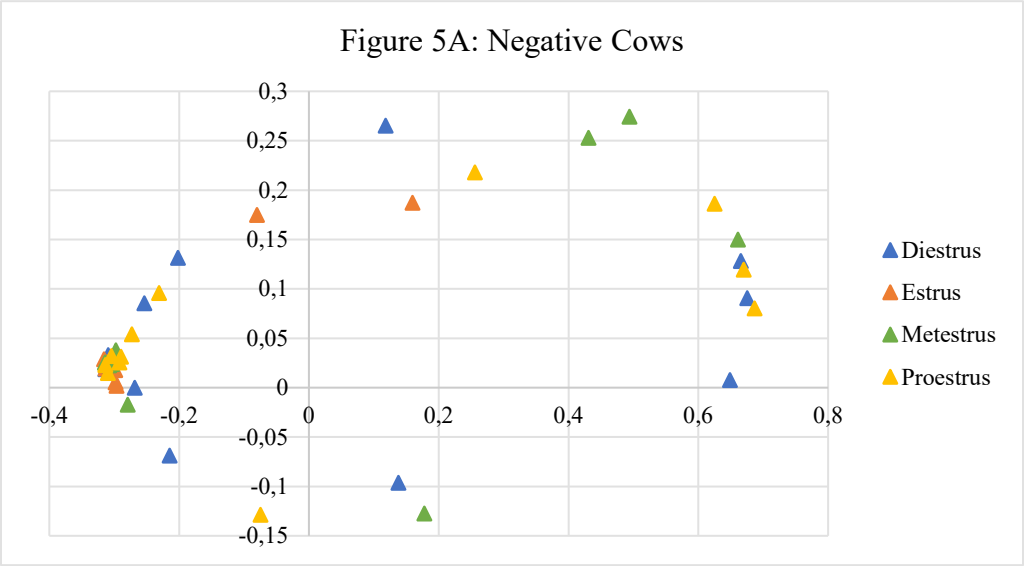
There was no evident clustering in microbiota structure through the estrous cycle in either group of cows as seen in PCoA graphs of Figure 5. P-values obtained show there was no statistical difference (all $P>0.05$) between pregnant and non-pregnant animals at the different sampling times, as seen in Table 2. There was a difference in structure in positive cows, between Day 1, before AI and Day 3 of sampling ($P=0.043$).

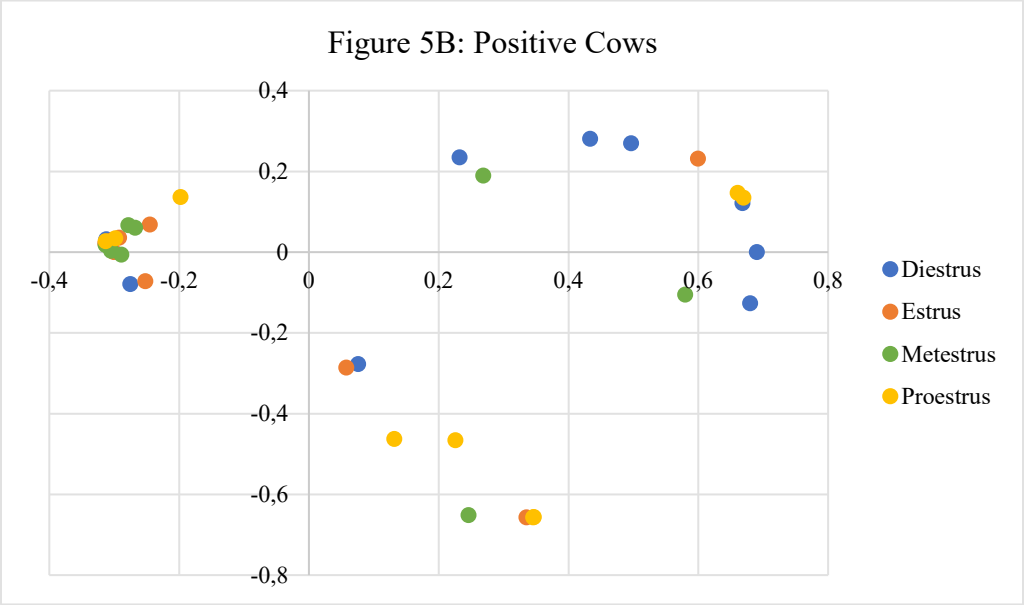
Figure 4 - PCoA graphs of membership of vaginal microbiota in cows that failed (4A) and succeeded (4B) to become pregnant showing no evident clustering.





Figures 5 - PCoA graphs of Yue and Clayton Index of Beta Diversity of structure of vaginal microbiota in cows that succeeded and failed to become pregnant showing no evident clustering during the four phases of the estrous cycle.





3.3 Relative Abundance of Vaginal Microbiota - Phyla

Of the 28 distinct phyla sequenced, only three phyla predominate throughout all phases of the estrous cycle: Firmicutes, Proteobacteria and Bacteroidetes, as seen in Figures 6. These three phyla combined account for over 80.735% or 4/5 of microbiota present during each phase. When comparing median results to averages, there are clear discrepancies, especially in pregnant cows during the final sampling phase, due to individual variances in microbiota membership and structure. (See Appendix 3)

A sample T-test was done to compare average phyla level variances over the four sampling phases in pregnant cows and non-pregnant cows. Results in 4 phyla do show some significant variations, of which all were $P < 0.05$. These were: Firmicutes at $P= 0.049$; Bacteroidetes, $P = 0.023$; Unclassified Bacteria $P = 0.012$; and Fusobacteria $P = 0.011$.

Firmicutes levels are higher in non-pregnant cows throughout all sampling phases, as compared to levels in those that became pregnant. Bacteroidetes levels were also consistently more elevated in non-pregnant cows throughout, though both groups showed a relatively steady decline in levels throughout the four sampling phases. Unclassified bacteria also decreased from the first to the last sampling phases in pregnant cows, while remaining relatively stable in non-pregnant cows. These relate inversely to increases in pregnant cows on day 19 in levels of Fusobacteria and Actinobacteria. Proteobacteria levels started relatively low in non-pregnant cows to increase sharply by Day 19, whereas levels in pregnant cows started and finished much higher than in non-pregnant cows. These fluctuations in Proteobacteria, though strong, were not determined as statistically significant, probably because of outliers since some cows had extreme levels of Proteobacteria when compared to others. (See section 3.5 on individual variances.)

Phyla membership varied greatly, which may explain some of the individual variances noted throughout. Proteobacteria were by far the most abundant in membership, with over 220 confirmed and unclassified genera, followed by 170 Firmicutes, 112 Actinobacteria, 80 Bacteroidetes, 16 Acidobacteria, 13 Verrumicrobia, 9 Planctomycetes, 8 Fusobacteria and 8 Tenericutes.

Upon further analysis of phyla results, there are clear fluctuations in microbiota membership at each sampling phase in individual cows. For example, in those cows that would become pregnant, Cow #955 had a great abundance of Firmicutes, and #1021 had more Tenericutes present than the

rest. Two days after AI, the Tenericutes present in #1021 have made way to a greater abundance of Firmicutes and #821 also shows more Firmicutes. Meanwhile, #955's high levels of Firmicutes have greatly diminished to be replaced by Proteobacteria at comparable levels to the rest of the pregnant cows. A great increase is seen in Proteobacteria in most pregnant cows at day 15, especially #1021, whose microbiota continue to undergo extreme fluctuations. Firmicutes gradually take over in cows #903 and 944, which only showed trace amounts during the first two sampling phases. On day 19, cows #1021, 1117 and 738 have a predominance of Proteobacteria while others show high levels of Firmicutes.

In cows that failed to become pregnant, fewer less extreme, individual fluctuations were seen. Firmicute levels are well distributed throughout all cows and phases, except for #1030 and #1039 which have important increases in Proteobacteria after AI and throughout the following phases. #882 also shows an increase in Fusobacteria at Day 15. Overall, levels on Day 19 do seem to return to comparable levels to those found at the beginning of the cycle, on Day 1, except for #1039, 1130 and 994 with higher-than-average Proteobacteria. (See Figures 7 A-H)

Table 1 - Mean, minimum, median and maximum values of most abundant phyla showing differences between pregnant and non-pregnant cows during the four sampling phases in percentages

		Estrus Day 1		Metestrus Day 3		Diestrus Day 15		Proestrus Day 19	
Bacterial Phyla	**Percentages**	**Neg**	**Pos**	**Neg**	**Pos**	**Neg**	**Pos**	**Neg**	**Pos**
Firmicutes	Mean	52	39	43	41	35	28	38	26
	Minimum value	36	7	10	16	0	3	1	1
	Median	54	43	47	49	38	32	48	10
	Maximum value	65	54	66	61	59	50	54	54
Proteobacteria	Mean	7	17	21	20	28	42	25	32
	Minimum value	2	2	2	3	2	1	2	2
	Median	5	9	7	6	13	43	8	10
	Maximum value	30	56	82	52	88	86	78	84
Bacteroidetes	Mean	28	20	21	20	19	11	19	12
	Minimum value	10	2	2	7	0	1	0	0
	Median	29	22	19	18	23	8	21	3
	Maximum value	43	40	39	35	50	31	32	29
Unclassified Bacteria	Mean	9	6	9	5	7	6	10	5
	Minimum value	4	1	1	2	0	0	0	0
	Median	9	5	10	5	7	4	10	1
	Maximum value	13	12	19	10	13	20	20	13
Fusobacteria	Mean	1	9	1	8	4	9	4	18
	Minimum value	0	0	0	0	0	0	0	0
	Median	0	0	0	0	0	0	0	2
	Maximum value	3	83	5	37	26	41	17	81
Actinobacteria	Mean	2	5	3	3	5	2	2	4
	Minimum value	0	0	0	0	0	0	0	0
	Median	1	5	2	2	1	1	1	2
	Maximum value	8	15	21	7	36	6	7	21
Tenericutes	Mean	0	2	0	0	1	1	1	1
	Minimum value	0	0	0	0	0	0	0	0
	Median	0	0	0	0	0	0	0	0
	Maximum value	1	19	0	1	12	4	6	10
Spirochaetes	Mean	1	0	1	1	1	0	1	0
	Minimum value	0	0	0	0	0	0	0	0
	Median	0	0	0	1	1	0	1	0

Figure 6 - Median and Mean comparisons of most prominent bacterial phyla between cows that became pregnant and cows that did not over the four phases of the estrous cycle.

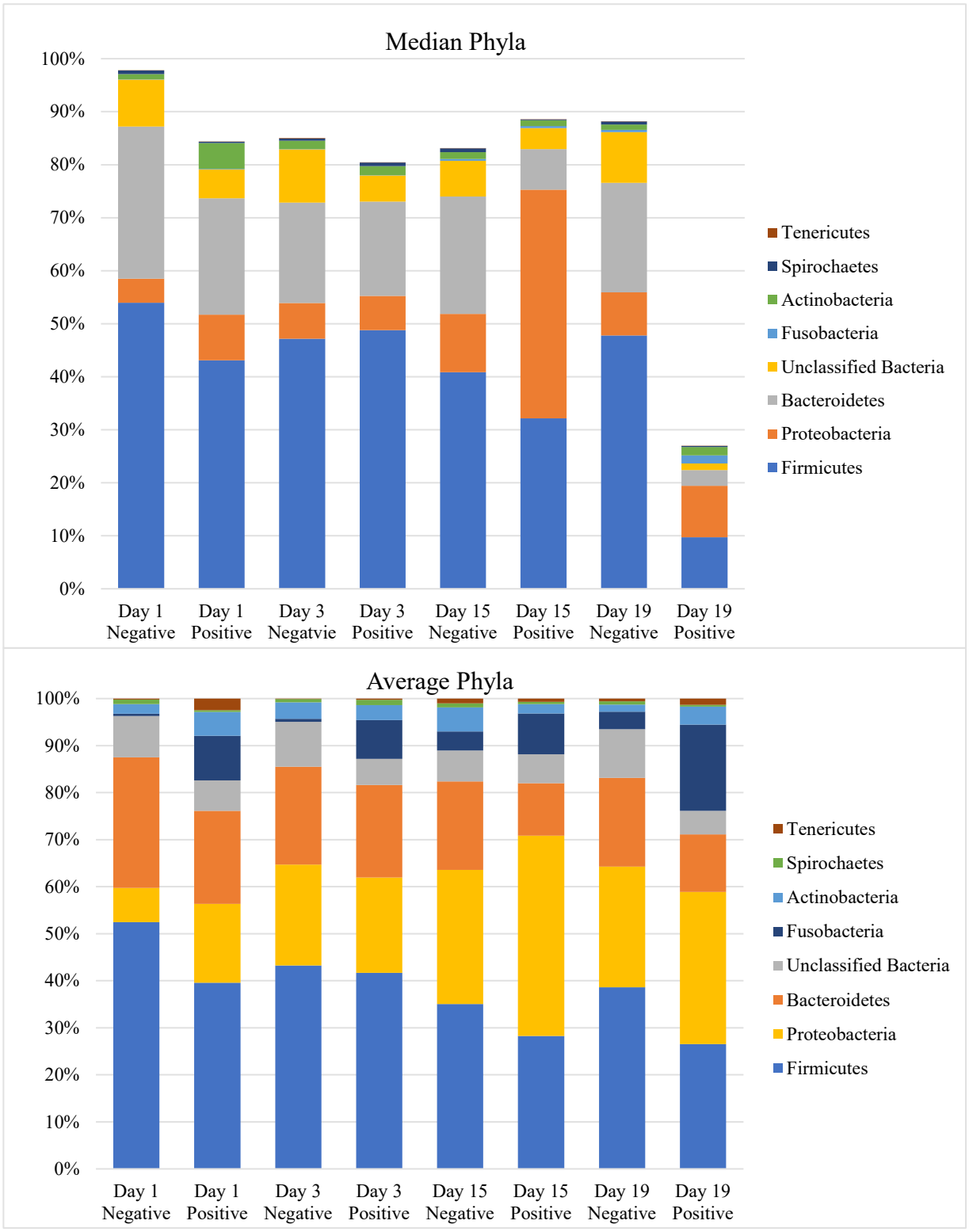
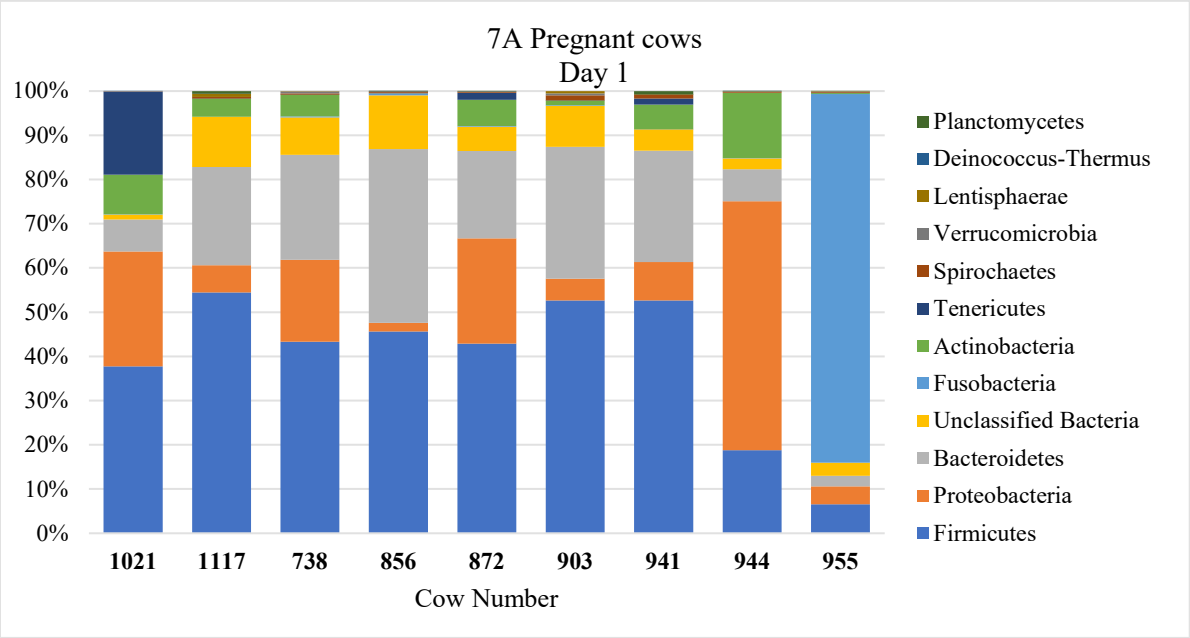
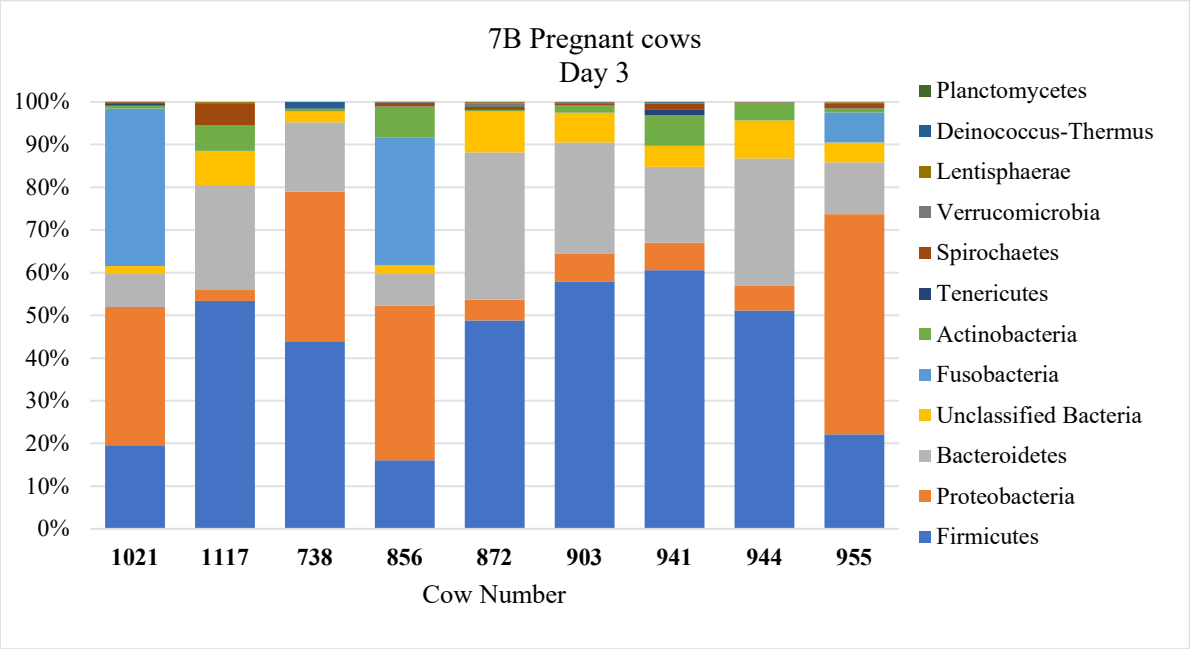
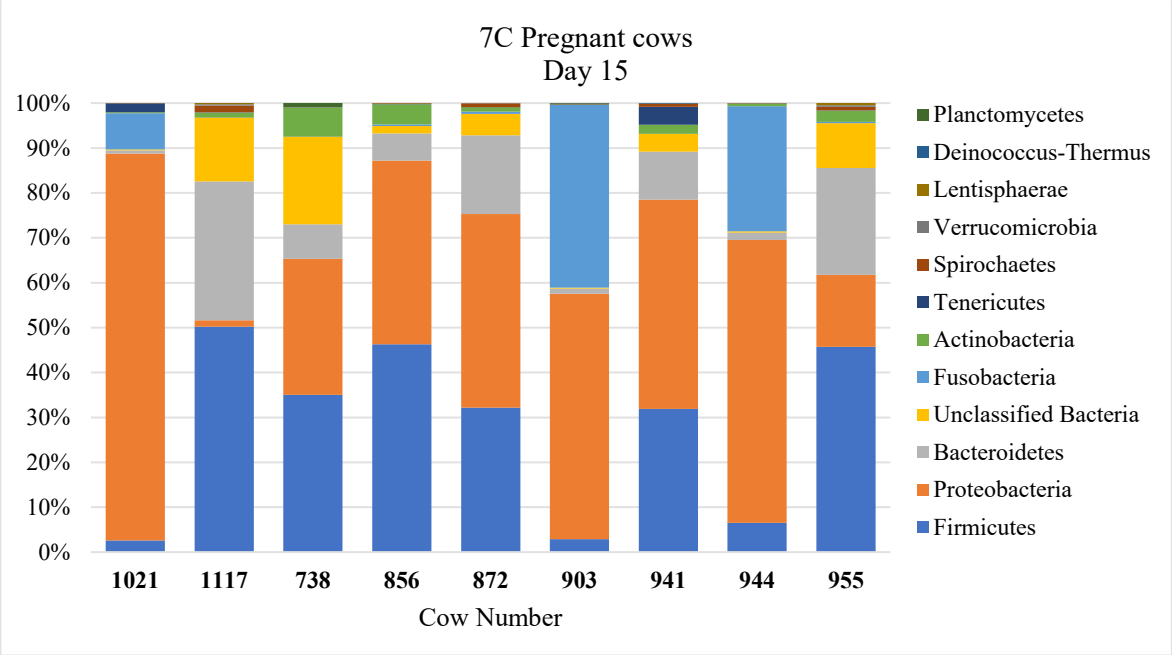
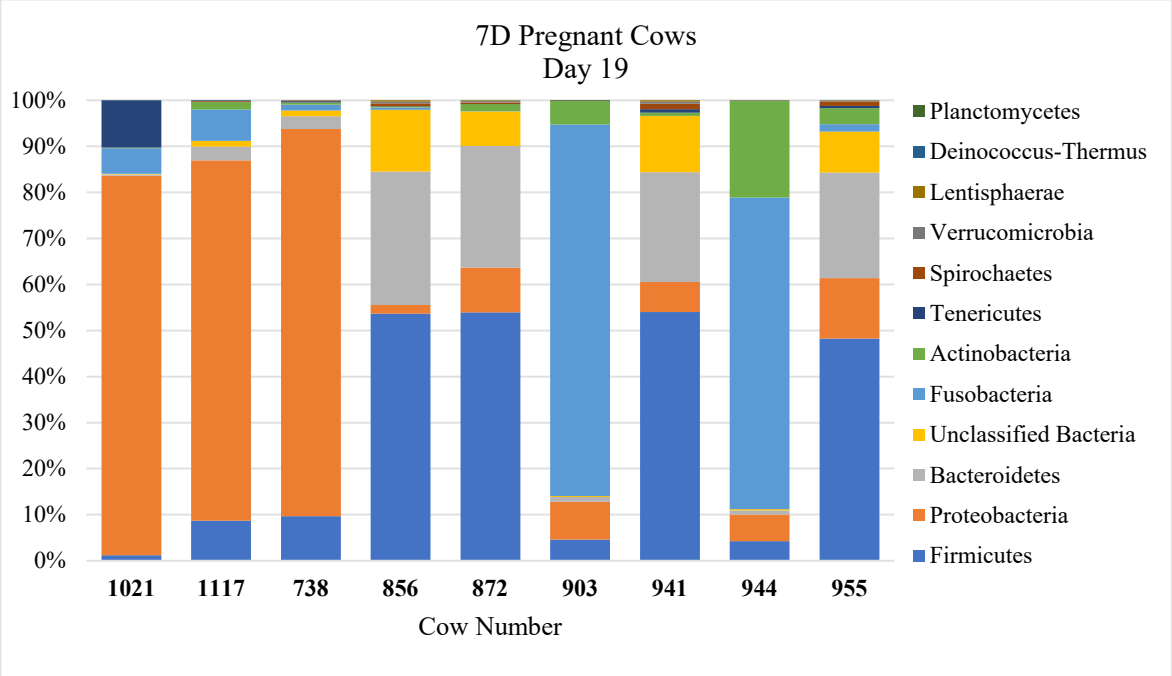


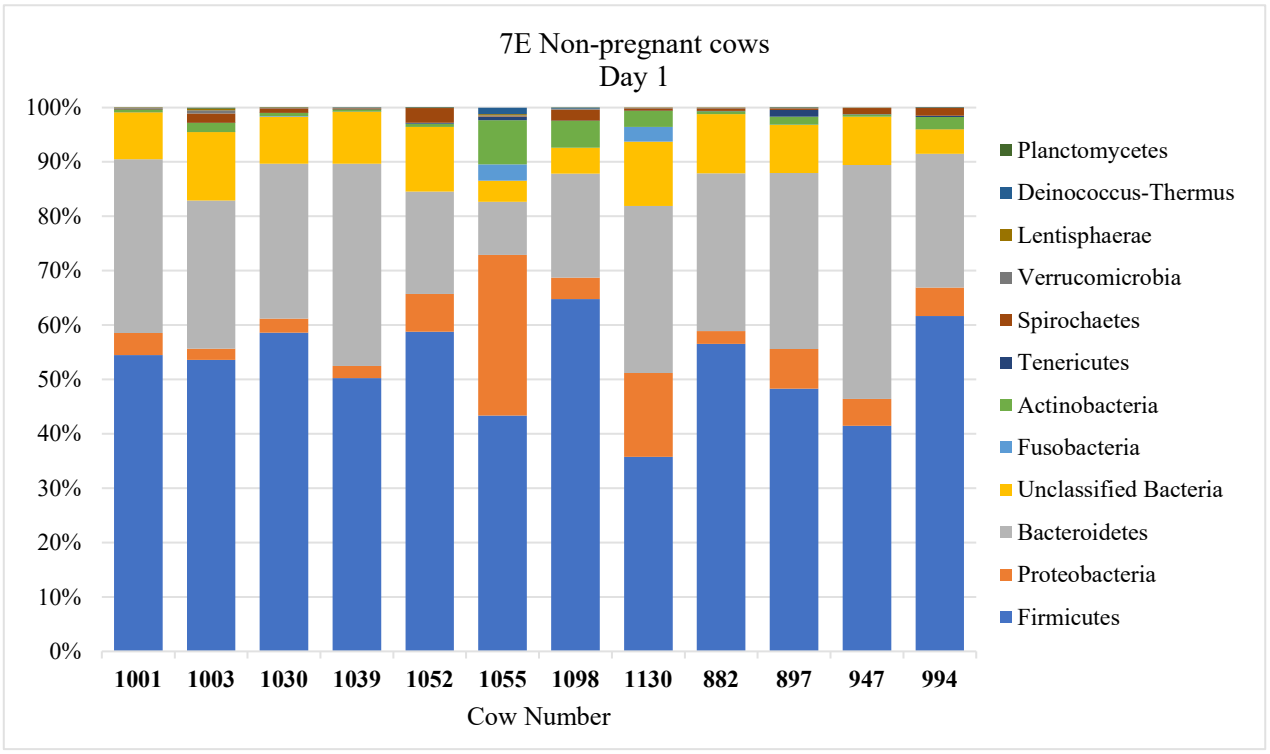
Figure 7 - Relative abundance of vaginal microbiota phyla during four sampling phases in cows that tested positive for pregnancy and in those that failed. A to H

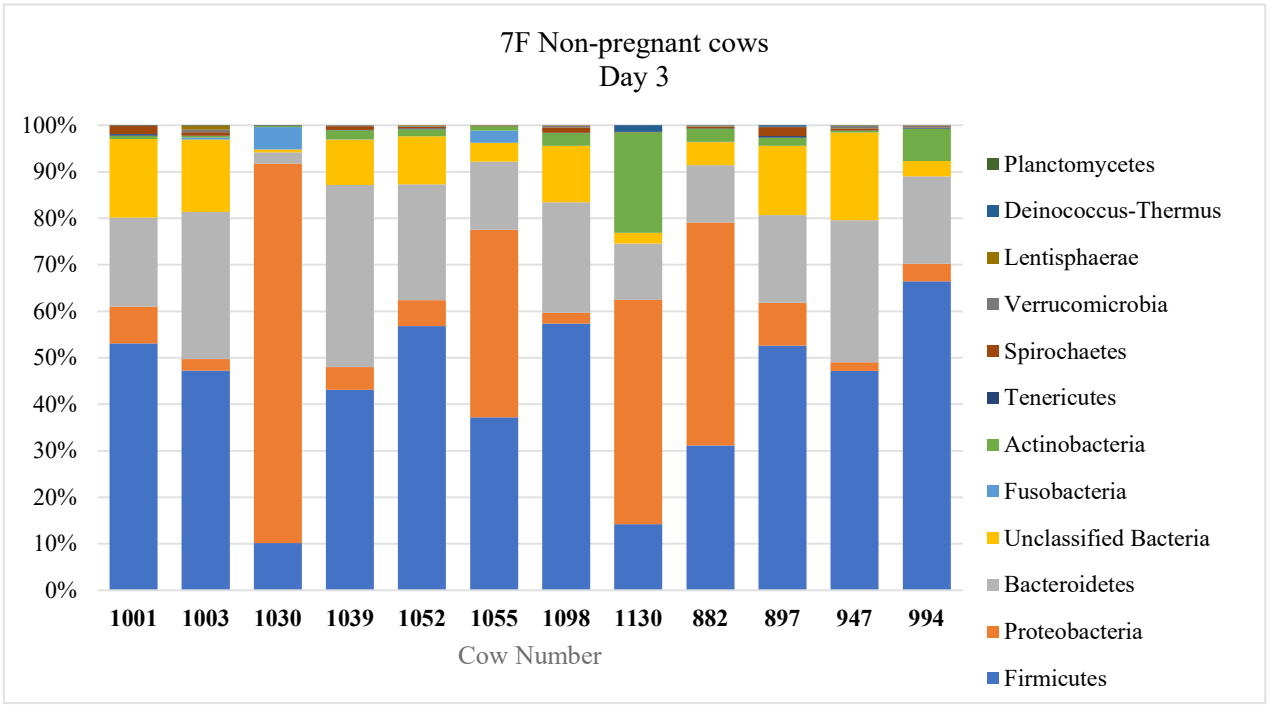


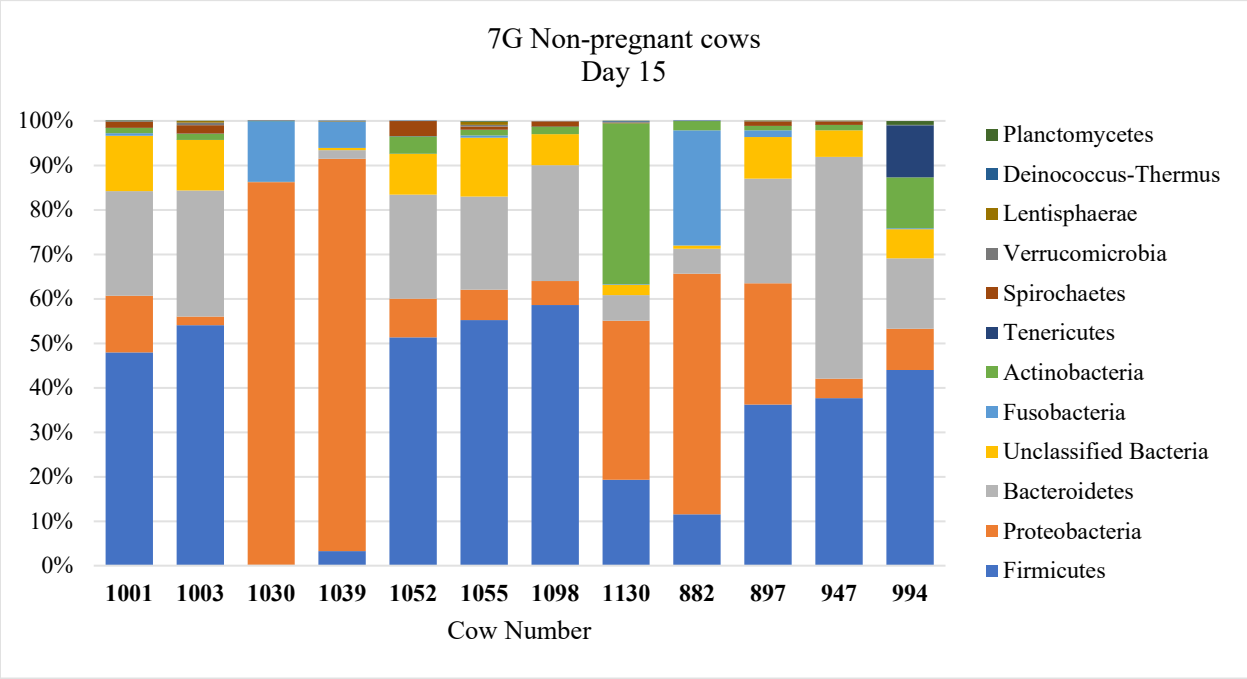


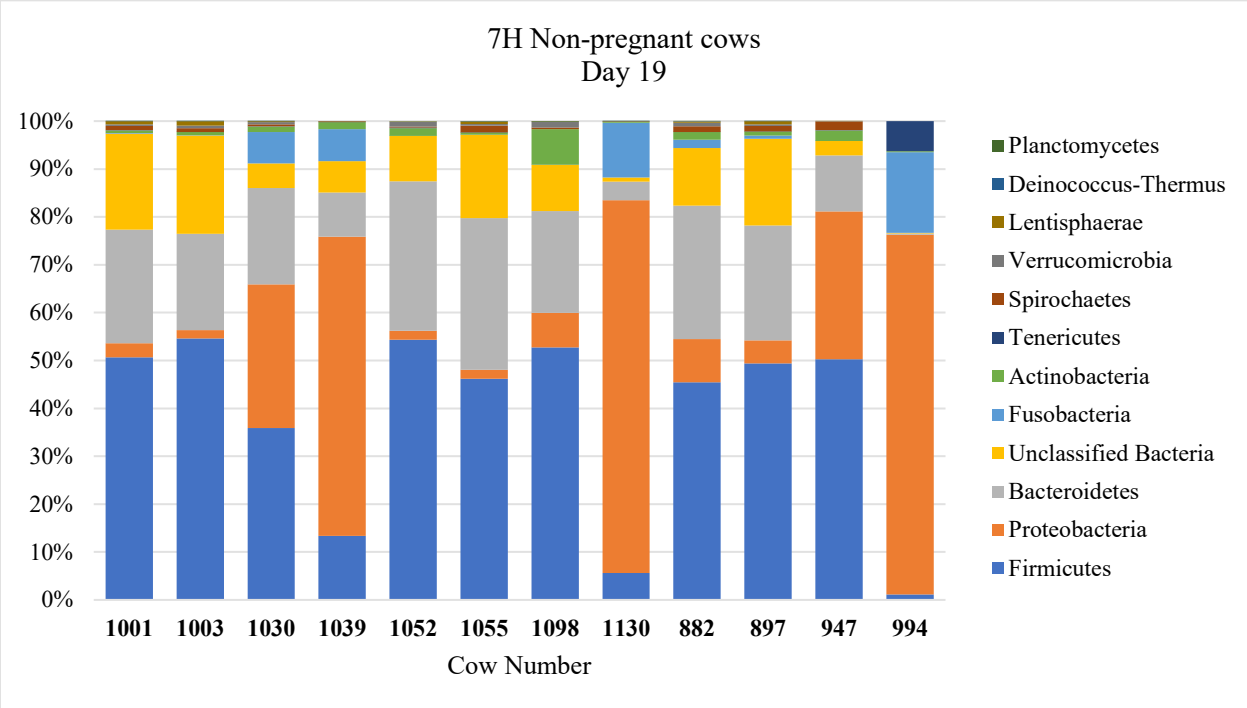

7C Pregnant cows
Day 15
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
1021
1117
738
856
872
903
941
944
955
Cow Number
Planctomycetes
Deinococcus-Thermus
Lentisphaerae
Verrucomicrobia
Spirochaetes
Tenericutes
Actinobacteria
Fusobacteria
Unclassified Bacteria
Bacteroidetes
Proteobacteria
Firmicutes


7D Pregnant Cows
Day 19
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
1021
1117
738
856
872
903
941
944
955
Cow Number
Planctomycetes
Deinococcus-Thermus
Lentisphaerae
Verrucomicrobia
Spirochaetes
Tenericutes
Actinobacteria
Fusobacteria
Unclassified Bacteria
Bacteroidetes
Proteobacteria
Firmicutes


7E Non-pregnant cows
Day 1
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
1001
1003
1030
1039
1052
1055
1098
1130
882
897
947
994
Cow Number
Planctomycetes
Deinococcus-Thermus
Lentisphaerae
Verrucomicrobia
Spirochaetes
Tenericutes
Actinobacteria
Fusobacteria
Unclassified Bacteria
Bacteroidetes
Proteobacteria
Firmicutes


7F Non-pregnant cows
Day 3
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
1001
1003
1030
1039
1052
1055
1098
1130
882
897
947
994
Cow Number
Planctomycetes
Deinococcus-Thermus
Lentisphaerae
Verrucomicrobia
Spirochaetes
Tenericutes
Actinobacteria
Fusobacteria
Unclassified Bacteria
Bacteroidetes
Proteobacteria
Firmicutes


7G Non-pregnant cows
Day 15
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
1001
1003
1030
1039
1052
1055
1098
1130
882
897
947
994
Cow Number
Planctomycetes
Deinococcus-Thermus
Lentisphaerae
Verrucomicrobia
Spirochaetes
Tenericutes
Actinobacteria
Fusobacteria
Unclassified Bacteria
Bacteroidetes
Proteobacteria
Firmicutes


7H Non-pregnant cows
Day 19
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
1001
1003
1030
1039
1052
1055
1098
1130
882
897
947
994
Cow Number
Planctomycetes
Deinococcus-Thermus
Lentisphaerae
Verrucomicrobia
Spirochaetes
Tenericutes
Actinobacteria
Fusobacteria
Unclassified Bacteria
Bacteroidetes
Proteobacteria
Firmicutes

3.4 Relative Abundance of Vaginal Microbiota - Genera

Sequencing classified 662 distinct genera with less than half, or 317, that were equal to or above 0.001%, and only 15 of those were equal to or above 1% relative abundance. The 15 genera in abundance greater than 1% are shown in Figure 8.

Although not statistically significant, *Histophilus* increased at Diestrus in pregnant cows, while unclassified *Clostridiales* were lower in pregnant cows throughout, with a marked decrease at Proestrus. Notable fluctuations in genera were seen in individual cows, as shown in Figure 10 and Figure 11. Pregnant cow #1021 showed high levels of *Histophilus*, while *Leptotrichiaceae* and *Pasteurellaceae* are more important in others, notably #1021M, 738, 903, 944 and 955. (See Figure 11.) Cows # 1030, 1039, 994 and 1130 in the non-pregnant group showed differences (non-statistically significant) from the rest. (See Figure 10.) The first three showed elevated levels of *Histophilus* and *Leptotrichiaceae* as compared to the rest, while #1130 showed very low abundance of all 16 genera at Metestrus and Diestrus.

During the estrus phase, LEfSe analysis identified 7 taxa significantly associated with cows that would become pregnant and 12 taxa more abundant in cows that failed to get pregnant.

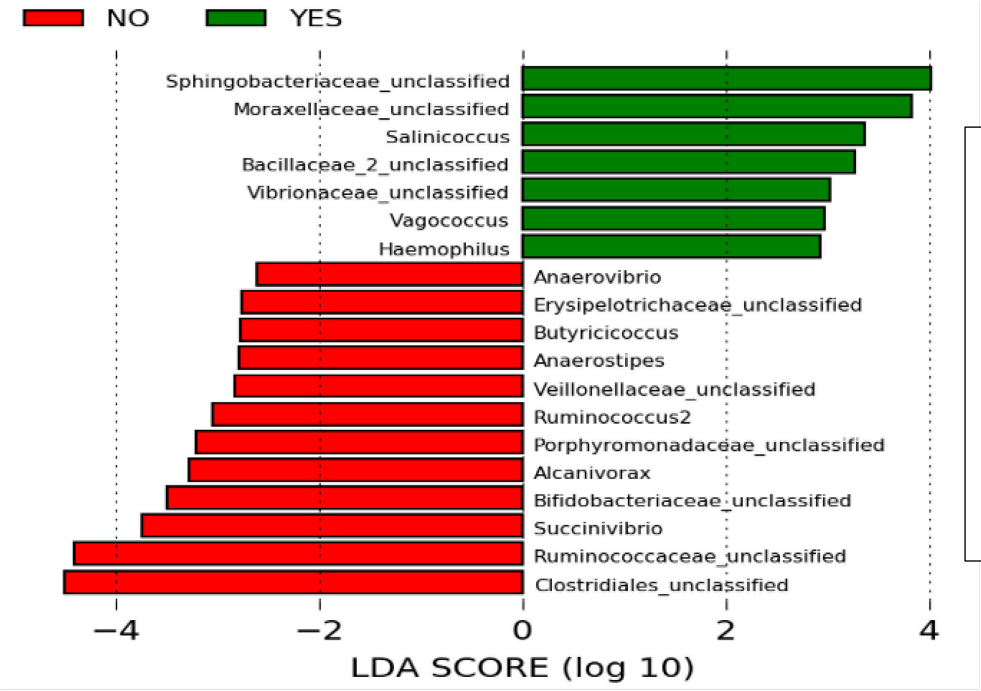


Figure 8-LEfSE graph of 7 taxa found in greater abundance in cows that would become pregnant, (in green) and 12 taxa found in greater abundance in non-pregnant cows (in red)

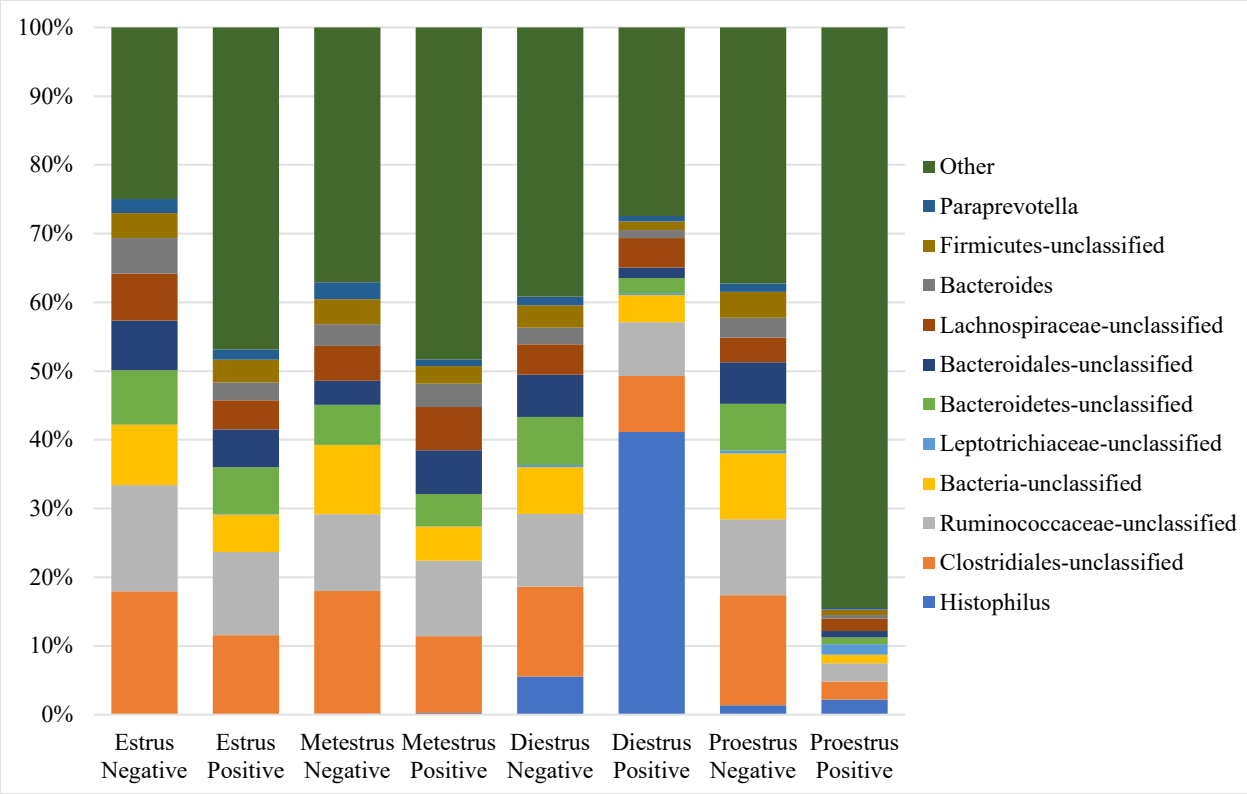


Figure 9 - Median of the most abundant genera of vaginal microbiota in pregnant and non-pregnant cows during the four phases of the estrous cycle. *Histophilus* increases significantly at Diestrus in pregnant cows, while unclassified *Clostridiales* are lower in pregnant cows throughout, with a marked decrease at Proestrus.

3.5 Individual Variances

Throughout the study, the presence of outliers made analysis challenging. Individual variances make mathematical and statistical calculations difficult. Therefore, a closer look at some of these variances seems important, as this would demonstrate why patterns and tendencies over the four phases cannot be quickly determined. Four cows stood out especially, as they seemed to show either consistently different microbiota from the rest or showed greater fluctuations in membership over the four phases. A more in-depth analysis of their genera may explain their particularities.

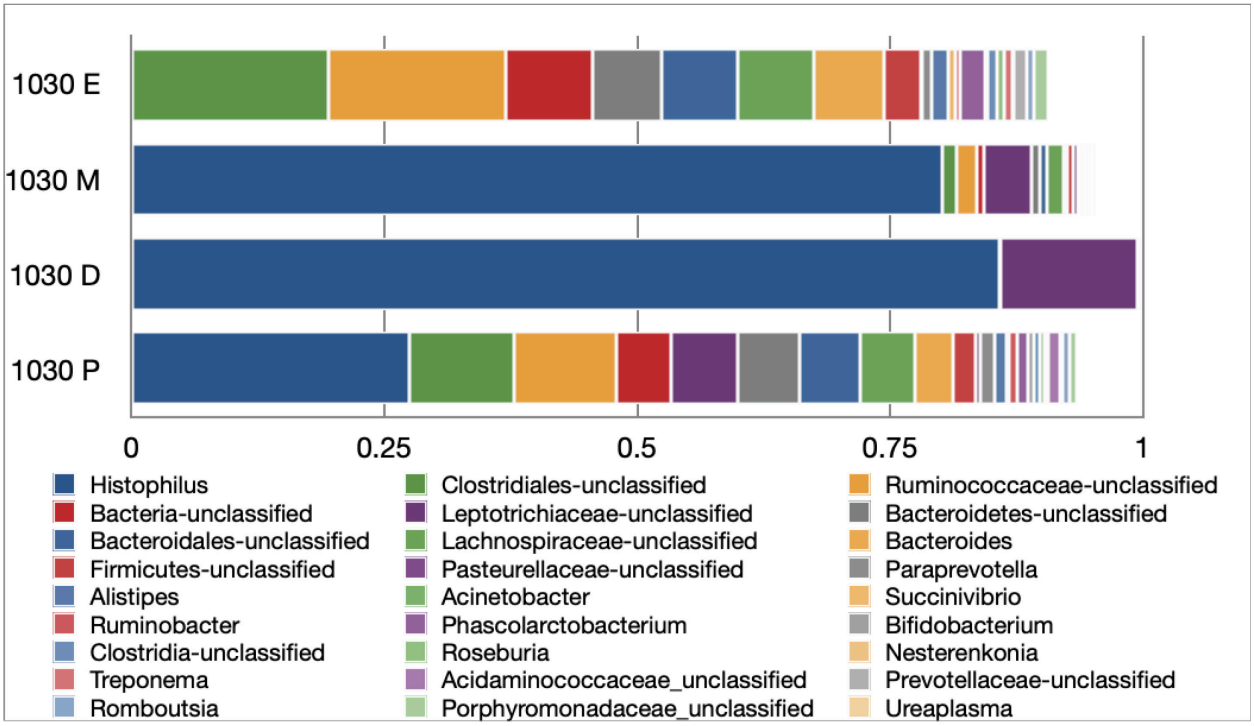
Two non-pregnant cows, #1030 and #1130, feature a vastly fluctuating membership over the four phases of the Estrous cycle. Based on the most prominent genera, there are inordinate changes at day 3 and day 15 of sampling in Cow #1130, and at day 19 in Cow #1030. Both have extreme spikes in *Histophilus* at these phases. #1030 shows very low diversity at the day 15, with a predominance of only 2 genera, *Histophilus* and *Leptotrichiaceae*, while #1130 shows low abundance of all 27 most prominent genera at both day 3 and day 15. While elevated levels of *Histophilus* at day 19 clearly overwhelm other genera.

Table 2 - Cow #1130 Phyla at Metestrus and Diestrus

1130	**Day 3**	**Day 15**
Firmicutes	14.18%	19.21%
Proteobacteria	47.98%	35.15%
Bacteroidetes	12.04%	5.85%
Bacteria-unclassified	2.29%	2.27%
Fusobacteria	0.00%	0.16%
Actinobacteria	21.85%	36.11%
Tenericutes	0.00%	0.00%
Spirochaetes	0.00%	0.15%
Verrucomicrobia	0.02%	0.17%
Lentisphaerae	0.00%	0.00%
Deinococcus-Thermus	1.46%	0.19%
Planctomycetes	0.00%	0.01%
Chloroflexi	0.08%	0.38%
Acidobacteria	0.0%	0.33%
Gemmatimonadetes	0.0%	0.01%

In cow #1130, relative abundance of most important genera drops significantly at both the Metestrus and Diestrus, from about 80 to 40%. Therefore, the next 55 genera were charted to look more deeply into these differences. The latter make up between 39% and 26% approximately. Indeed, though structure of microbiota phyla showed moderate variance with less Proteobacteria and Bacteroidetes and an increase in Actinobacteria and Firmicutes, these all fall well within the average, as seen in the table to the right. Nonetheless, these findings confirm that membership is extremely fluid throughout, as prominent genera are replaced by others during these two phases.

Figure 10A and B - Vaginal microbiota diversity fluctuations in two non-pregnant cows during the four phases of Estrous: cow #1030 and # 1130, with Histophilus levels, in blue, clearly dominate at days 3 and 15 in #1030, and 19 in #1130.



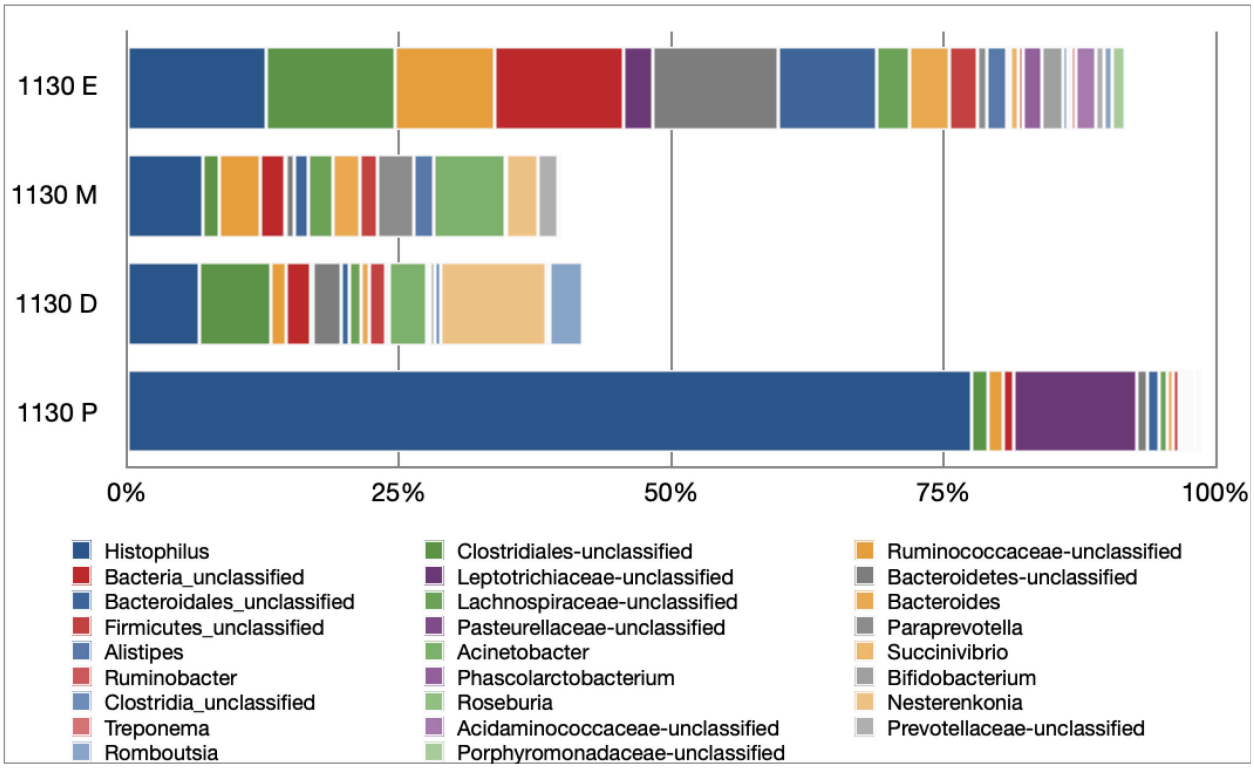
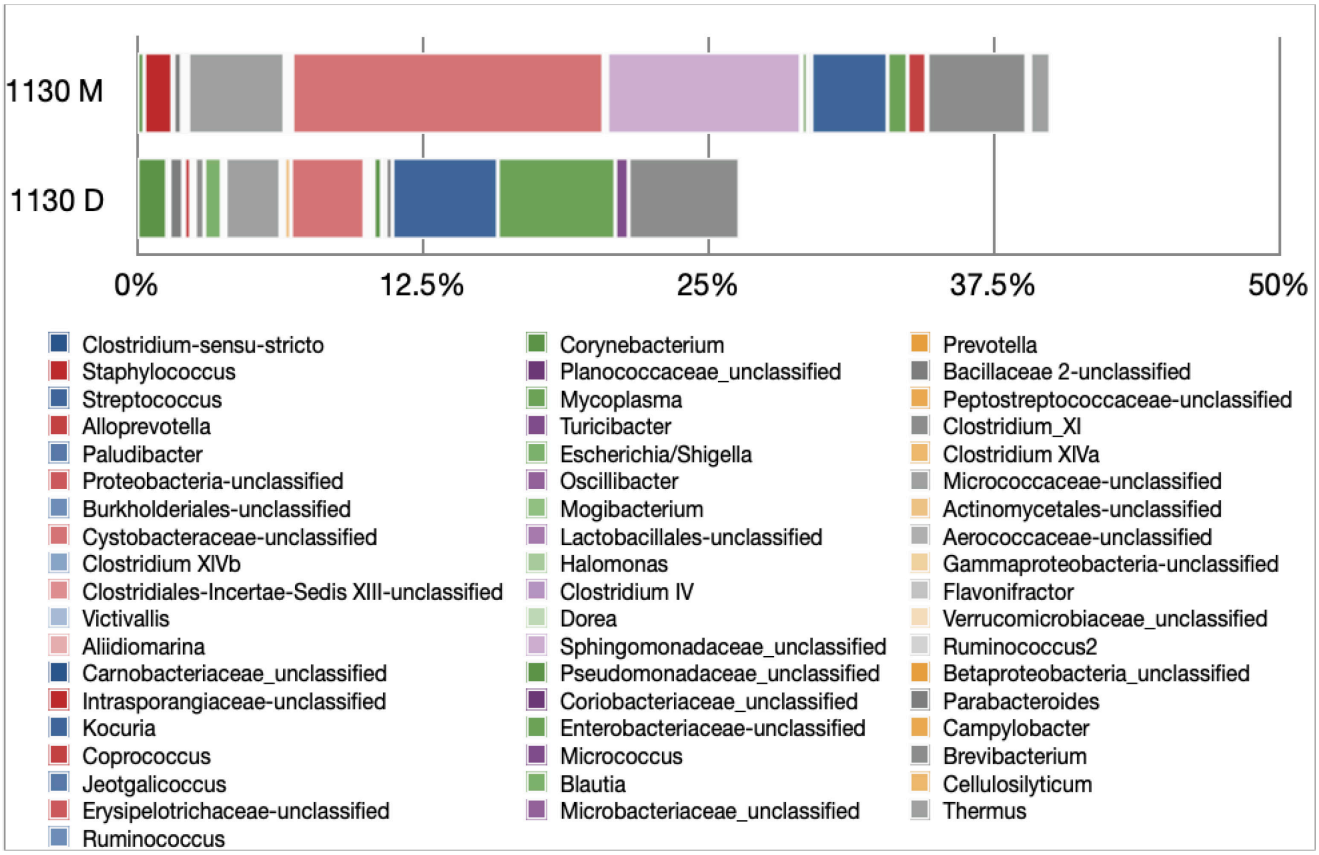


Figure 11 - Genera fluctuations in cow #1130 during the metestrus and diestrus phases: Abundance of other genera total over 38%, with a marked increase in *Clostridiales* and *Clostridium* at metestrus. Trace genera total over 26% of relative abundance at diestrus.



Two pregnant cows also strayed from the norm. Cows #955 and #1021 showed clear distinctions from other pregnant cows. Cow #955 showed very different microbiota genera at the first day of Estrous compared to others. She had an extreme abundance of unclassified *Leptotrichiaceae*, a Fusobacteria, at Day 1, followed by large amounts of *Phascolarctobacterium*, a Firmicutes, at Day 3. This last Genus is usually found within healthy gut microbiota (Wu et al., 2017). Like with other pregnant cows, her microbiota diversity seems to show much greater evenness with greater biodiversity on Days 15 and 19. Cow #1021 shows significant spikes in *Histophilus* on Day 15 and 19, along with a great abundance in unclassified *Leptotrichiaceae* and *Pasteurellaceae* at Day 3. She also shows less abundance of these genera at the start of Estrous. Like with Cow #1130 at metestrus and diestrus, this cow has a much richer diversity than others at Day 1, with many inferior genera playing an important role in the overall membership of her vaginal microbiota. Of note is the steady decline of potentially harmful bacteria, like *Ureaplasma* for instance, throughout the four sampling periods (see Figure 11).

Figure 12 A and B - Vaginal Microbiota Diversity Fluctuations in two pregnant cows: cow #955 and cow #1021, during the four sampling periods.

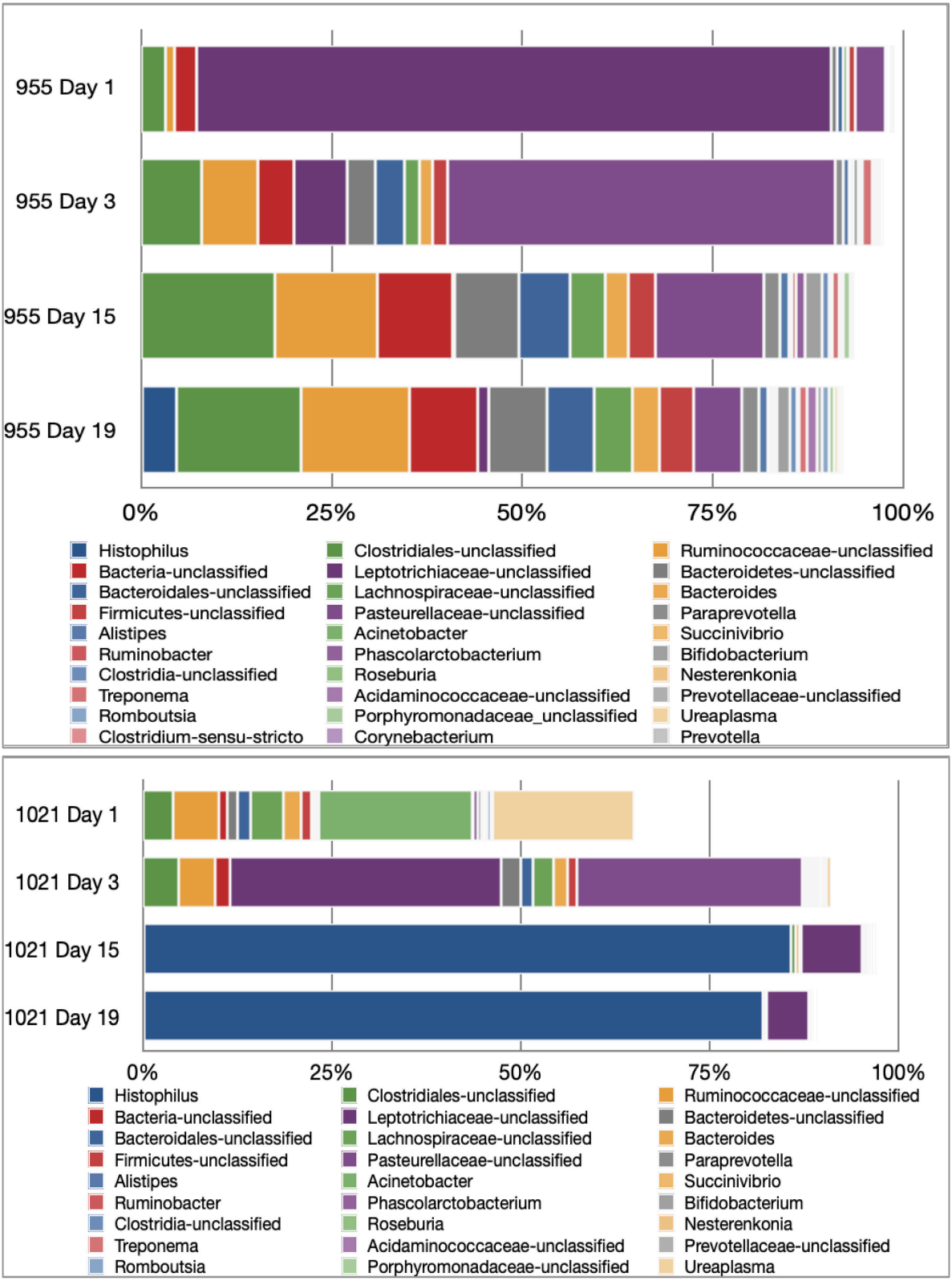
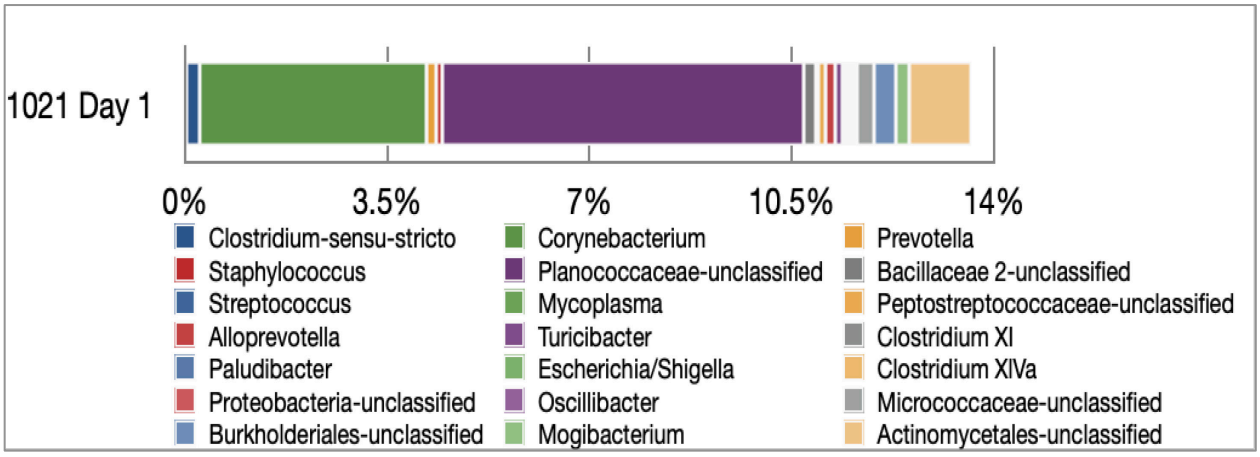


Figure 13 - Other Genera Present at Day 1 of Estrous in # 1021: High levels of *Corynebacterium* and unclassified *Planococcaceae.*



Section 4 - Discussion

4.1 Hormonal fluctuations

This analysis did not show statistically significant changes in microbiota, though variances in the microbiota of individual cows were extreme, to the point of making analysis difficult. Nonetheless, there were still some clear fluctuations overall during the four phases of the cycle. These could relate to probable hormonal changes in both groups of cows. Proteobacteria levels in pregnant cows did greatly fluctuate. This phylum seemed to increase in some animals between days 3 and 15, from a median value of 6% to 43%. A reversal occurs at day 19, where Proteobacteria levels drop to only 9.7% in pregnant cows, as compared to elevated rates of 47.8% in non-pregnant cows. This change may be related to higher progesterone levels present in pregnant cows around days 18-21, since the CL would be increasing in size to support the pregnancy (Scully et al., 2014). Similarly, Bacteroidetes medians were more elevated in non-pregnant cows throughout, whereas pregnant cows showed medians of 21.9% on Day 1, to drop to only 2.9% on day 19. It can be hypothesize that progesterone concentration differences played a role over this particular phylum as well.

Similar trends were also seen in genera. For instance, in some animals *Histophilus* increased at day 15 in pregnant cows while at day 19 *Clostridiales* decreased dramatically. It can be hypothesize that rising progesterone levels affected these genera as well. Further research is required to investigate these observations.

4.2 Comparison with other research

This study did obtain comparable results with other research. A study by Quadros et al., 2020, found similar levels of vaginal microbiota in dairy cows. Firmicutes, Proteobacteria and Actinobacteria showed comparable relative abundance to those found in these cows. It found an average of 38% Firmicutes as did this study, while Protobacteria at estrus in cows that would become pregnant was at 17%, also equivalent to the percentages found in the Quadros study.

Another study, by Deng et al., 2019, also had similar findings at the phylum level, where Firmicutes were the most dominant at 31.57%, followed by Proteobacteria (24.08%) and

Bacteroidetes (12.96%). Similarly, research done by Laguardia-Nascimenta et al., 2015, also obtained many equivalent results. The most prominent bacteria found were in the same order of importance as this study's findings: They noted a predominance in Firmicutes, followed by Proteobacteria, Bacteroidetes and unclassified phyla. Laguardia also found many bacterial genera of comparable importance as in this study. They saw a predominance of only ten OTUs through about half of their samples. Like our study, *Bacteroides*, *Clostridium* and *Ruminococcus* are especially significant. One notable difference was the strong presence of *Aeribacillus*, a Firmicutes Genus which was actually absent from our OTU data, unless it may have been present in the 3% of unclassified Firmicutes found. They were nonetheless unable to associate vaginal microbiota composition to pregnancy status in their sampling.

The results from this study differ from those from a study of vaginal microbiota in heifers by Quereda et al., 2020, since this stufy used multiparous Holstein cows. The most notable differences were with Tenericutes. Their findings showed a large abundance of Tenericutes (35.6%), but this phylum was in very low relative abundance in this study, with a mean of only 0.81% over all four phases. Other phyla included Firmicutes at 25.2% as compared to 37.95% overall in this study, and Bacteroidetes at 14.9%, compared to 18.53% here. Finally, though Proteobacteria remains in high abundance throughout the estrous cycle with our cows, the Quereda group found that Proteobacteria was in lower abundance. These differences may be explained by the physiological differences between heifers and multiparous cows.

Of greater interest were observations by Quereda et al., suggesting changes in lesser taxa may actually play an important role in the vaginal health of cows during the estrous cycle. This agrees with our study as well: differences in less abundant taxa between pregnant and non-pregnant cows were found. Many other studies also note an increase in rare or unclassified bacteria, however, in pregnant cows, relative to a decrease in more prominent bacterial genera. A study by Deng et al., 2019, found that vaginal microbiota was dominated by an unclassified Enterobacteriaceae (21.05%), followed by *Ureaplasma* (4.37%) and an unclassified Bacteroidaceae (2.49%). Another study by Ault et al., 2019 showed that there seems to be a decrease in species richness and phylogenetic diversity within the microbiota of the uterus which may lead to issues with fertility (Ault et al., 2019).

Many other animals showed very similar tendencies to the microbiota found in the present study. For instance, a study of microbiota during estrous in wild buffalo done by Mahalingam et al., 2019, had many comparable results. They found that Proteobacteria, Actinobacteria, Firmicutes, Bacteroidetes and Tenericutes were the most abundant phyla. These bacterial phyla also predominate dairy cow vaginal microbiota in this study. The clearest differences were with Actinobacteria and Tenericutes, which were not as abundant in our dairy cows. The different physiological and metabolic demands of Holstein dairy cows and wild buffalo, along with their completely different habitats, would readily explain these differences (Franzolin & Alves, 2010).

Horses also share a similar number of phyla as our cows with equally high diversity, as per a study done in 2020 on Arabian mares, by Barba et al. Researchers also noted a very low percentage of *Lactobacilli,* which is also comparable to low levels found in our study.

A study done on wild baboons by Miller et al., 2017, also found similar predominant vaginal microbiota. Another study done on rodents by Levy et al., 2020, also found similar values of Firmicutes and Proteobacteria during the different phases, comparable to those found in our non-pregnant cows. In Göttingen Minipigs, similar bacterial phyla were found during the estrus phase (Lorenzen et al., 2015). Finally, a study by Lynman et al., 2019 found the same most abundant phyla within both the uterus and the vaginas of bitches. Of course, these findings do not preclude differences in genera and species, which are to be expected, given the various physiologies and diets of these different animals.

These studies do show much greater vaginal microbiota diversity in ruminants as compared to primates and carnivores. Humans especially tend to have much less diversity than other animals. This greater diversity also seems to relate to higher pH levels in ruminants. The average vaginal pH for cows is around 7.5 (Beckwith-Cohen et al., 2012), while that of a human female remains between 4.2 and 5.0 (Ravel et al., 2011). Indeed, in a study by Swartz et al., 2014 sheep are the only other ruminant with a higher diversity than cows, while humans have the least.

Comparably, most of the cows that did become pregnant in our study also tended to have the richest microbiota diversity, with a tendency to have a greater prevalence of lesser genera. In fact, one cow, #738, was the eldest of our subjects at 108 months, and had already given birth to 7 healthy calves. As with many of the other pregnant cows in this study, roughly 1/4 of genera at different

sampling stages was made up of a wide variety of lesser genera. As previously shown in results, other cows that became pregnant, like #955 and #1021, also showed important levels of lesser genera. This is further indication that vaginal biodiversity may be an important factor to ensuring high fertility rates.

4.2 Limitations of this study

Some limitations in this study were that neither pH nor hormone levels were tested during sampling. Ideally, additional swabs could be taken to map microbiota changes over time with more details. Following cows over two cycles would also help confirm these findings. Finally, using a larger sample group would help balance individual variances to minimize their impact on the final results. Eventually, studies at the species level will help eliminate any lingering questions as to the impact of particular genera or possible pathogenic bacteria on other microbiota populations and finally confirm the link of certain bacteria on pregnancy rates.

Section 5 - Conclusion

In conclusion, this study investigated the vaginal microbiota during the different phases of the estrous cycle following AI in multiparous Holstein dairy cows. Firmicutes, Proteobacteria and Bacteroidetes proved to be the most abundant phyla found in both pregnant and non-pregnant dairy cows. Although there were no significant differences between the different phases of the estrous cycle, inter-individual variations were vast. Over time variation of membership (Day 15 and 19) was observed in pregnant cows.

Significant differences between pregnant and non-pregnant dairy cows of certain low abundance taxa at the moment of IA deserve further investigation to determine what changes would affect reproductive health and fertility.

Section 6 - Acknowledgments

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Chapter 4 - General Discussion

4.1 Individual variances in pregnant and non-pregnant cows

Fluctuations of microbiota over the 4 phases make generalisation difficult. Individual variances between cows are significant, also making analysis challenging. In pregnant cows, some stand out with clear differences in their microbiota makeup. Cows #1021, 1130 and 944 especially, show interesting dissimilarities with the other cows' microbiota structure. For instance, at Estrus before AI, cow #1021 shows higher than average levels of Tenericutes, while cow #955 shows extreme levels of Fusobacteria. On day 3 of sampling, there are many fluctuations as compared to the first sample, with cows #1021 and # 856 showing an increase in Fusobacteria, while cow #955 shows less, with a substantial increase in Proteobacteria. At day 15, cow #1021 again shows many fluctuations as compared to its last sampling in all phyla. Proteobacteria in this cow has overtaken other phyla, and account for nearly 90% of microbiota. Fusobacteria levels have increased in 2 other cows and cannot be seen in cow #944, a significant change to her high levels at Day 1. Finally, at day 19, Proteobacteria dominate in cows #1021, 1117 and 738, while Fusobacteria have taken over in cows #903 and 944. High Firmicutes levels are seen in cows #856, 872, 941 and 955.

In cows that failed to become pregnant, cows #1030, 1130 and 994 show the greatest variance in microbiota as compared to the rest. At Estrus, cow #1130 shows the lowest abundance of

Firmicutes, while cow #947 shows the highest levels of Bacteroidetes, with only 4 cows showing levels of Actinobacteria above 2%. At day 3, cow #1030 shows marked increase in Proteobacteria, while #1130 and #994 show an increase in Actinobacteria. At day 15, three cows, #1030, 1039 and 882, show high levels of Proteobacteria while #1130 shows an increase in Actinobacteria along with #994, though to a lesser degree. At day 19, high levels of Firmicutes are seen in 8 of 12 cows, with high levels of Proteobacteria in cows #1039, 1130 and 994. #994 also shows a notable increase in Fusobacteria from day 15, when only trace amounts appeared.

4.2 Higher diversity of vaginal microbiota in cows

Analysis of samples over the four phases of estrous have shown an immensely complex and diverse microbiota in dairy cows. Other evidence supports these findings. A paper by Swartz et al., 2014, actually compared vaginal diversity in many species. It established that ruminants showed much higher diversity than other species, with cows figuring second only to sheep. Human microbiota was at the opposite end of the spectrum, as it shows a very restricted microbiota diversity. This greater diversity also seems to relate to higher pH levels in ruminants. Average bovine vaginal pH levels remain consistently around 7.5 (Beckwith-Cohen et al., 2012), while that of a human female stays between 4.2 and 5.0 (Ravel et al., 2011).

Similar levels of richness and complexity of the vaginal microbiota in buffalo (Franzolin & Alves, 2010), baboons (Miller et al., 2017), minipigs (Lynman et al., 2019), and dogs (Lorenzen et al., 2015, also confirm that maintaining microbiota diversity, through the creation of a neutral (non-acidic) environment is essential to the overall health of the reproductive tract.

Research done by Rodrigues et al., 2015, also states that the presence of *Lactobacilli* and other acidifying bacteria may lead to acidic environments in the vaginal canal of cattle that can cause dysbiosis. Our cows all showed low abundance of *Lactobacilli* and other acidifying bacteria, since they were scrupulously chosen for their health.

The only exception to these findings in our study were elevated rates of *Histophilus*, especially in some pregnant cows. This contradicts the Rodrigues study, since *Histophilus* strains were linked to disease through his research. Two non-pregnant cows however, (#1030 and #1130), did show a rise in *Histophilus* on days 3, 15 and 19. The abundance of *Histophilus* became so important, it

actually dominated other bacteria during these time points. Yet in pregnant cows, *Histophilus* levels did tend to drop by day 19. More research into this at the species level may confirm its link to infertility.

Given all these comparisons, we may look at the vaginal microbiome of human females to compare them to that of cows. This may be an important step to developing a sustainable evidence-based probiotic regimen aimed at promoting an optimal, healthy vaginal microbiome to improve pregnancy rates in cows. For instance, studies like the one done by O'Hanlon et al., 2013, show that a woman's microbiota is dominated by *Lactobacilli.* In humans, high abundance of *Lactobacilli* is crucial to avoid BV (Lev-Sagie et al., 2019). However, this strain was rarely seen in the cows' vaginal microbiota. When present in any abundance, it is often related to disease, such as metritis (Wang et al., 2013).

These bacterial imbalances can again be related to hormonal fluctuations and pH levels. Beckwith-Cohen et al., 2012, established the benchmark of 7.5 pH as observed in his research in healthy peri and postpartum heifers and cows. Therefore, contrary to the acidic pH levels in humans (O'Hanlon et al., 2013) a near-neutral vaginal pH is optimal to maintaining a healthy microbiome in cattle (Swartz et al., 2014).

There is mounting evidence that lower pH may lead to dysbiosis in cows, as many bacteria cannot survive in a more acidic environment. Such observations give still more importance to research such as this one, since we must be able to map the vaginal microbiota in cattle in order to establish a clear link between pH, hormones, vaginal microbiota populations and fluctuations and fertility rates. Only then may we be able to consider future evidence-based microbiota manipulation to establish an optimal environment for pregnancy.

4.3 Hormonal studies and further links to pH

A great deal oof research has confirmed that hormone changes also affect pH. Estrogen especially has an acidifying effect on pH levels, as seen in studies done on women (Gorodeski et al., 2005). Other studies also note a decrease in pH at estrus, probably related to a release of estrogen. (Schilling et al., 1968) Therefore, high levels of estrogen and a lower pH before AI may also relate

to the variations in phyla and genera found in our cows that became pregnant. Unfortunately, no pH samples were taken during this study to confirm this

Previous studies have also shown that vaginal microbiota is highly influenced by changes in hormonal profiles. Deng et al., 2019 noticed significant increases of vaginal microbiota diversity through estrous and early pregnancy and a decrease of diversity in the third trimester, while Ault et al., 2019 saw fluctuations at the onset of estrous.

A recent study by Messman et al., 2020, also showed a strong correlation between estradiol levels at estrus before AI, and a greater abundance of certain microbiota species, while lower levels of estradiol would promote other bacterial species to flourish. This research also pointed to the possible link of these species to pregnancy rates. This is an important step in developing a clearer understanding of the vital relationship between hormones, microbiota and pregnancy.

In the future, it would be beneficial to consider that the vaginal microbiota present in dairy cows may be a clear indicator of the reproductive state of the animal. Fluctuations in microbiota may be used as a diagnostic tool for veterinarians and farmers, to better determine how to proceed with cows with reproductive failure. A study done by Šuluburic et al., 2017, looked into how a lack in progesterone (P4) might affect early embryonic development in dairy cows. Since the Šuluburic study showed a clear impact of hCG and P4 treatments following AI, it would be beneficial to trace the impact such hormonal treatments and changes may have on vaginal microbiota, given that the most significant changes in microbiota populations in this study did occur at Day 15, while there were noticeable increases in Proteobacteria at Metestrus, only 2 days after AI. In addition, Firmicutes declined in non-pregnant cows and increased in those that were fertilized at that time. Also, though these variations were not statistically significant, non-pregnant cows saw a contrasting increase in unclassified Bacteria, Fusobacteria, Acidobacteria and Chloroflexi, while these all declined in cows that would test positive for pregnancy.

Finally, another study by Quadros et al., 2020 looking into the effects an intra-vaginal progesterone device would have on the vaginal microbiota in Holstein dairy cows, found that multiparous cows had greater bacterial diversity than primiparous cows. This holds true with results in this study. Though all cows in this study were multiparous, the nine that did become pregnant did show greater diversity and abundance of certain bacteria compared to those that did not, with

substantially less of more prominent bacterial genera and an increase in less abundant phyla and genera. One of the pregnant cows, #738, was actually the eldest at 108 months old, and had already given birth to 7 calves. This cow did show some differences in microbiota as compared to the rest: she had about 13% less of the three most abundant phyla at the onset of Estrous as compared to average levels in pregnant cows, with more abundance in lesser phyla. She also had a clear absence of those bacteria that are considered potentially pathogenic, with 0% Chloroflexi, Chlamydiae, Fibrobacteres, Planctomycetes and Deinococcus-Thermus at the onset of estrous. All these studies suggest a correlation between vaginal microbiota abundance and diversity, hormones and pregnancy rate.

4.4 Limits of this Study

If this study were to be repeated, a few changes would be beneficial. Vaginal swabs should be taken concurrently with pH levels and hormonal plasma samples to confirm the links between these factors and vaginal microbiota fluctuations. Ideally, additional swabs could be taken to map microbiota changes over time. Following cows over two cycles would also help confirm findings. Finally, using a larger sample group would help balance individual variances to minimize their impact on the final results. Eventually, study at the species level will help eliminate any lingering questions as to the impact of particular genera, or of certain potentially pathogenic bacteria.

Section 5 - Future Perspectives

5.1 Microbiota Manipulation

One of the goals of this study was to gather descriptive information of vaginal microbiota populations and their fluctuations during the estrous cycle to one day be able to manipulate the vaginal microbiota of dairy cows to improve fertility. A clear difference between the microbiota of pregnant and non-pregnant cows during the cycle would give definite indications of the impact of microbiota on reproduction rates. This data would also give us pathways for further exploration to attempt to correct any microbiota changes that could be at the source of reproductive failure. At

the very least, this information can also serve to enhance a healthy vaginal microbiome based on those found in healthy multiparous cows.

Our research did find some promising results. Levels of Proteobacteria in pregnant cows seemed to fluctuate much more than those in non-pregnant cows. While Tenericutes, Actinobacteria and Fusobacteria showed a much wider spread in pregnant cows than in non-pregnant cows, between insignificant minimum and extreme maximum values. There was a statistically significant change (P=0.043) between Estrous and Day 15 of sampling in pregnant cows, only a few days after AI. This may show that the vaginal microbiota was influenced by hormonal changes associated with fertilisation. Anecdotal evidence does point to greater vaginal microbiota diversity in pregnant cows than those who did not become pregnant, since most had a greater number of less prevalent genera. Though this observation was not determined as statistically significant, it still brings forward the notion that greater diversity is essential to a healthy reproductive tract in cows.

5.2 The Use of Probiotics in Cattle

5.2.1 Local vaginal probiotic uses

The only vaginal probiotic that has been tested so far on cattle includes using various *Lactobacilii spp.* This strain was tested to determine its effectiveness in preventing diseases like metritis and other purulent vaginal conditions. One study by Ametaj et al., 2014 applied 3 strains of Lactic acid bacteria (LAB), intravaginally into transition dairy cows around calving. The control groups in the study were treated with 1mL of sterile skim milk while the treatment groups were treated with 1mL of a mixture of 3 *Lactobacilli*. The results showed that the use of intravaginal probiotics could lower purulent vaginal discharges (Ametaj et al., 2014).

Another study done by the same lab group, Ametaj, 2005, showed that applying an intravaginal probiotic of LAB prepartum and then another dose after calving decreased the incidence of the cows developing mastitis and other uterine infections. It was also shown that intravaginal LAB expedited uterine involution 14 days postpartum. The study goes on to claim that there was an increase in milk yield and feed efficiency in the treated groups.

Although these papers seem promising, it is unclear how the cows faired long-term. Many other recent studies have mentioned that the use of Lactic acid bacteria in cattle can be devastating to the vaginal microbiota if the native bacteria do not recover over a period of time to re-establish the natural order and pH levels in the vaginal canal (Khafipour et al., 2016), (Quereda et al., 2020), (Rodrigues et al., 2015) & (Wang et al., 2013). It has also been shown that on a long-term basis, even lactic acid bacteria found in feed can not only affect the pH and microbiota in the rumen, but inversely effect vaginal microbiota as well.

5.2.2 Long term probiotic uses in cattle and their effects on vaginal microbiota

Cattle feed is already enhanced by the addition of prebiotics in the form of calorie-rich grains and partially fermented fodder. Some yeasts are also prevalent in feed as probiotic supplements, since they are a readily available source of fermentation. Their benefits have already been demonstrated through attentive research. These supplements do encourage weight-gain in calves and increase milk production in cows (Adjej-Fremah et al., 2018). Nonetheless, little is known of the long-term effects of these enhancements on the overall health of the animal and its microbiota. Indeed, a growing body of research indicates that these probiotics and prebiotics may have adverse effects on the development of a healthy gut microbiota in calves and are also detrimental when given to healthy cattle (Uyeno et al., 2010).

Caution must be taken when changing feed or adding a high grain concentrate diet to cows because this will cause an imbalance in the microbiota in the rumen and cause a release of neuroactive chemicals, which can change the hormonal profile. This change will cause disruptions in the gut-brain axis affecting other populations of bacteria around the body. This in turn will also change the pH in other places of the body, including the reproductive tract, which can lead to reproductive diseases (Khafipour et al., 2016).

Metabolic disease, such as Subacute ruminal acidosis (SARA), is another cause for concern when feeding a high grain concentrate diet to cattle. This metabolic disease will depress the ruminal pH below 5.6, leading to very acidic conditions that will harm the rumen microflora. SARA will effect feed intake, milk production, rumen microbiota, rumen digestion, and can lead to lameness, rumenitis and liver abscesses. With the gut-brain axis in mind, this will inevitably also affect the vaginal pH and microbiota as the disease progresses (Zhao et al., 2018).

Research studying the possible links between maintaining a healthy vaginal microbiota through probiotic supplementation to improve reproductive health in dairy cows is ongoing. A recent paper by Quereda et al., 2020, concluded that "the discrimination of beneficial bacterial groups can also lead to the utilization of probiotic-based treatments. Moreover, if a relationship is to be discovered between the vaginal microbial composition of healthy animals and fertility rates in cows, biomarkers for reproduction can also be revealed." (Quereda et al., 2020)

Probiotic use of *Lactobacilli* and other acidifying bacteria have been of great interest since they do have a proven inhibitive effect on pathogens, like *E. coli* (Otero et al., 1999). They also dominate the vaginal microbiota of human females and play an essential role in maintaining homeostasis by lowering pH (Ravel et al., 2011). However, ruminants have a much higher overall microbiota diversity than humans (Swartz et al., 2014). Bovine vaginal pH levels are also much higher than in humans: the average vaginal pH for cows is around 7.5 (Beckwith-Cohen et al., 2012), while that of a human female remains between 4.2 and 5.0 (Ravel et al., 2011). These two species are also physiologically distinct with different metabolic needs and functions. Any supplementation regimen should therefore be based on the specific physiology of cows and that of their microbiota.

The Otero group has been studying the effects of acidifying bacteria, especially *Lactobacilli*, since 1999, and are still attempting to create a reliable probiotic with these bacterial strains. (Otero et al., 1999 and 2006) The addition of *Lactobacilli* in cattle feed is now a common practice (Uyeno et al., 2015). Patents are now registered with the Canadian government for intra-uterine probiotic devices that contain *Lactobacillus* and other acidifying bacteria to be used prophylactically to prevent vaginal tract infections in cows (Ametaj et al., 2014). However, the findings from the study by Wang et al., 2013 associated high levels of *Lactobacillus* to disease in cows.

Similarly, this present study also found very low levels of *Lactobacilli* in both groups of cows, since they were chosen precisely because of their proven reproductive health and good physical condition. In addition, the study by Quereda et al., 2020, also mentioned that though *Lactobacillus* spp. is abundant in the human vaginal microbiota, they are scarce in cattle vaginal microbiota. Therefore, their role in maintaining eubiosis by inhibiting the proliferation of undesirable microorganisms in humans may be taken by another, yet unknown bacteria taxa in the cow's

vaginal microbiome. They also confirm that "more research is needed to elucidate this possible role of other beneficial vaginal bacteria in ruminants." (Quereda et al., 2020)

These are sharp indicators of the need for further research in probiotic supplementation. Though acidifying bacteria have a clear effect on pathogens, they also disrupt the natural balance and pH levels of the cow's microbiome. Therefore, any further attempts to develop a reliable microbiota manipulation strategy to improve overall reproductive health of cows must keep the health of their whole vaginal microbiota at the forefront.

Section 6 - Conclusion

In conclusion, the vaginal microbiota in cows fluctuates greatly, and though some clear generalizations can be made, each cow's microbiome is unique. In fact, some cows showed an extremely different vaginal microbiota from the norm. It is a rich and diverse microbiome that is nonetheless influenced by environmental, physiological and dietary changes. Three distinct phyla: Firmicutes, Proteobacteria and Bacteroidetes, were shown to dominate this environment, though there were strong fluctuations of these and other phyla during the four phases of the estrous cycle, which we theorize may be linked to hormonal and pH changes. Therefore, our first hypothesis, that bovine vaginal microbiota changes during the four phases of estrous, is confirmed.

Our second hypothesis, that bovine vaginal microbiota composition would differ in pregnant and non-pregnant cows, was also confirmed by the differences in some taxa before artificial insemination, along with the presence of certain genera in pregnant cows that were absent in non-pregnant cows, even though these were only in trace amounts. We also observed the tendency oof greater biodiversity in the vaginal microbiota of pregnant cows throughout. Comparing phyla fluctuations over the four phases of the cycle in pregnant and non-pregnant cows also showed some statistically significant differences in Firmicutes, Bacteroidetes, Unclassified Bacteria and Fusobacteria. More research is required to further investigate these findings and to pursue further analysis at the genera and species levels. Nonetheless, this study adds to the growing body of research demonstrating the vast richness and fluidity of vaginal microbiota while confirming the importance of maintaining this diversity to ensure reproductive health in cattle.

Section 7 - Bibliography

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Section 8 - Appendix

Appendix 1 - Description of subjects used in this study

Cow number	Intensity of heat	Age (months)	Total precedent births	Milk production (L)	Pregnancy status
882	100	78	4	50.5	NO
897	100	72	4	49.0	NO
947	84	71	4	46.6	NO
994	84	64	4	51.5	NO
1001	52	50	3	45.4	NO
1003	68	61	3	33.9	NO
1030	80	54	3	38.5	NO
1039	92	53	2	37.9	NO
1052	88	57	3	53.1	NO
1055	100	51	3	44.6	NO
1098	84	46	2	34.5	NO
1130	100	37	2	38.9	NO
738	76	108	7	49.2	YES
856	72	86	4	25.4	YES
872	84	81	2	18.9	YES
903	96	75	4	40.5	YES
941	92	73	4	45.1	YES
944	75	68	3	36.2	YES
955	100	62	3	31.8	YES
1021	84	55	3	64.8	YES
1117	76	37	2	50.7	YES

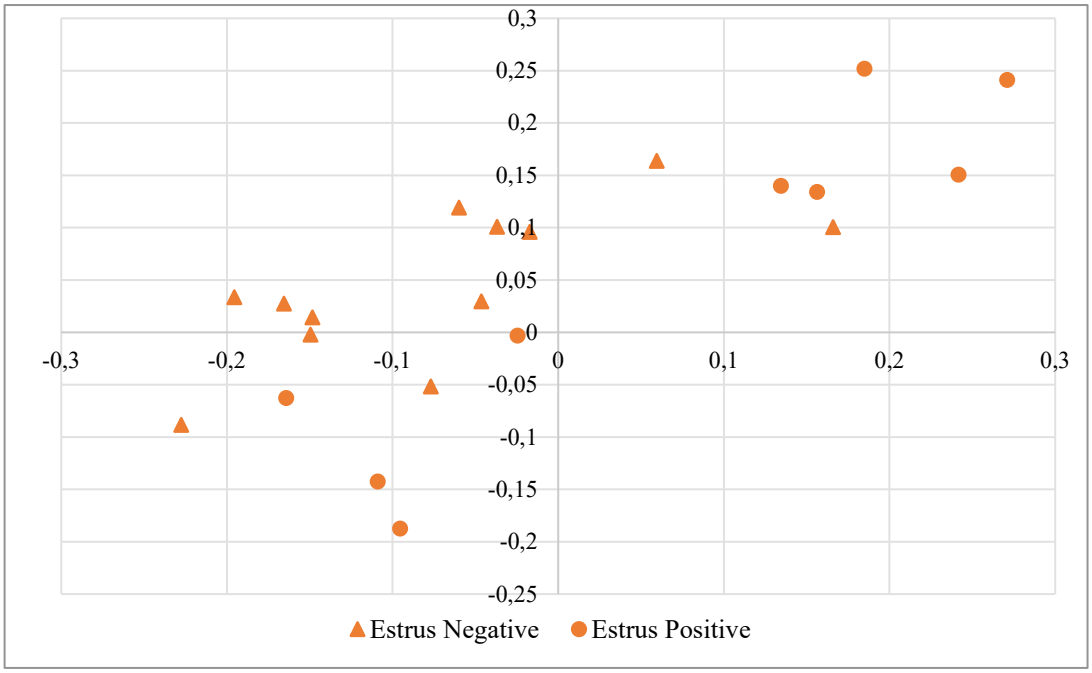
Appendix 2 - P values of AMOVA and ANOVA Statistical tests

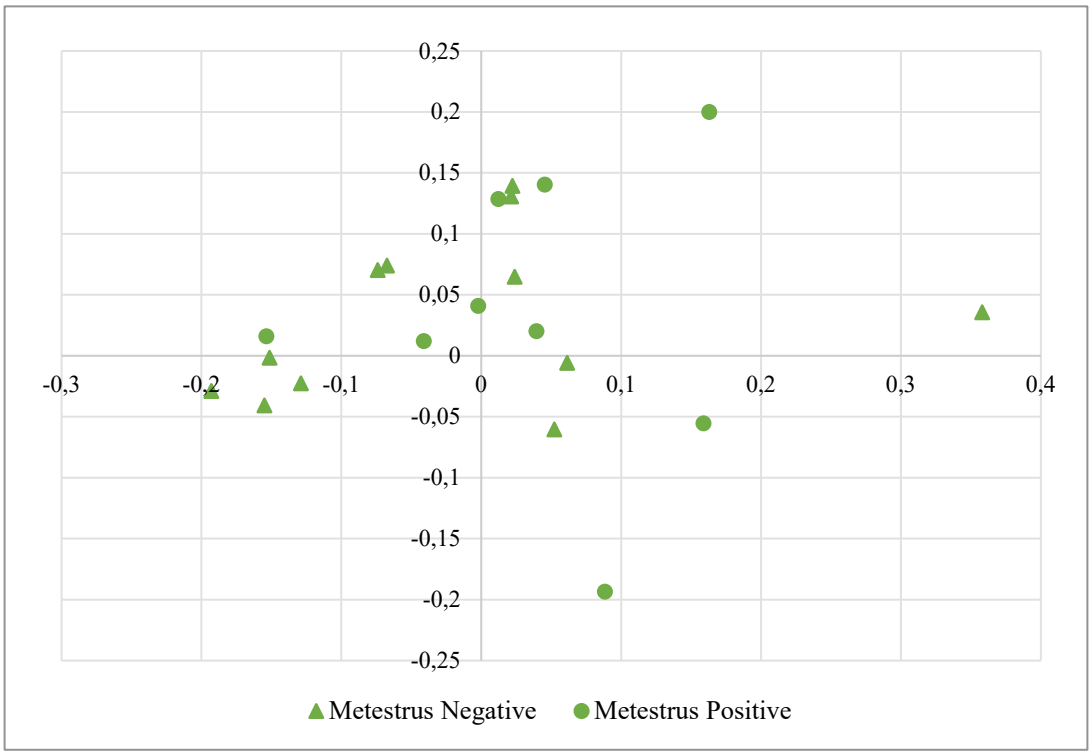
Comparison	Membership	Structure
Non pregnant cows		
Estrus-Metestrus	0.391	P>0.05
Estrus-Diestrus	0.108	P>0.05
Estrus-Proestrus	**0.028**	P>0.05
Metestrus-Diestrus	0.633	P>0.05
Metestrus-Proestrus	0.093	P>0.05
Diestrus-Proestrus	0.314	P>0.05
Pregnant cows		
Estrus-day 3	P>0.05	0.688
Estrus- day 15	P>0.05	**0.043**
Estrus-day 19	P>0.05	0.407
Day 3 - day 15	P>0.05	0.073
Day 3 - day 19	P>0.05	0.658
Day 3 - day 19	P>0.05	0.412

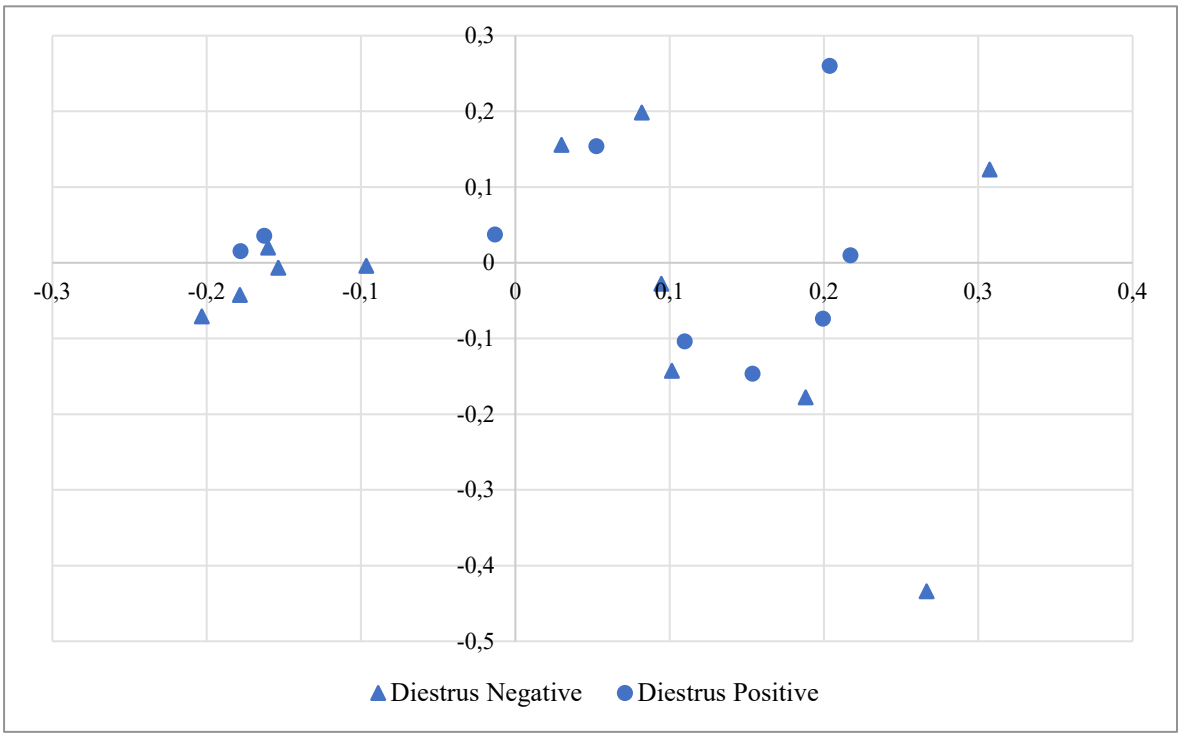
Appendix 3 - Averages and standard deviations of alpha diversity indices with P-values of the vaginal microbiota at 4 sampling phases of the estrous cycle in dairy cows that were positive and negative for pregnancy after AI.

	Number of Genera	Chao	Simpson	Shannon
Estrus				
Day 1 Positive (Before AI)	122 (45)	143 (49)	11 (5)	3 (1)
Day 1 Negative	102 (18)	127 (24)	10 (2)	3 (0)
P-values	0.90523	0.164271	0.421896	0.447959
Metestrus				
Day 3 Positive	105 (25)	125 (31)	9 (4)	3 (0)
Day 3 Negative	102 (16)	120 (18)	10 (5)	3 (1)
P-values	0.372028	0.326122	0.424282	0.499669
Diestrus				
Day 15 Positive	103 (32)	129 (39)	7 (5)	2 (1)
Diestrus Negative	88 (37)	109 (46)	10 (6)	3 (1)
P-values	0.163769	0.20862	0.139929	0.298902
Day 19 Positive	82 (20)	103 (23)	6 (5)	2 (1)
Day 19 Negative	88 (20)	108 (22)	8 (4)	2 (1)
P-values	0.244806	0.317462	0.16092	0.089013

Appendix 4 A to D - PCoA graphs of Jaccard Index of Beta diversity of membership of vaginal microbiota in cows that succeeded and failed to become pregnant showing no evident clustering during the four phases of the Estrous cycle






0,3
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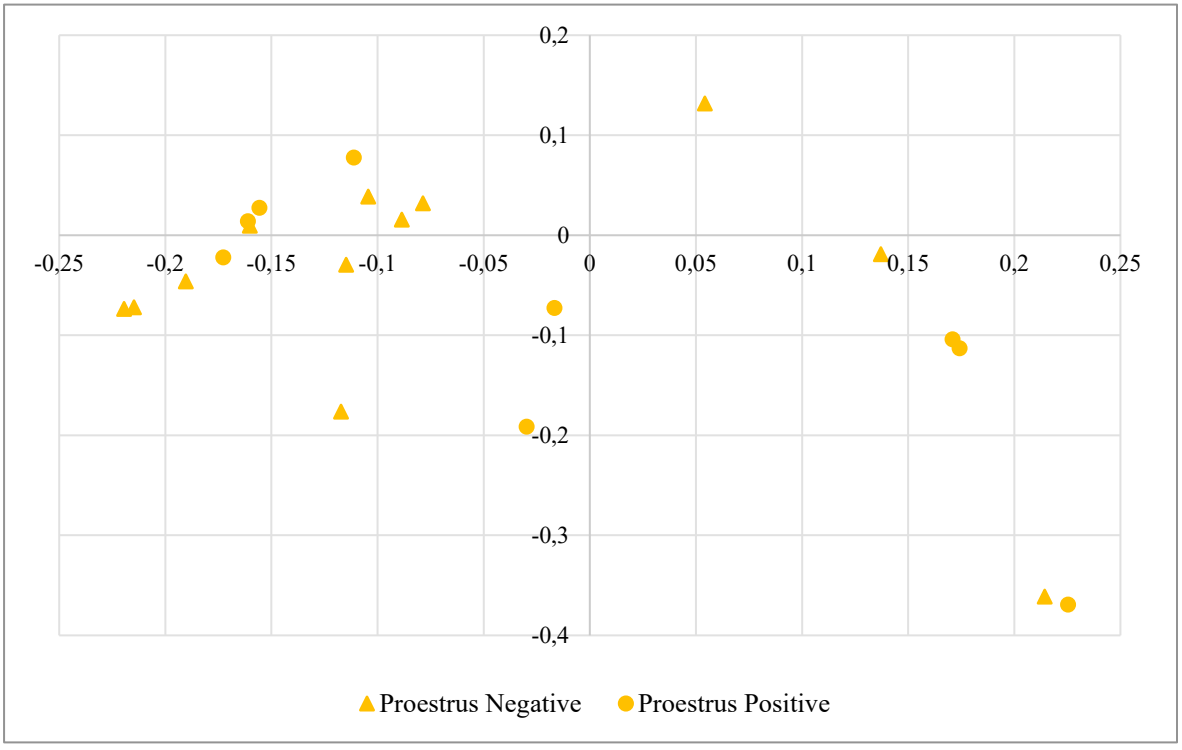
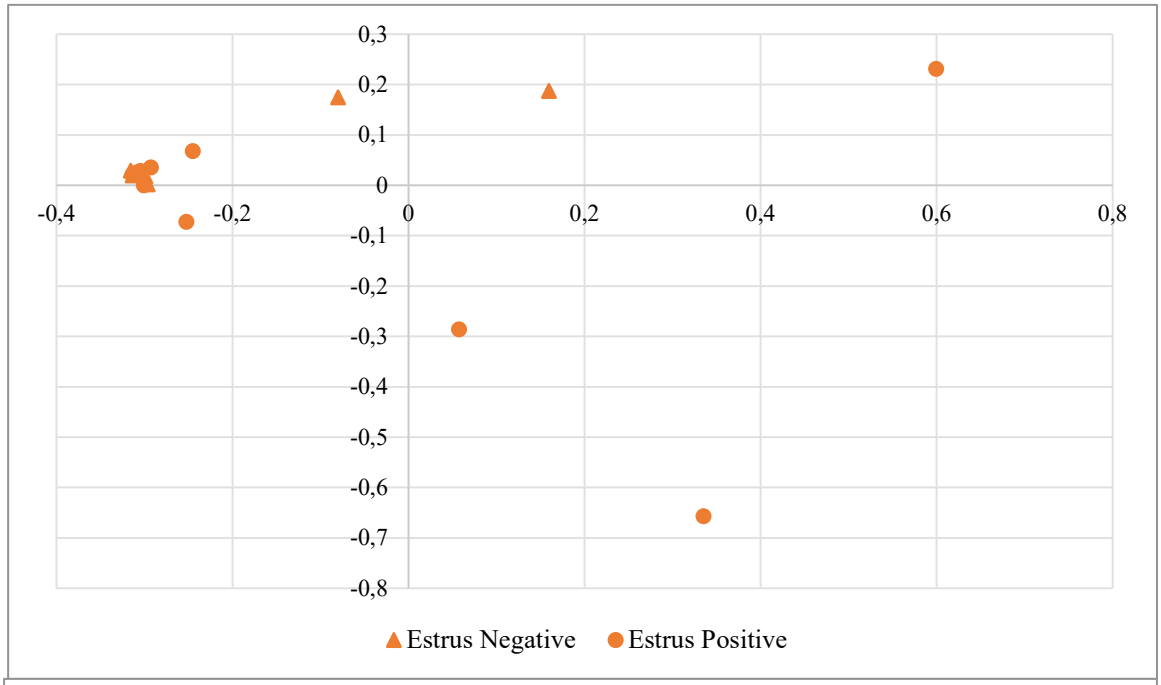
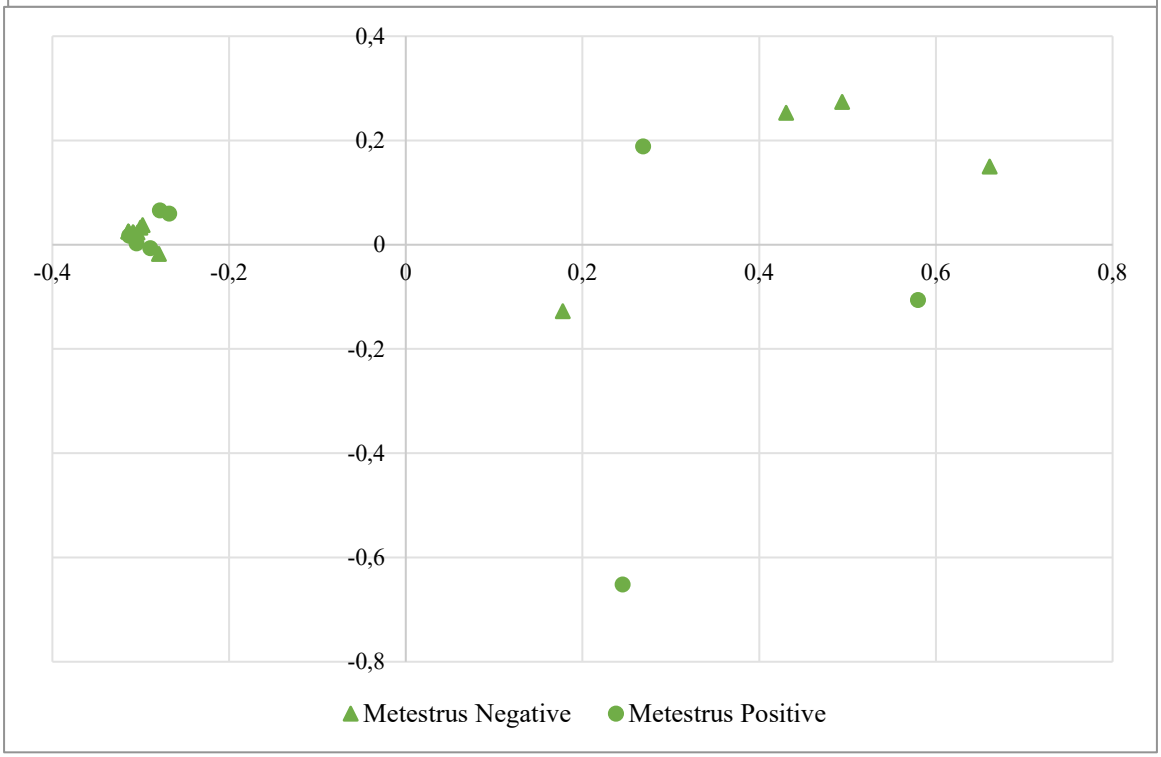
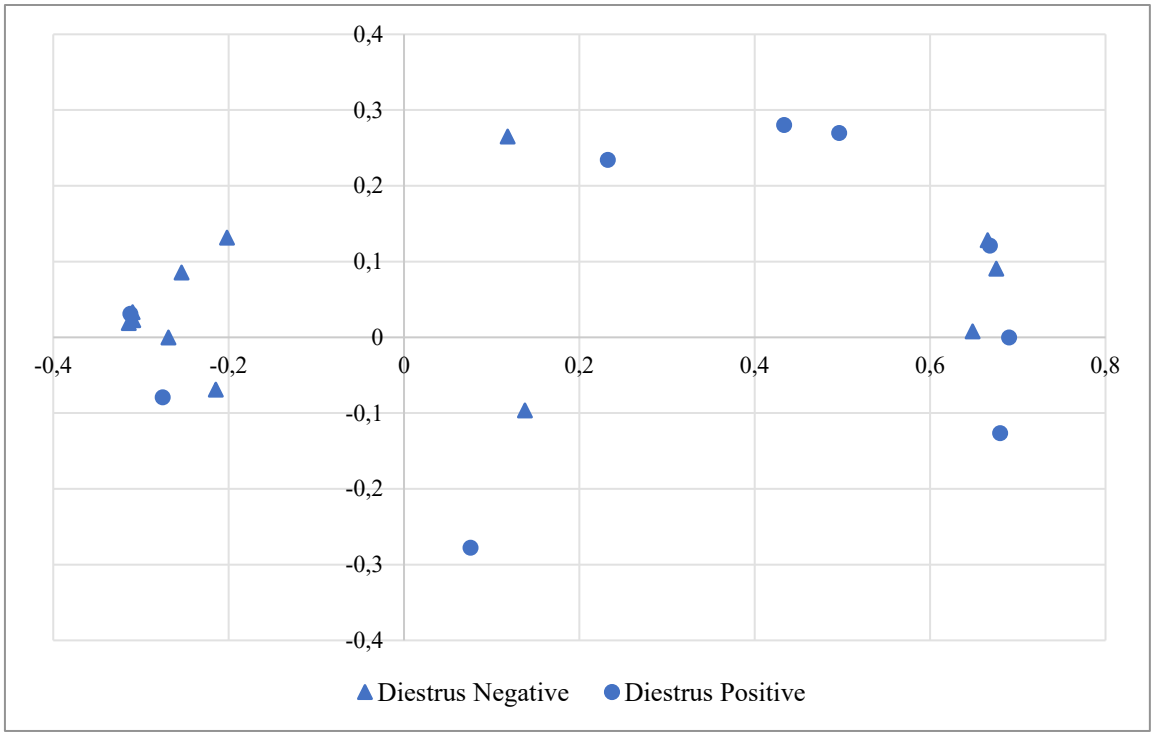
0,1
0
-0,1
-0,2
-0,3
-0,4
-0,5
-0,3
-0,2
-0,1
0
0,1
0,2
0,3
0,4
Diestrus Negative
Diestrus Positive


0,2
0,1
0
-0,1
-0,2
-0,3
-0,4
-0,25
-0,2
-0,15
-0,1
-0,05
0
0,05
0,1
0,15
0,2
0,25
Proestrus Negative
Proestrus Positive

Appendix 5 A to D - PCoA graphs of Yue and Clayton Index of Beta Diversity of structure of vaginal microbiota comparing cows that succeeded and failed to become pregnant during each phase of the estrous cycle, showing no evident clustering.






0,4
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0,2
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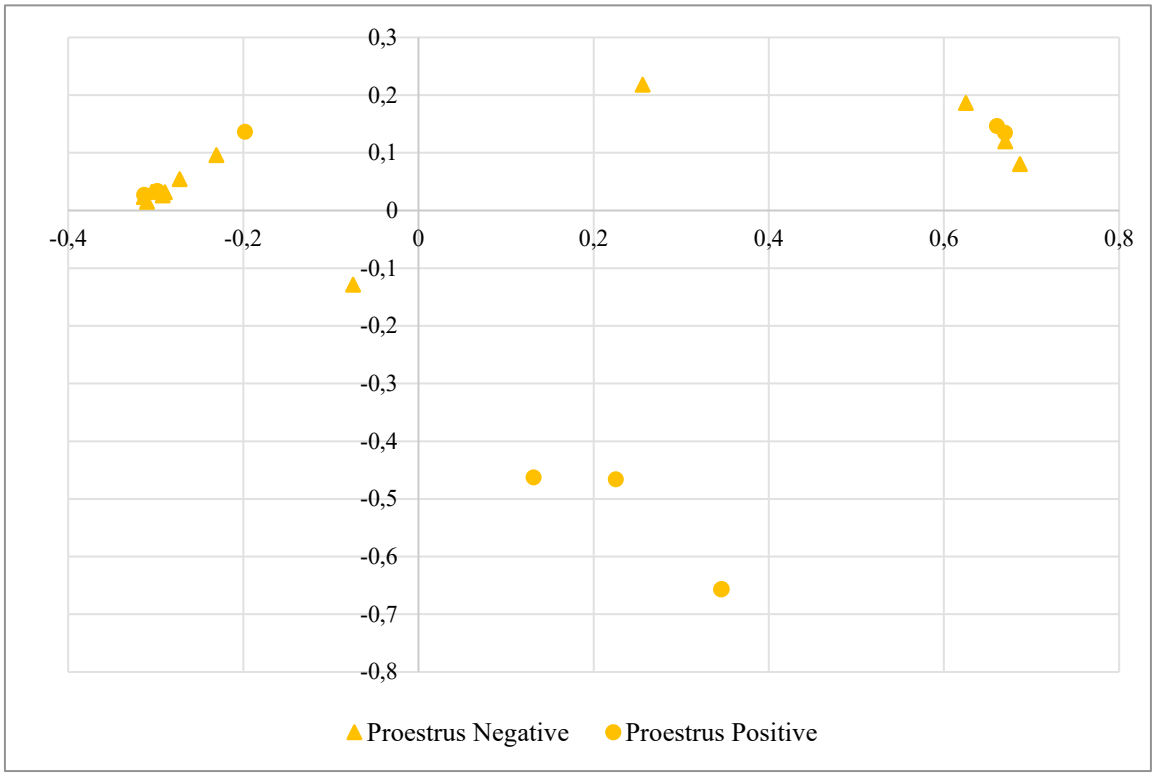
0
-0,1
-0,2
-0,3
-0,4
-0,4
-0,2
0
0,2
0,4
0,6
0,8
Diestrus Negative
Diestrus Positive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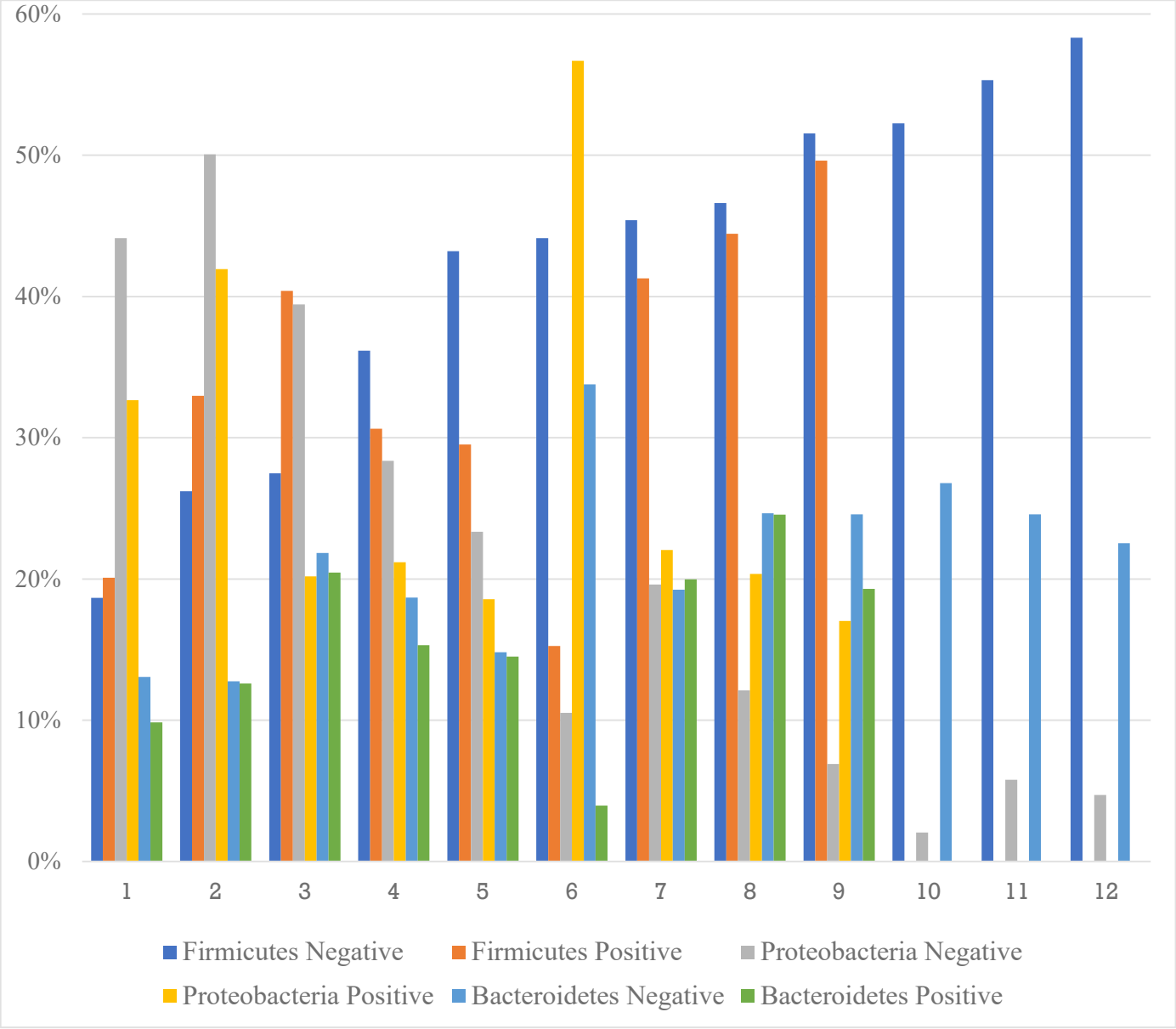
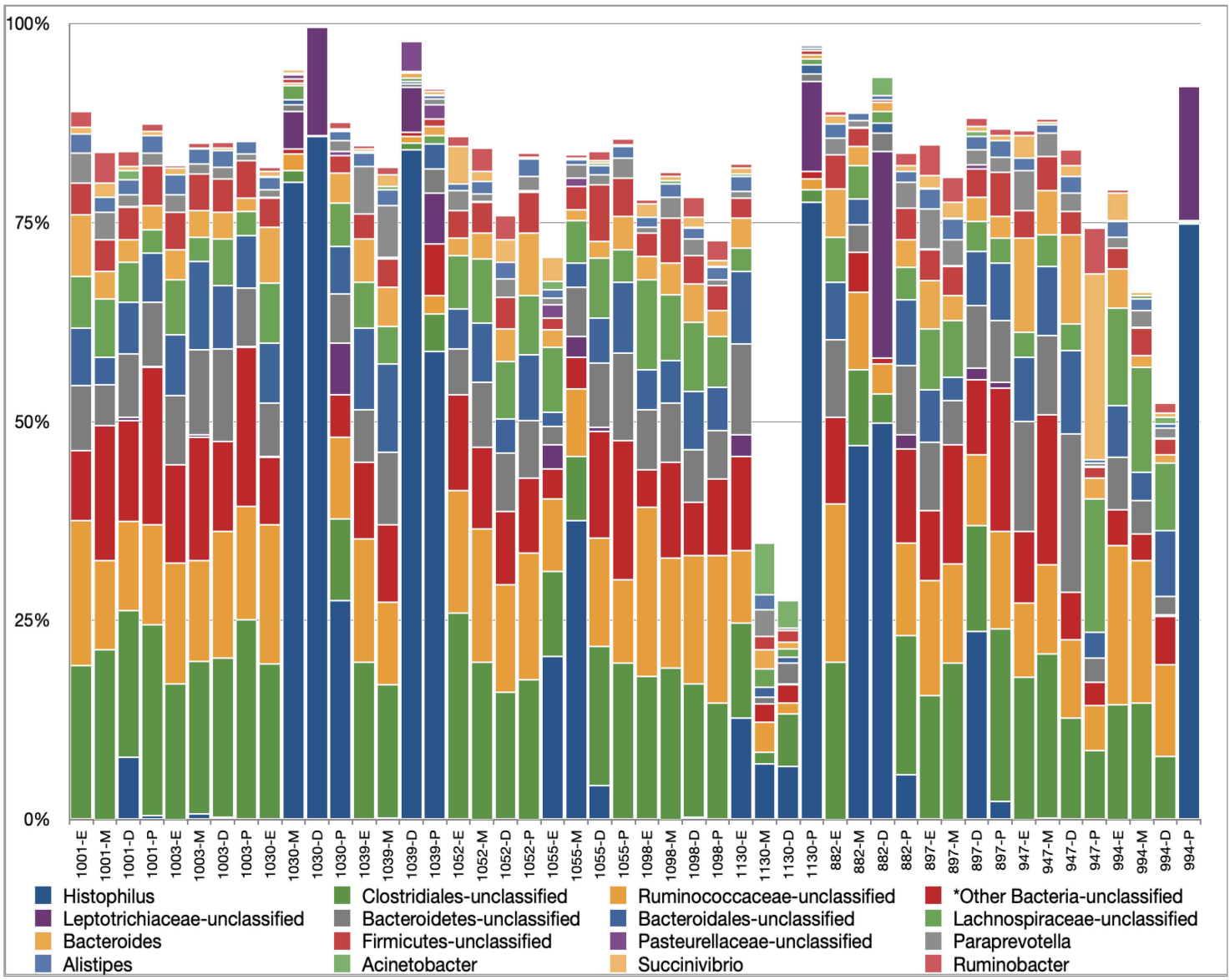
-0,4
-0,5
-0,6
-0,7
-0,8
-0,4
-0,2
0
0,2
0,4
0,6
0,8
Proestrus Negative
Proestrus Positive

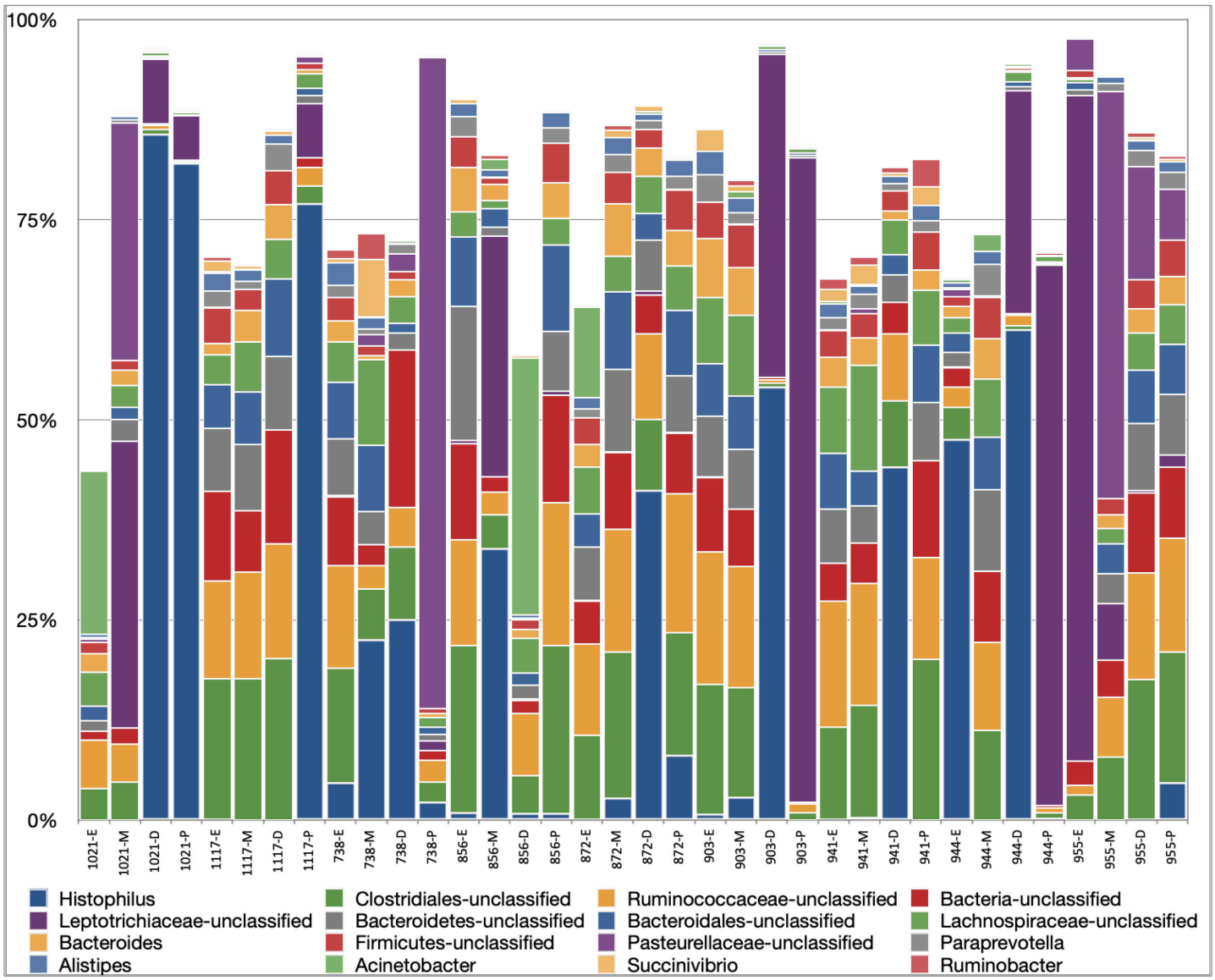
Appendix 6 - Comparative graph of the mean of three most abundant vaginal microbiota phyla in each cow



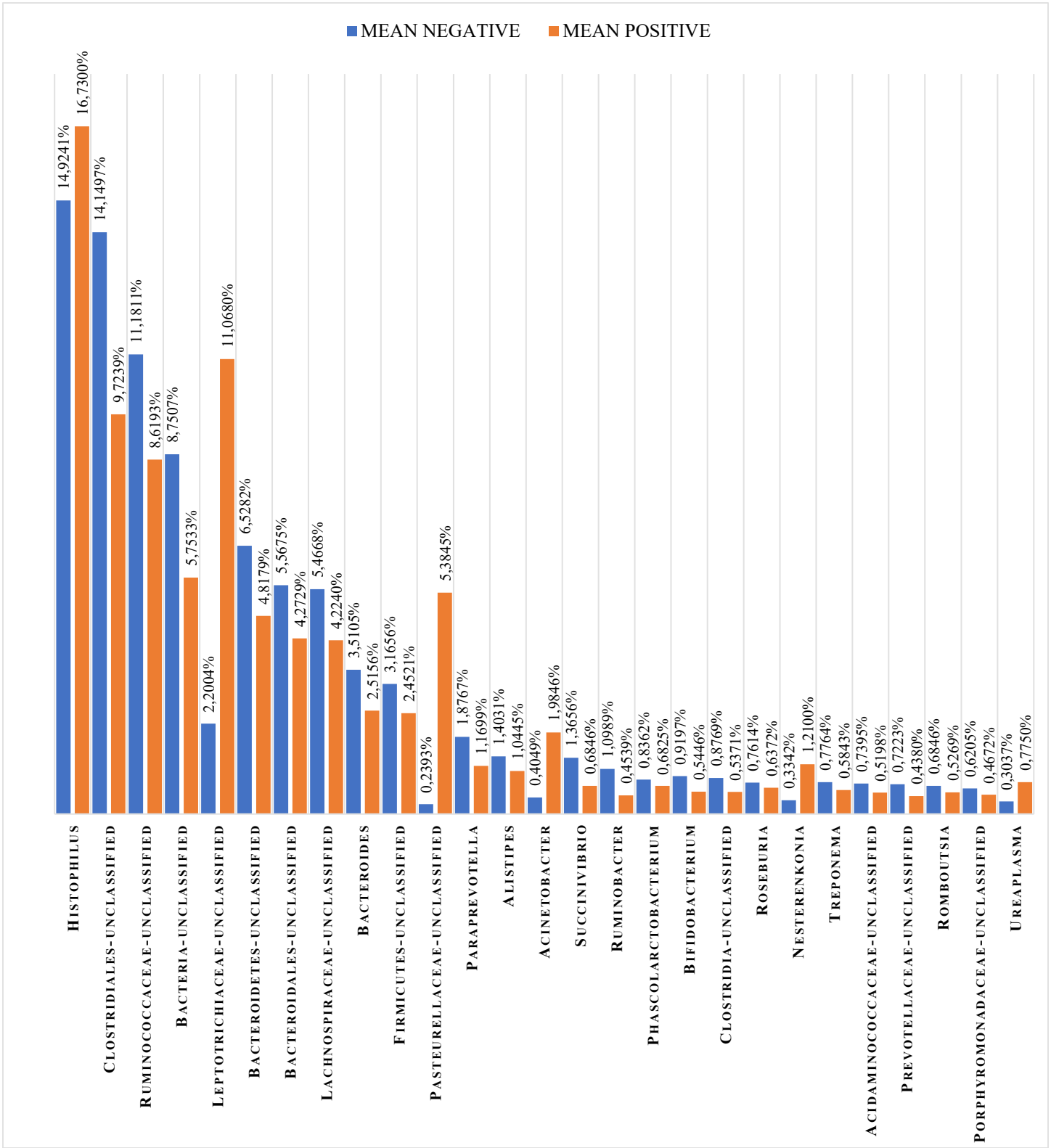
Appendix 7A - Most abundant genera in non-pregnant cows over the four phases of the Estrous cycle. Note low abundance of these genera in cow #1130 and abundance of Histophilus in cows #1030, 1039, 1130P and 994P.



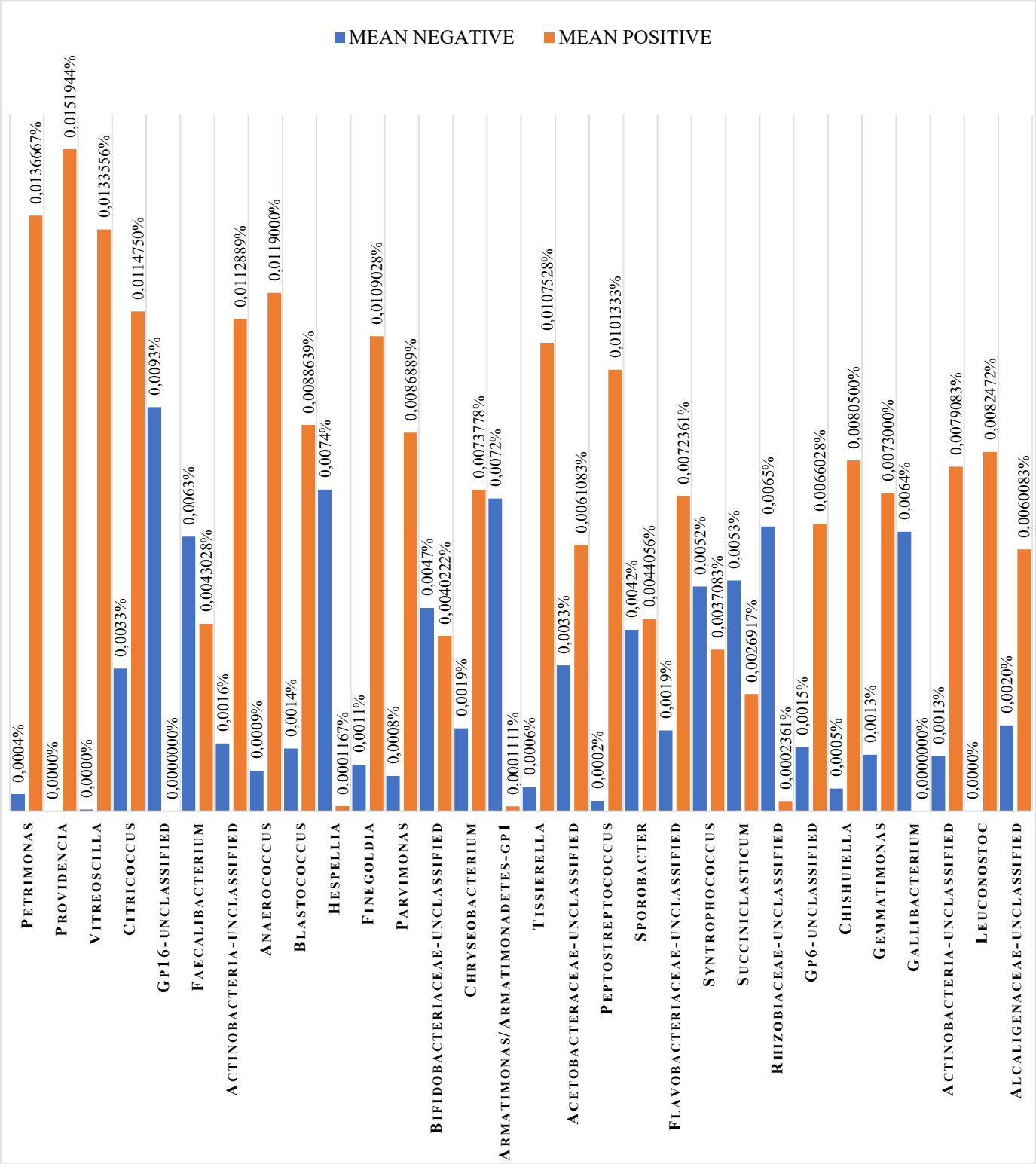
Appendix 7B - Most abundant genera in pregnant cows over the four phases of the Estrous cycle. Note high abundance of Histophilus in cows #1021 and 903, and of Leptotrichiaiaceaae in cows #903, 944 and 955, with high levels of Pasteurellaceae in #738 and 955M.



Appendix 8A - Comparison of mean genera populations in cows that tested negative and cows that tested positive for pregnancy (> 0.7%)



Appendix 8B - Comparison of mean genera populations in cows that tested negative and Cows that tested positive for pregnancy (>0.005%)



Appendix 8C - Comparison of mean genera populations in cows that tested negative and cows that tested positive for pregnancy (> 0.0003%)