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EFFECT OF *TRITRICHOMONAS FOETUS* INFECTION ON THE VAGINAL BACTERIAL MICROBIOTA OF HEIFERS

by

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Declaration

I, Isabela Salles Martins, student number 14451001, hereby declare that this dissertation, “*Effect of Tritrichomonas foetus infection on the vaginal bacterial microbiota of heifers,*” is submitted in accordance with the requirements for the Masters in Veterinary Science degree at University of Pretoria, is my own original work and has not previously been submitted to any other institution of higher learning. All sources cited or quoted in this research paper are indicated and acknowledged with a comprehensive list of references.

.....

Isabela Salles Martins

.....

Date

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Abstract

EFFECT OF *TRITRICHOMONAS FOETUS* INFECTION ON THE VAGINAL BACTERIAL MICROBIOTA OF HEIFERS

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Bovine trichomonosis is a venereal disease responsible for extensive economic losses. *Tritrichomonas foetus*, deposited during copulation, adheres to the vaginal mucosa where it proliferates. The vaginal bacterial microbiota protects the mucosa by colonization resistance. We sought to describe the bovine vaginal microbiota using metagenomics; contrasting between oestrus and dioestrus; and the interaction between the pathogen and the resident bacterial community.

Six heifers were divided into control and infected groups. Using endometrial guarded swabs, three cranial vaginal samples were taken on days 1, 11 and 21 of their reproductive cycle, totalling eighteen samples. The DNA of the samples were extracted and the V3-V4 region of the 16S rRNA gene amplified prior to next-generation sequencing, which provided over a million high-quality reads.

The vaginal bacterial microbiota of heifers were populated by organisms of the phyla *Tenericutes*, *Proteobacteria*, *Firmicutes*, *Actinobacteria* and *Bacteroidetes*. The control oestrus heifers were dominated by the phyla *Tenericutes* and *Proteobacteria*, represented by the families *Mycoplasmataceae* and *Pasteurellaceae*, respectively. The Shannon entropy (alpha diversity) were higher in oestrus than in dioestrus. Analysis of beta diversity via PERMANOVA did not reveal significant differences between heifers in oestrus and in dioestrus. However, the abundance of the family *Mycoplasmataceae* appeared to be affected by cycle stage ($p < 0.05$).

Analysis of the microbiota in the *T. foetus* infected heifers revealed a significantly higher alpha diversity compared to the controls in the corresponding stages of the oestrus cycle. Analysis of beta diversity via PERMANOVA revealed significant ($p < 0.1$) differences between the two sample groups. The infected groups were found to have significantly less *Mycoplasmataceae*, and an increase in the families *Bacillaceae*, *Ruminococcaceae*, *Propionibacteria*, *Lachnospiraceae*, *Paenibacillaceae* and *Prevotellaceae*.

The use of a culture-independent method expanded the knowledge of, and illustrated the substantial influence of, a few organisms on the bovine vaginal microbiota. The sex hormones may have influenced the microbiota. The introduction of *T. foetus* caused a dramatic change to the vaginal microbiota and an increase in the bacterial diversity.

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List of abbreviations

16S rRNA	16S ribosomal RiboNucleic Acid
ANOSIM	ANalysis Of SIMilarities
ARC	Agricultural Research Council
BVEC	Bovine Vaginal Epithelial Cells
DGGE	Denaturing Gradient Gel Electrophoresis
DNA	DeoxyriboNucleic Acid
DPP	Days Post Partum
GRAS	Generally Recognized As Safe
HC	Hormonal Contraception
HIV	Human Immunodeficiency Virus
LSU	Large Sub-Unit
NGS	Next Generation Sequencing
NMDS	Non-metric Multi-Dimensional Scaling
OTU	Operational Taxonomic Unit
PAMPs	Pathogen-Associated Molecular Patterns
PAS	Periodic Acid-Schiff (a staining method)
PAST 3.16	PAleontological STatistics 3.16
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PERMANOVA	PERmutational Multiple ANalysis Of VAriance
RNA	RiboNucleic Acid
SIMPER	SIMilarity PERcentage
SSU	Small Sub-Unit
STI	Sexually Transmitted Infection
<i>T. foetus</i>	<i>Tritrichomonas foetus</i>
<i>T. vaginalis</i>	<i>Trichomonas vaginalis</i>

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1 Introduction

The upper female genital tract, consisting of the uterus, uterine tubes and ovaries, is considered to be a near-sterile environment. The continuity with the cervix, vagina and external genitalia allows the migration of the foetus during birth to the exterior, and the introduction of semen to the interior. During these windows of exposure to the external environment, there is a heightened risk of harmful microorganisms entering the internal genital tract. The external genital tract (vulva), the caudal and cranial vagina and cervix together separate the lower genital tract from the upper or internal genital tract, acting as physical barriers between the external environment and the uterus. The defence mechanisms in the genital tract are composed of physical barriers and immunological responses (Sheldon, 2014).

In contrast to the uterus, the normal vagina supports a community of microorganisms. The bovine vaginal bacterial microbiota is composed of a dynamic mixture of aerobic, facultative anaerobic and strictly anaerobic organisms. The resident vaginal microbiota has been recognized as playing an important competitive role by maintaining a healthy environment and preventing proliferation of pathogens foreign to the vagina, thereby preventing the establishment of infection (McVey et al., 2013). The successful competition between these microorganisms determines the normal microbiota that consequently protect the underlying epithelial cells through the mechanism of “colonization resistance” (McVey et al., 2013). Proliferation of exogenous pathogens or endogenous opportunistic bacteria can disturb the stability of this bacterial community, thereby causing an infection and stimulating a local inflammatory and immune response (McVey et al., 2013). Although the normal bacterial microbiota is not directly associated with specific immunity, their competitive role in preventing colonization by pathogenic microorganisms can be considered part of a physical barrier and beneficial to their host (Quinn and Markey, 2003).

The protozoa *Tritrichomonas foetus* is an exogenous primary pathogen of significant importance to the cattle industry due to its high prevalence and the insidious nature of the disease it causes. Transmitted venereally, *Tritrichomonas foetus* results in trichomonosis – characterised by vaginitis, endometritis, placentitis, abortion, long intervals between parturition and reproductive failure – leading to substantial economic losses (Bartlett, 1947, Parsonson et al., 1976). The economic impact of *Tritrichomonas foetus* cannot be overstated as it essentially exists in every country in the world where cattle are raised extensively. In South Africa, it has been described as a major cause of infertility in beef cattle, with devastating consequences related to the resultant economic losses (Irons, 2004).

Previous studies of the microbiota of the human vagina indicated that the bacterial community in the normal vagina has an important role in preventing colonization by invasive nonindigenous organisms,

including those responsible for bacterial vaginosis, sexually transmitted diseases, yeast infections and urinary tract infections (Zhou et al., 2004, Witkin et al., 2007). Among the sexually transmitted diseases, *Trichomonas vaginalis*, the causative of human trichomoniasis, is believed to compete directly with the normal bacterial flora, composed mostly of lactobacilli, to colonize this environment. The proliferation of *T. vaginalis* is marked by the growth of opportunistic bacteria, reduction in the lactobacillus population and an increase in the vaginal pH which, together, initiate an inflammatory and immune response (Jirovec and Petrů, 1968, McGrory et al., 1994).

Considering the relevance of competitive normal vaginal microbiota in promoting mucosal protection, the need for a more in-depth understanding of the resident bacterial microbiota in the vagina of cows, as well as the pathogenesis of *T. foetus* within this environment, is evident. This work presents a study of the effect of the protozoa *Trichomonas foetus* on the resident vaginal bacterial microbiota of heifers in an attempt to expand the understanding of the pathogenic mechanisms by which this protozoon successfully achieves infection.

2 Literature review

2.1 *Tritrichomonas foetus*

Trichomonads represent a group of protozoan flagellates adapted for living in anaerobic or micro-aerobic environments. Most of these organisms inhabit the intestines of a variety of vertebrate and invertebrate hosts. Only a few trichomonad organisms have been shown to have pathogenic potential for mammals and birds. Among them are two significant pathogens that are sexually transmitted through the genito-urinary tract of cattle and humans, *Tritrichomonas foetus* and *Trichomonas vaginalis*, respectively (BonDurant, 1994; Petrin et al., 1998). The protozoa *T. foetus* has also been found as an important agent causative of chronic diarrhoea in domestic felines. The pathogen is transmitted through the oral-faecal route and is more commonly found in multi-cat environments. Stockdale et al. (2007) demonstrated that virgin heifers, infected with *T. foetus* isolated from cats, developed vaginitis and endometritis. The cat and the bovine isolate can be genetically distinguished even though they share the same nomenclature (Reinmann et al., 2012).

Tritrichomonas foetus is an obligatory inhabitant of the bovine reproductive tract. The parasite is approximately 8 to 18 μm long and 4 to 9 μm wide, and can be identified by the presence of three anterior flagella, a characteristic undulating membrane reaching the posterior end of the body and a recurrent flagellum continuing beyond the undulating membrane as a free-trailing portion (Figure 1). Internal organelles include the nucleus, hydrogenosomes, and the axostyle – a rigid structure that runs longitudinally from one end of the cell to the other (Benchimol, 2005).

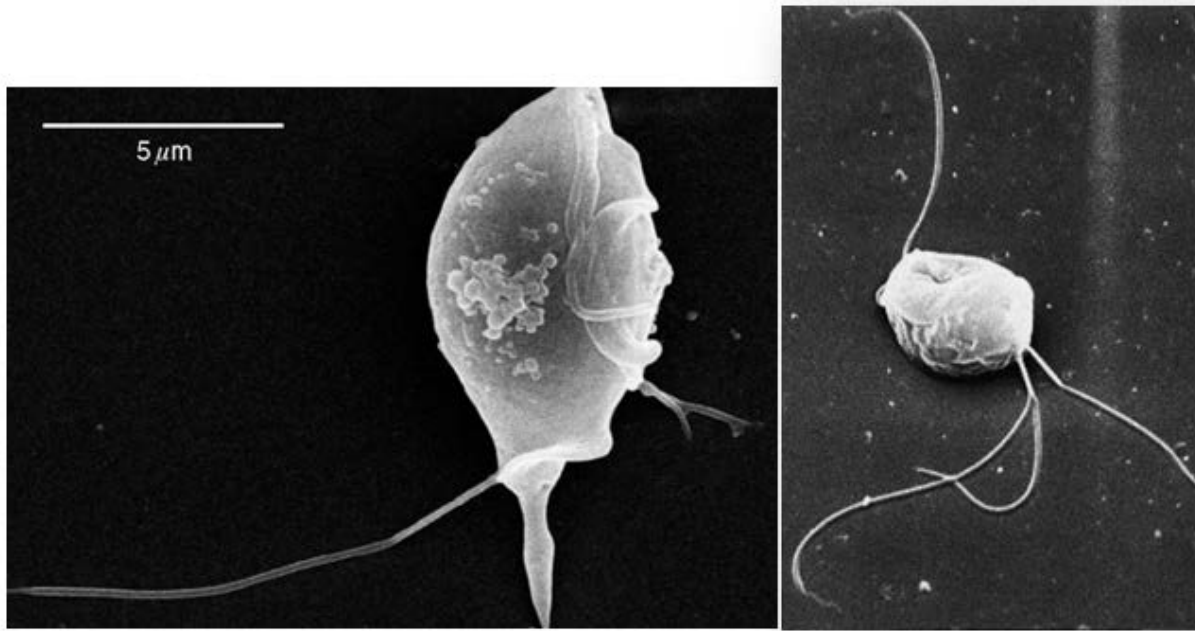


Figure 1: Electromicrograph of the *Tritrichomonas foetus* organism.

Figures taken from (Noakes, 2001, Lim et al., 2010). The parasite is approximately 8–18 µm long and 4–9 µm wide. The presence of three anterior flagella, a characteristic undulating membrane reaching the posterior end of the body and a flagellum beyond the undulating membrane, are some features used in its identification (Benchimol, 2005).

Trichomonad organisms obtain energy by utilizing exogenous and endogenous carbohydrates that are degraded under both anaerobic and aerobic conditions into organic acid, in most cases acetate, succinate and carbon dioxide. Molecular hydrogen is also produced in anaerobic conditions (Lindmark and Müller, 1973). In contrast to aerobic eukaryotic organisms in which the mitochondria is the respiratory organelle, the anaerobic protozoa *T. foetus* contain hydrogenosomes, unique energy-producing organelles capable of catalysing the oxidation of pyruvate to acetate, CO₂ and H₂ (Steinbüchel and Müller, 1986).

T. foetus can display a pyriform body or exhibit a rounded or elongated shape. When a fresh specimen obtained from an infected animal is examined, the protozoa tends to be pyriform and elongated, but in culture it exhibits pleomorphism and a tendency to become spherical (Irons, 2004). The existence of a pseudocyst form, thought to be stimulated by adverse conditions, has been described and appears to be an active form also capable of mitosis and cytoadherence (Meyer Mariante et al., 2004).

Tritrichomonas foetus organisms move with a jerky, rolling motion and are observed in cultures of preputial samples of infected bulls, vaginal washings of cervical-vaginal mucus of infected cows and in aborted fetuses. During natural infection, the trophozoite is transmitted during coitus. This gives rise to a population in the lumen and in the mucosal surfaces of the urogenital tract of cattle that proliferates through a simple life cycle by longitudinal binary fission (Schwebke and Burgess, 2004).

Bulls are long-term, asymptomatic carriers of the organism. *T. foetus* thrives in an environment with low oxygen levels, most commonly found deep within the bull's preputial crypts. It has been suggested that, as bulls age, they can be more susceptible to infection because the preputial and penile epithelium develops more mucosal folds and deeper crypts with reduced levels of oxygen, an ideal environment for *T. foetus*' growth (Rhyan et al., 1999). The transmission of the infection occurs in most cases via coitus, but can also occur by gynaecological examination of cows, or even by artificial insemination, as the protozoa can survive the standard method of semen freezing (Monke and Mitchell, 1998). However, given the stringent biosecurity measures in the artificial insemination industry, production systems utilising natural service strategies, used mainly in the beef industry, are more at risk of infection (BonDurant, 2005).

Medical treatment of infected bulls is prolonged, expensive and labour intensive, without the certainty of a cure. Because of the protozoa's location within the deeper crypts of the prepuce, local topical treatment might not be successful, requiring systemic treatment with an antiprotozoal agent. Oral administration of antiprotozoal agents interferes with ruminal protozoa and carries a risk of causing ruminal stasis, a life-threatening condition (Irons, 2004). Besides this risk, these agents are not registered to be used in food-producing animals in some countries.

2.1.1 The pathogenesis in the female genital tract

Sexually-active females of all ages are susceptible to *T. foetus*. Natural infection occurs when the highly transmittable *T. foetus* is deposited in the cranial vagina from the infected bull's preputial cavity. It initially adheres to the vagina, colonizes it and then migrates through the cervix to the uterus and uterine tubes, producing vaginitis and, in some cases, cervicitis, endometritis and salpingitis (Irons, 2004). Colonization of the entire reproductive tract with *T. foetus* occurs between one to two weeks after infection (Rae and Crews, 2006, Skirrow and BonDurant, 1990).

The vagina does not play a passive, inconsequential role in the progression of the disease. The colonization of the vagina appears to be an important platform enabling subsequent migration into the uterus. This colonization was demonstrated by Hammond D.M (1945) through daily collection of cervico-vaginal mucus of artificially-infected cows, in which large numbers of *T. foetus* organisms appeared from the twelfth to the nineteenth day after infection. A marked decrease in *T. foetus* numbers after the third week of exposure was associated with the development of a local resistance. In another study, *T. foetus* was shown to persist in the cervix and vagina for periods of up to 95 days (Parsonson et al., 1976).

Repeated oestrus, pyometra, low pregnancy rate, abortion and extended calving intervals are the first physical signs of *T. foetus* infection in a herd. The parasite does not prevent fertilization, but causes embryonic or foetal death, resulting in irregular and extended returns to oestrus. Abortions commonly

occur between the second and fourth months of gestation, but very few occur after the fourth month (Noakes, 2009).

The development of endometritis is closely associated with embryonic or foetal death (Parsonson et al., 1976), likely due to interference with nidation or nutrition of the conceptus. However, the exact mechanism is not known. It has also been suggested that embryonic death occurs as a result of damage to the developing placentomes (BonDurant, 1997). Foetal death can be accompanied by the development of pyometra in up to 10 percent of the cases in which the persistent corpus luteum maintains the closure of the cervix, which leads to the uterus being filled with enormous quantities of trichomonads (Noakes, 2009).

Parsonson et al. (1976) identified histological evidence of endometritis and lesions in the genital tract after 60 days of infection, thus distinguishing this moment as critical to determining the outcome of the pregnancy. In this regard, Anderson et al. (1996) immunized 24 heifers with TF 1.17, a purified superficial antigen from *T. foetus* strain D1, and then infected the heifers with the D1 *T. foetus* strain to evaluate their immune and inflammatory responses. It was demonstrated that the immunization did not offer complete protection, but did accelerate the clearance of the trichomonads to 60 to 70 days after infection, consequently minimizing the foetal loss. Herr et al. (1991) produced a vaccine with the antigen used to infect heifers. Vaccinated and non-vaccinated groups, with 12 individuals each, were kept with the infected bull for 60 days of the breeding season. The heifers were mated and infected (confirmed via culture of vaginal samples), but only three were pregnant 4 to 5 months after the removal of the bull, with no abortions observed. Even though the heifers did not conceive, the research revealed the vaccination to be reasonably effective, as the vaccinated group were found to be free of infection after 7 weeks on average, while the placebo group were free after 14 weeks on average.

A *T. foetus* whole cell vaccine is currently available (Trichguard® V5L, Zoetis 2017¹) and can be used in males and females. Females vaccinated twice, two months prior to the breeding season, do not become immune but transmission of the disease is reduced, along with a decreased chance of foetal losses. Edmondson et al. (2017) experimentally tested the efficacy of the vaccine by vaccinating 20 cows prior to the breeding season under a natural mating regime. Fifty percent of the treated cows gave birth to a live foetus when compared to 20 percent of the control group. The vaccine improved

¹ Trichguard® V5L contains killed, concentrated cultures of *Trichomonas foetus*, *Campylobacter fetus*, *Leptospira canicola*, *L. grippotyphosa*, *L. icterohaemorrhagiae* and *L. pomona* organisms suspended in a special oil adjuvant, Zoetis (2017).

pregnancy and birth rates when compared to the control animals; however, the losses caused by *T. foetus* are still considerable.

Sufficient immunity can develop after abortion to maintain a pregnancy to term (Noakes, 2009, Skirrow and BonDurant, 1990). However, this immunity is short-term, probably lasting no more than 15 months, and cows are fully susceptible to infection in successive seasons (Clark et al., 1983). Most cows that calve can successfully eliminate the infection and not become long-term carriers, but some animals become persistently infected throughout gestation and into the following breeding season, acting as a reservoir of infection to the herd (Rae and Crews, 2006). Regarding the time for clearance of the infection, sexual rest for a period of three months is considered adequate; however, the females must be cycling regularly to ensure elimination of the protozoa. The majority of infections will spontaneously resolve with the return to oestrus (Irons, 2004). Exceptions are following foetal mummification or pyometra, both of which require medical treatment and extra time for the infection to clear. Clearance from the vagina, cervix and uterus occurs simultaneously (Gault et al., 1995).

2.1.2 Host-parasite interaction

The clinical features and epizootiology of *T. foetus* infection in cows have been adequately described, but studies that describe the pathology and pathogenesis in detail are limited (Parsonson et al., 1976). Efforts have been dedicated to developing a better understanding of how *T. foetus* interacts with different tissues in the vagina, and the mechanism by which it can cause disruption of the host tissue to establish the infection.

The host-parasite interaction is highly complex. Much of the data available in this regard is related to *Trichomonas vaginalis*. Trichomonosis in women is caused by the protozoa *Trichomonas vaginalis* which is classified as the most common sexually transmitted infection (STI) in the world, with 276 million new cases registered annually (WHO, 2008). It is associated with adverse birth outcomes vis-à-vis premature rupture of foetal membranes, low birth weight and premature birth. Similarly to other STIs such as gonorrhoea, chlamydia, and syphilis, *T. vaginalis* considerably enhances the predisposition of an infected person contracting human immunodeficiency virus (HIV) (Thurman and Doncel, 2011). The protozoa, when introduced to the genital tract, causes disruption of the genital cell membranes, which leads to mucosal immune response and localized inflammation. Migration of lymphocytes and macrophages to the site increases the number of potential cells for HIV to invade and proliferate (Petrin et al., 1998, Thurman and Doncel, 2011). This correlation between *T. vaginalis* and HIV transmission has drawn attention to the reduction of trichomonosis as a strategy for HIV prevention (WHO, 2008).

Despite the host-parasite species specificity (Singh et al., 1999), *T. foetus* and *T. vaginalis* possess several factors in common related to their morphology, mechanisms of adhesion and cell apoptosis.

These attributes of *T. foetus* and *T. vaginalis* interaction with the female host are briefly discussed below.

2.1.2.1 Mucus interaction of trichomonads

Mucus is a viscoelastic substance secreted by the secretory cells of the endocervix, which represents either a mechanical barrier or a transport medium to spermatozoa. The physical characteristics of mucus can be influenced by the sex hormones (Roberts, 1956). Under the influence of oestrogen, i.e. during oestrus, the cervical glands' activity is increased by the secretion of a translucent mucus with a fluid consistency. This mucus acts to protect the lower genital tract during copulation through lubrication, and as a physical barrier by covering the underlying epithelial mucosa while serving as a spermatozoa transport medium to the uterus. During pregnancy, the mucus is thick, scanty and opaque, and serves to seal the cervix, which prevents pathogens from getting into the uterus (Roberts, 1956). Besides physical characteristics, the major proteinaceous constituent of the mucus is mucin. Mucins are large glycoproteins, with a gel-like property and heavy glycosylation, which makes mucin resistant to proteolytic activities produced by pathogens (Rose, 1992).

Tritrichomonas foetus is deposited in the cranial vagina of the cow during copulation when the female is in oestrus. The mucus layer is the first physical barrier surrounding the host epithelial surface encountered by the pathogenic organism (Lehker and Sweeney, 1999). Studies of the interaction between mucin and *Trichomonas vaginalis* showed that the parasite initially adheres to mucin as a mechanism to remain in the reproductive tract for prolonged periods of time after coitus. Adherence is then followed by a mucolytic activity: a cysteine proteinase secreted by the parasite solubilises the mucus and detaches the parasite from the mucus layer. Subsequent flagellar movements allow the parasite to penetrate the soluble mucus and successfully attach to the epithelial cell layer (Lehker and Sweeney, 1999).

2.1.2.2 Trichomonads' interaction with vaginal epithelial cells

The vagina extends from the cervix to the vestibule. Histologically, it is composed mostly of a stratified squamous epithelium that varies in thickness under the influence of sex hormones. In the cranial vagina, a surface layer of columnar and goblet cells containing PAS-positive mucosubstances is present in the stratified squamous epithelium. PAS, i.e. "Periodic acid-Schiff", is a staining method used to detect polysaccharides and mucosubstances in tissues (Eurell et al., 2006).

The vaginal epithelial cells act as a physical barrier and as a sentinel of the genital tract. Toll-like receptors on their cell surface can recognize the proliferation of pathogens by the expression of pathogen-associated molecular patterns (PAMPs). These toll-like receptors react to these PAMPs with

the activation of innate immune and inflammatory responses in an effort to maintain a balanced, healthy microbiome (Tizard, 2009).

The immune response in the upper and lower genital tract is also controlled by sex hormones. Studies in the genital tract of women have shown that estradiol and progesterone change epithelial cell permeability, microbicide activity and cytokine secretion to allow procreation. In the lower genital tract, a high level of estradiol during the follicular phase is associated with a decrease in inflammatory and immune response (Wira et al., 2015).

Tritrichomonas foetus deposited in the lower genital tract during copulation uses its posterior flagellum and adhesion proteins to adhere to the vaginal epithelium. Epithelial adhesion is necessary for *T. foetus*' survival. Cytotoxicity is a major consequence of *T. foetus* infection. However, this is only achieved by a contact-dependent mechanism. The parasite secretes cysteine proteinases that induce apoptosis of the bovine vaginal epithelial cell (Singh et al., 2004, Singh et al., 1999). Transmission electron microscopy has shown that *T. vaginalis* is also capable of *in vitro* phagocytosis of small portions of the epithelial cell membrane. The degradation of the cell was confirmed through the identification of acid phosphatase activity inside the phagocytic vacuoles (Rendón-Maldonado et al., 1998).

Keratinization of the squamous epithelial cells occurs in the vagina of cows during oestrus (Roberts, 1956). Trichomonads can interact with keratin and ingest keratin particles as a source of protein and nutrition, as well as a way to access the epithelium lining (Vilela and Benchimol, 2011).

T. vaginalis infection is very rarely found in girls or children's genitalia (Jirovec and Petrů, 1968). The reason for this is that the environment is unsuitable for the establishment of the protozoa, due to the absence of glycogen in the vaginal epithelial cells, which only occurs under oestrogenization. The implication of this finding is that oestrogenization is a key nutritional factor for *T. vaginalis*' viability.

2.2 The vaginal bacterial microbiota

The vulva, vagina, and cervix represent successive physical barriers to protect the body against microorganisms gaining access to the uterus. The vagina plays an important role by protecting the genital tract through mucus secretion, the competition of its healthy microbial community and by the immune and inflammatory responses to pathogenic organisms provided by the epithelial cells (Sheldon, 2014).

The vaginal microbiota is the result of a dynamic interaction of organisms with constant replacement of microbes more capable of living in a particular site (niche) (McVey et al., 2013). Microorganisms that compete successfully for particular sites gradually form a stable normal flora (Quinn and Markey, 2003). This "occupation" results in a barrier to colonization (infection), hence the term "colonization

resistance” (McVey et al., 2013). In healthy individuals, the bacteria living in the vaginal microbiome is composed of “commensal bacteria”, a term that refers to the relationship between bacteria and the host in which no discernible harm is caused to the host. On the other hand, pathogenic organisms which are capable of producing a disease can be from exogenous or endogenous sources. *Trichomonas foetus* is an example of a pathogenic organism of exogenous origin. Pathogens of endogenous origin can be commensal bacteria responding opportunistically when epithelial barriers are damaged, or when immunity is weakened (Quinn and Markey, 2003).

The normal bacterial microbiota can compete with, and sometimes prevent establishment of, pathogenic microorganisms. This may be achieved by competition for nutrients, formation of inhibitory substances, or by the attachment to receptors on the cell surface, thereby preventing colonization by invading pathogens. Although the normal flora are not directly associated with non-specific immunity, their competitive role can be considered beneficial for the host (Quinn and Markey, 2003).

In women, the vaginal bacterial flora are populated by a range of lactic-acid-producing bacteria. *Lactobacillus* types predominate, promoting an acidic environment that prevents the proliferation of “undesirable” organisms (Zhou et al., 2004, Donders, 2007). *Lactobacillus*-deficient conditions, representing abnormal vaginal bacterial microbiota, are associated with conditions such as bacterial vaginosis, aerobic vaginitis and the transmission of STIs such as gonorrhoea, chlamydia, syphilis, HIV as well as trichomonosis (Donders, 2007, Donders et al., 2000).

Jirovec and Petru (1968) studied the vaginal bacterial flora in women using simple stain smear techniques that demonstrated the competitive relationship between the *Lactobacillus* population and *T. vaginalis*, with a significant reduction or absence of *Lactobacillus* in the acute and persistent stages of infection. A significant increase in other types of bacteria, as well as many trichomonads, was also observed in the persistent phase. McGrory et al. (1994) subsequently demonstrated *in vitro* the deleterious effect of *T. vaginalis* on the numbers of *Lactobacillus acidophilus* as well as on its phagocytic characteristics.

Bacteria do not only use animals as hosts; they can also lodge inside protozoal organisms as a mechanism of survival. This is the case in the symbiotic relationship existent between the two sexually-transmitted microorganisms *Mycoplasma homini* and *Trichomonas vaginalis*, whereby the protozoa provide an environmental reservoir for the pathogenic bacteria (Vancini and Benchimol, 2008).

No studies of the interactions between the Trichomonads and the bacterial vaginal microbiota in cattle have been published.

2.3 Bovine vaginal bacterial microbiota

In a healthy cow, the vaginal bacterial microbiota is composed of a wide spectrum of organisms, which can be described as a dynamic mixture of aerobic, facultative anaerobic and strictly anaerobic microorganisms, with new strains being constantly introduced (Noakes, 2009). Many studies related to this topic have given special attention to the uterine microbiota of post-partum healthy and metritic cows (Santos and Bicalho, 2012, Santos et al., 2011, Machado et al., 2012). Panangala et al. (1978) collected cervico-vaginal samples from 72 fertile and 70 repeat-breeder cows to assess the micro-flora (bacteria, fungi and mycoplasma) resident in normal fertile and repeat-breeder cows. The cows selected were in different reproductive stages, and media culturing for anaerobes, aerobes and mycoplasma were included. The results found that the mean number of different types of bacteria per sample was 2.89 in normal-fertile animals, and 3.26 in repeat-breeder cows. There was no significant difference in the bacterial species between the two groups. Bacteria of the families *Enterobacteriaceae*, *Streptococcaceae*, *Micrococcaceae*, *Bacillaceae* and *Corynebacterium* were found in both groups. An increased number of Enterobacteriaceae was detected in the repeat breeders. Twenty one percent of the animals were positive for T strain *Mycoplasma* (*Ureaplasma urealyticum*) and 8.5 percent for large colony *Mycoplasma* (mostly *M. bovis genitalium*).

Otero et al. (1999) developed an experiment to evaluate the variation of the normal bacterial flora during the cow's oestrous cycle, giving emphasis to the *Lactobacillus* and *Enterococcus* populations with the aim of creating a probiotic product for veterinary use. These two bacteria were chosen because they are classified as GRAS (Generally Recognized As Safe). The authors suggest that there is no difference in the diversity of the bacteria population during the pro-oestrous, oestrous, metestrous and dioestrous periods, but that an increase in the total bacteria count of *Lactobacillus* and *Enterococci* was observed during periods of increased oestrogen. In the following year, the same authors studied the qualitative and quantitative changes in the aerobic vaginal micro-flora over the growth period of heifers, with the aim of determining the colonization kinetics and the predominant genera of species (Otero et al., 2000). They collected 10 sets of samples from 15 heifers from weaning until the age of 23 months. Coagulase-negative *Staphylococcus* and α -haemolytic *Streptococcus* were markedly present in the aerobic culture. The main species belonging to the *Enterobacteriaceae* which were isolated was *Escherichia coli* (Otero et al., 2000). Sexual maturity and copulation were found to impact on the bacterial community of heifers and cows. Low rates of bacterial isolation in heifers suggest that sexual maturity and copulation are important contributors to the bacterial flora of the genitalia.

Husted (2003) described the vaginal microbiota in healthy animals, compared with those suffering from vaginitis, using a culture-based methodology. Culture media for aerobic, anaerobic, microaerophilic, *Mycoplasma* and *Ureaplasma* were used. Common isolates of both groups included: *Acinetobacter lwoffii* (*Proteobacteria*), *Trueperella pyogenes* (*Actinobacteria*), *Escherichia coli* (*Proteobacteria*),

Corynebacterium spp. (Actinobacteria), *Streptococcus spp.* (Firmicutes) and *Staphylococcus spp.* (Firmicutes). Animals with vaginitis had more isolates than healthy animals in both aerobic and anaerobic culture. Anaerobic culture revealed *Peptostreptococcus spp.*, *Prevotella spp.*, *Fusobacterium spp.*, and *Clostridium perfringens*. *Ureaplasma* and *Mycoplasma* were not detected in either group.

Zambrano-Nava et al. (2011) described the vaginal bacterial microbiota in Criollo Limonero cows. Samples from 51 healthy cows were collected for the culture of aerobic and anaerobic organisms. The observed results showed some similarities with the other studies already mentioned. The predominant bacteria isolated were *Trueperella pyogenes*, *Staphylococcus aureus*, *Staphylococcus coagulase-negative*, *Bacteroides spp.*, *Peptostreptococcus* and *Erysipelothrix rhusiopathiae*. The authors also reported 46.1 percent of the samples did not have bacterial growth. Of those with bacterial growth, 58.18 percent of isolates were anaerobic bacteria, while 41.82 percent were aerobic.

These papers reveal the diversity of bacterial species existent in the bovine vagina, identified through the traditional culturing method. Sample collection from animals with different health statuses and from different breeds, nutrition, reproductive stages, ages and environmental factors could potentially have contributed to this diversity. Despite the differences between the vaginal bacterial compositions observed, some genera appeared to be isolated more commonly, such as *Staphylococcus spp.*, *Streptococcus spp.*, *Arcanobacterium*, *Bacillus spp.*, *Micrococcus spp.*, and *Enterococcus spp.*.

Though many studies on the bovine vaginal microbiota were based on the traditional bacterial culturing method, culture-independent methods relying on DNA analysis are increasingly being used. Santos et al. (2011) pioneered the investigation of the bovine post-partum uterine microbiota through the use of metagenomics. In their work, the microbiota of healthy and metritic cows from two different farms of origin were described. Five cows from each group and from each farm made up a total of 20 animals evaluated. However, the uterine samples were pooled according to their grouping, resulting in 4 samples explored using 16S rRNA group-specific primers. The bacterial flora identified showed similarities between samples from cows presenting the same uterine status but from different environments. The healthy uteri of cows 10 days post-partum presented organisms belonging mostly to the phyla *Proteobacteria* (Gammaproteobacteria) and *Tenericutes* (*Mycoplasma* and *Ureaplasma*). The metritic cows showed a high number of *Fusobacteria*, followed by *Bacteroidetes* and *Proteobacteria*. Among the organisms identified in the healthy uteri were *Mannheimia varigena* and *Pasteurella hemolytica* in the phylum *Proteobacteria*, as well as *Ureaplasma diversum* from the genus *Mycoplasma* (phylum *Tenericutes*). The authors highlighted the absence of the organisms *E. coli* and *Arcanobacterium pyogenes* in any of the healthy or diseased animals, as they are pathogens known for causing post-partum metritis.

In the subsequent year, Santos and Bicalho (2012) used DGGE (Denaturing Gradient Gel Electrophoresis) and DNA pyrosequencing of the V1-V2 regions of the 16S rRNA gene to study the post-partum uterine bacterial microbiome. Uterine fluid was collected from dairy cows from the same farm at three different times after calving, namely 1-3 days, 8-10 days and 34-36 days, and with different health statuses, namely healthy cows (n=4), metritic (n=4), metritic followed by endometritis (n=4) and endometritis (n=4). A dramatic shift in the microbiota was observed over the three sampling intervals within the same group, which demonstrated the microbial community dynamics. In terms of the bacterial phyla, regardless of the health status, most of the sequences belonged to: *Bacteroidetes*, *Fusobacteria*, *Firmicutes*, *Proteobacteria* and *Tenericutes*. The authors once again re-emphasized the low detection of the organisms *E. coli* and *Arcanobacterium pyogenes*.

Machado et al. (2012) performed metagenomic pyrosequencing of the V1-V2 region of the 16S rRNA gene for the investigation of the bacterial diversity of the uteri of cows 35 days post-partum. Bacterial communities were appraised using uterine lavage sample scores, metric and non-metritic cows, retained placenta and those without retained placenta, as well as trace mineral supplementation in the pregnant females. The authors did not describe age, parity, and individual findings. Bacteria of the phyla *Firmicutes*, *Bacteroidetes*, *Proteobacteria*, *Tenericutes*, *Fusobacteria*, *Actinobacteria* and *Spirochaetes* were found throughout the groups analysed.

Contrasting to the works of the previous authors on uterine flora, Swartz et al. (2014) reported the vaginal bacterial microbiota of cows (n=20) and ewes (n=20). The 16S rRNA gene (regions V3-V4) was sequenced with an Illumina MiSeq sequencer. The cows were of variable pregnancy status. Bacterial diversity did not differ among age groups, reproductive status and species. The bovine bacterial communities were dominated by *Proteobacteria* (mostly *gamma-proteobacteria*), *Bacteroidetes*, *Fusobacteria*, *Actinobacteria* and *Tenericutes* phyla. At genus level, the major genera in cow samples were *Aggregatibacter spp*, *Streptobacillus spp*, *Phocoenobacter spp*, *Sediminicola spp*, and *Sporobacter spp*. Bacteria detected in similar culture-based studies were identified; however, in very low abundance. The study emphasised the high abundance of genera encountered using metagenomics when compared to the culture-based studies.

Studies utilising culture-independent methods to study the vaginal microbiota as it is affected by the hormonal status of the animal in different stages of the oestrus cycle are lacking.

2.4 Metagenomics circumventing culture biases

Most microorganisms cannot be grown effectively in pure culture. In the mid-1980s, a few scientists studying uncultured microorganisms persisted with the suggestion that culture did not capture the full spectrum of microbial diversity (Handelsman, 2004). In 1985, direct analyses of the 5S and 16S rRNA genes were used to describe the diversity of organisms in an environmental sample relying on direct

sequencing of RNA or sequencing of reverse transcription-generated DNA copies (Stahl et al., 1985). The terminology allocated to genomic analysis of a population of microorganisms, i.e. “Metagenomics”, also referred to as “Community Genomics”, first appeared in a publication in 1998. It was then described as the study of genetic material recovered directly from environmental samples. More recently, the term Metagenomics has been defined as: “The application of modern genomics techniques to the study of communities of microbial organisms directly in their natural environments, bypassing the need for isolation and lab cultivation of individual species” (Chen and Pachter, 2005).

2.4.1 16S rRNA usage in bacterial identification

The 16S rRNA (16S ribosomal RNA gene) is extensively used for the reconstruction of environmental bacterial phylogenies because of its slow rate of evolution and its presence in all bacteria (i.e. it is part of a section of circular prokaryotic DNA found in all bacteria and archaea). Additionally, its relatively short size of 1.5 kb makes it cheaper to sequence than many other unique bacterial genes (Greengenes, 2014). The 16S rRNA gene codes for rRNA, which in turn makes up part of the ribosome. The ribosome is composed of two subunits, the large subunit (LSU) and the small subunit (SSU). These two subunits sandwich the mRNA (messenger RNA) as it feeds through the ribosome during translation. In bacteria, while there are other associated proteins which help to make up the functional units of the ribosome, generally the SSU is coded for by the 16S rRNA gene, and the LSU is coded for by the 23S and 5S rRNA genes (Greengenes, 2014).

The DNA that codes for the ribosome, eg 5S, 16S and 23S rRNA, has been mostly conserved due to its important function in translating mRNA into proteins, and therefore life. But even within this gene, there are parts that have been conserved more than others. This is due to the structure of the ribosome itself. The way that the ribosome folds – creating bonds with itself in some places (conserved regions) while other portions are looped and unbounded (hyper variable regions), such that the degree to which any portion of the gene is subject to mutation – varies (Greengenes, 2014). Although the absolute rate of change in the 16S rRNA gene sequence is not known, it does mark evolutionary distance and relatedness of organisms and can therefore be used in species identification (Clarridge, 2004).

The 16S gene contains nine hyper-variable regions, i.e. V1 to V9, which can be targeted with different primer sets. These shorter regions are used by next-generation sequencing platforms in metagenome studies. However, the specific region to sequence is still the subject of debate, since the choice among the regions of the 16S gene may vary according to the taxa under investigation, experimental objectives, published and unpublished recommendations and/or experimental familiarities with a certain region in a particular author’s laboratory (Claesson et al., 2010). The attempt to determine which region of the gene is most used in studies related to the vaginal bacterial flora revealed a variety of choices by different authors (Santos and Bicalho, 2012, Hummelen et al., 2010, Huse et al., 2008). In the present

experiment, we elected to sequence the V3-V4 variable regions of the 16S rRNA gene, as it is adequate for identification of the expected organisms in the vaginal microbiota at family and genus level (Swartz et al., 2014).

3 Aims and hypothesis

The hypothesis for this study was as follows: *Tritrichomonas foetus* causes alterations to the normal vaginal bacterial flora in female cattle in the process of colonization as part of successfully establishing an infection. This study thus sought to prove that conjecture.

3.1 Introduction

Tritrichomonas foetus causes damage to the bovine female reproductive tract and extensive economic losses to farmers due to early foetal death and infertility. The vaginal bacterial microbiota has an important role in protecting the mucosa and maintaining a healthy environment through habitat competition. With this in mind, the null hypothesis of this project was that the bovine microbiota does not differ depending on the stage of the reproductive cycle and that *Tritrichomonas foetus* does not affect the vaginal bacterial microbiota of heifers.

3.2 Aims of the study

1. To describe the composition of the bovine vaginal bacterial microbiome using 16S rRNA metagenomics.
2. To study the changes in the microbiome between oestrus and dioestrus.
3. To describe the impact of *Tritrichomonas foetus* infection on the vaginal bacterial microbiota during the period of the establishment of infection.

3.3 Expected benefits arising from the work

This work aimed to contribute scientifically by exploring two avenues of research in the field of *Tritrichomonas foetus* pathogenesis and its influence on the vaginal microbiota of heifers. Firstly, with the application of metagenomics, this project gives a description of the vaginal bacterial microbiota of heifers, which provides insight into the bacterial communities present in the vagina and expands what was previously described by the traditional method of culturing. The investigation of the vaginal bacterial microbiota of control heifers during the follicular and luteal stages of the reproductive cycle, enables an assessment of the influence imposed by the sex hormones on the vaginal bacterial population. The second and novel contribution from the project is an improved understanding of how the parasite *Tritrichomonas foetus* influences the vaginal bacterial microbiota as colonization progresses. This will give insight into the *T. foetus* pathogenesis in terms of how it affects or is affected by the host bacterial microbiota, which is believed to offer protection to the vaginal tract epithelium. The identification of a bacterial component of the microbiota associated with the protozoa or with the host immunity could possibly provide information which may in time contribute to the development of preventive measures.

4 Materials and methods

4.1 Experimental design overview

A control-cohort study was designed to test the influence of *Tritrichomonas foetus* on the vaginal bacterial flora during the early stages of infection. The natural process was simulated *in vivo* by infecting heifers during oestrus. Animals were allocated into two groups: an uninfected (control) group and an infected (experimental) group, with each group consisting of three animals (after a process of random selection from a larger group of participants). The vaginal bacterial microbiome was studied using the V3-V4 region of the universal 16S ribosomal RNA (rRNA) gene. The control group was designed to provide a comparative dataset to demonstrate the bacterial microbiome at different stages of the reproductive cycle as a point of comparison with that of the infected heifers at the same stages of the cycle.

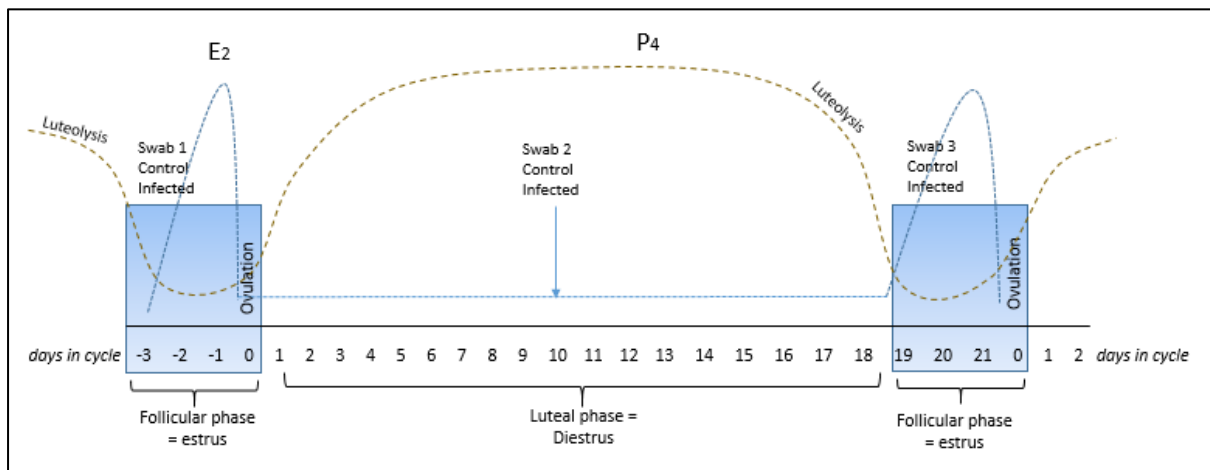


Figure 2: Experiment in relation to bovine reproductive cycle, adapted from Senger (1997).

The figure presents the bovine oestrus cycle relative to when the swabs were collected in the infected and control groups.

4.2 Ethical approvals for the study

Approval was obtained prior to the execution of the experiment from the following authorities:

1. The South African Government through The Department of Agriculture, Forestry and Fisheries (DAFF). Permission was provided for the project under section 20 of the Animal Disease Act, 1984;
2. The University of Pretoria's Animal Ethics Committee as the host organisation for the work, and
3. The Taaiboschbult feedlot consented for the procedures to be performed. The consent was obtained under the authority of the Animal Use and Care Committee (AUCC).

4.3 Animal origin, selection and maintenance

The Taaiboschbult feedlot, located near the town of Potchefstroom (North West Province, South Africa), made 50 Bonsmara heifers from the same origin available for the experiment. It was a prerequisite for selection that all females were clinically healthy, virgin and reproductively cyclic. The animals were kept in the same feedlot pen, but separated from other animals, for the duration of the experiment. Considering the many opportunities for attrition, the project started with 50 animals and followed a course in which animals that did not meet the specified criteria at each step (Figure 3: Experimental layout – Field work) were excluded.

4.4 Section A: Field work

4.4.1 Control and infected group treatments: an overview

Trichomonosis is transmitted mainly during natural mating. To simulate the natural mechanism of infection, oestrus was induced through two injections of 500 mcg of cloprostenol (Prostaglandin, Estrumate – MSD Animal Health), administered intramuscularly, 12 days apart (refer to Figure 3 for the experimental layout and activities). Four days later, which was designated as “Day 1”, the females which were confirmed to be in the follicular phase of their cycle by rectal and ultrasonographic examination, were selected and allocated randomly into the two different groups: the infected group and the uninfected control group.

The project began with twenty nine (29) heifers that satisfied the selection prerequisites and were found to be in the follicular phase, with 10 allocated to the uninfected control group and 19 to the infected group (refer to Figure 3 at 06-March-15 “Day1”). A vaginal sample was collected from both groups as stated in section 4.4.3 Sampling method. *Tritrichomonas foetus* were cultured as described in section 4.4.2 *Trichomonas foetus* culturing for *in vivo* infection. Females in the infected group were inoculated intra-vaginally with 13.6×10^6 *T. foetus* organisms, an adequate concentration to induce infection (BonDurant et al., 1993), diluted in 1 ml *modified Diamond's medium* (Onderstepoort Bacteriology Laboratory), while the control group received the same volume of the pure medium excluding the organism. *Modified Diamond's medium* is used for clinical diagnosis of Trichomonads organisms. The composition is enhanced to optimize protozoal survival and replication. The composition of *modified Diamond's medium* is as follows: Casein peptone 24g; Yeast extract 12g; Maltose 6g; Streptomycin 1.5g; Cysteine L-hydrochloride 1.2g; L-Ascorbic acid 0.24g; Amphotericin B 2mg; Penicillin 1.000.000U; Horse serum 120ml and demineralized water (900ml).

A second swab collection was performed ten days later on “Day 11” (Figures 2 and 3) after the heifers were confirmed to be in dioestrus by means of transrectal palpation and ultrasound examination. This date was determined based on the research in which Hammond (1945), demonstrated a significant

parasite proliferation approximately 10 to 12 days post-infection. The third and final swab collection was performed a further ten days later on “Day 21” when the animals were expected to be in the follicular phase. Females that were found not to be in the expected reproductive stage were eliminated.

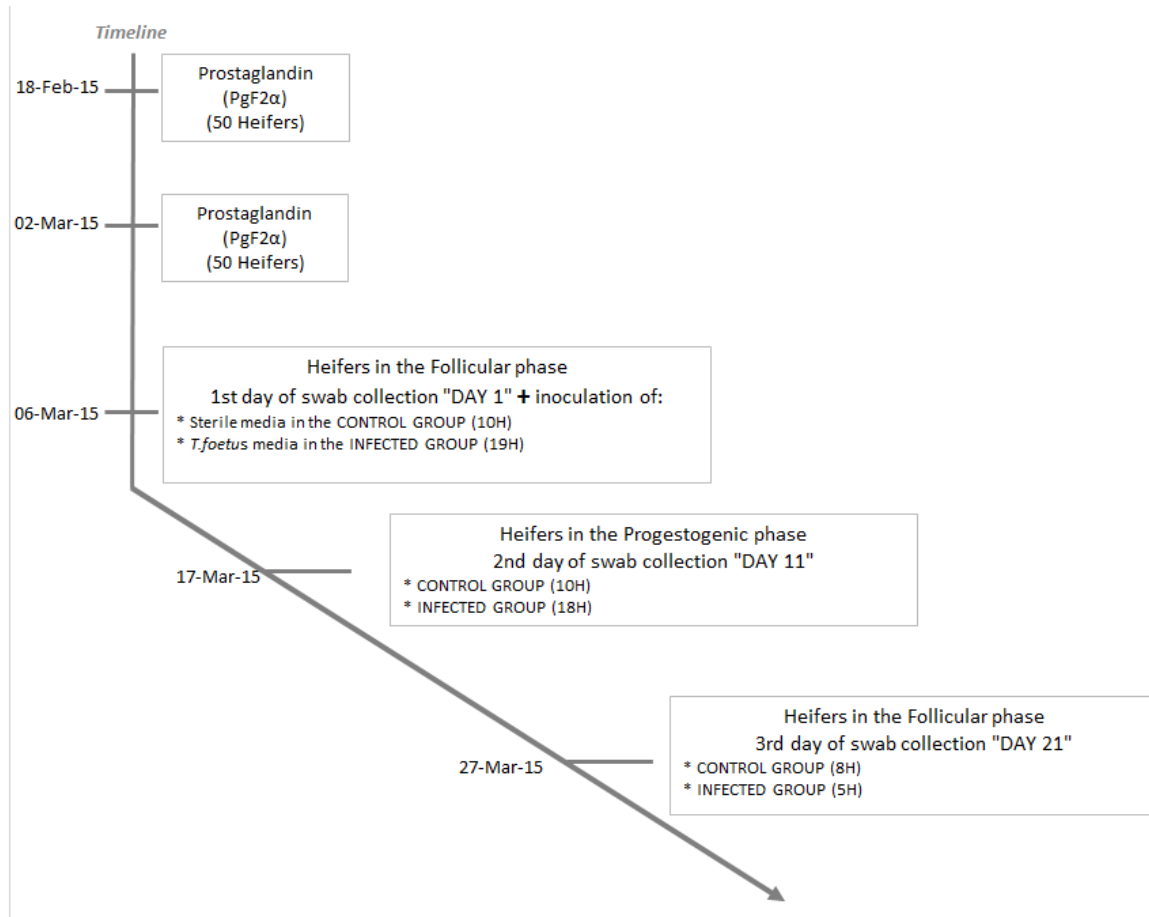


Figure 3: Experimental layout – Fieldwork.

The figure shows the timeline of the fieldwork carried out during the experiment, with two prostaglandin injections to synchronise cycling, followed by three sample collections.

4.4.2 *Tritrichomonas foetus* culturing for *in vivo* infection

A field strain of *Tritrichomonas foetus* was isolated from diagnostic material from Stellenbosch University by Dr Annelize Jonker (Helderfontein – Provincial Veterinary Laboratory) and kindly provided for this study. The isolate’s identity was confirmed morphologically and cultured as previously reported (BonDurant et al., 1993) for three days in *modified Diamond’s medium* at 37°C until a dose rate of 10⁶ organisms per cow was achieved.

4.4.3 Sampling method

The females selected had a three-week acclimatisation period from the arrival in the feedlot until the first swab collection to allow their vaginal bacterial microbiota to adapt in order to mitigate any environmental influences. The swab used for the sample collection was a guarded endometrial cytobrush designed for equine endometrial samples (Equine guarded endometrial cytobrush, designed

and manufactured by the Section of Reproduction, Department of Production Animal Studies, Faculty of Veterinary Science, Onderstepoort Campus, University of Pretoria). This specialized swab is 72 cm in length, contains a guarded double cytobrush of 2 cm in length, protected by a hard cannula, with the last section covered by a plastic sheath (depicted in Figure 4).



Figure 4: Equine guarded endometrial cytobrush. The swab contained a double cytobrush, guarded by a hard cannula and covered by a plastic sheath.

The vulva was cleaned with a paper towel, and the swab introduced deep into the cranial vagina. The plastic sheath was ruptured and retracted, and then the cytobrush was exposed. Through rectal palpation of the cervix, the double cytobrush was guided into the cervical opening and cervical fornix, and rotated a number of times to ensure a good sample. The double cytobrush was then retracted into the cannula prior to the removal of the swab from the vagina. The swab was handed to the second operator in a field laboratory adjacent to the crush (Figure 5).



Figure 5: Field laboratory for examination and sample collection.

The table pictured shows the equine endometrial guarded swabs, paper towel used to clean the vulva, rectal gloves, swab container prior to animal identification, stethoscope, thermometer used for the clinical examination, a marker, as well as a board with the animal identifications.

4.4.4 Sample treatment

Inside a closed room which served as a field laboratory, an operator separated the two cytobrushes (duplicated samples). One sample was placed in a sterile micro tube for genomic evaluation, and a second sample was kept for either bacterial culture, in the event of the molecular methods failing to yield satisfactory results, or as a possible means of verifying the results (Figure 6). The containers were marked with the cows' numbers and transported to the laboratory on ice.

To determine infection status in the infected group and to exclude cross-contamination between the groups, wet preparations of vaginal secretions were examined microscopically for the presence of *T. foetus* organisms. *Tritrichomonas foetus* infection was diagnosed by means of immediate direct examination using phase contrast microscopy. Once the sample collection was completed, samples were immediately transferred on ice to the University of Pretoria and stored in a freezer at -70°C .

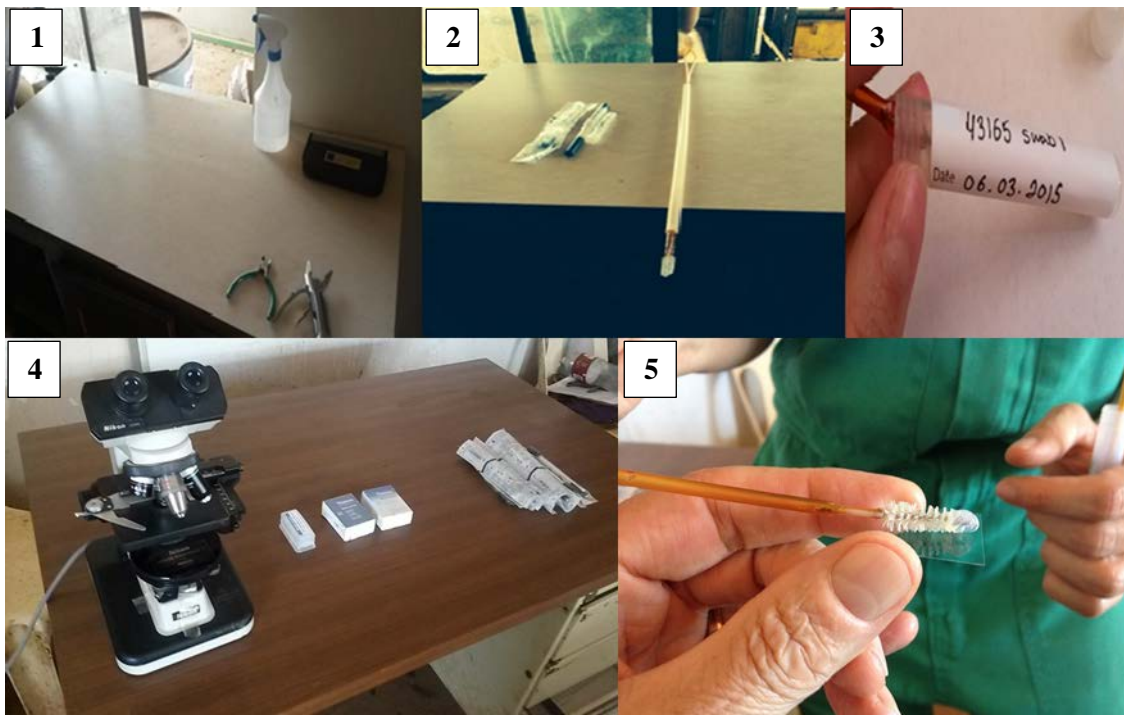


Figure 6: Sample collection and handling setup.

Insert 1: Table to receive the swab; Inserts 2 and 3: Demonstrating the handling of the specimen; Insert 4: Table with microscope for *T. foetus* examination; Insert 5: Preparing the “wet preparation” by touching the swab onto the cover slip and placing it on the slide for *T. foetus* visual confirmation.

All the procedures and processes followed throughout the experiment are presented in Figure 7 in the form of a flow diagram. The flow diagram is divided into three sections A, B and C depicting the progression of the project. Section A refers to the fieldwork which encompassed the processes of preparing the enrolled heifers for the trial, infecting a certain number of them and taking samples from them in oestrus, dioestrus and the following oestrus. This section resulted in three heifers being selected from the healthy animals and three from the infected animals whose samples progressed to the next

section. Section B refers to all the laboratory work conducted on the samples, from extraction of the DNA to sequencing the reads into interpretable data to be fed into the next section. Section C firstly refers to processes applied to the data in order to first clean it for poor quality reads, which was then followed by statistical analysis and presentation of the results in a number of forms (refer to the appropriate sections in the materials and methods for further details).

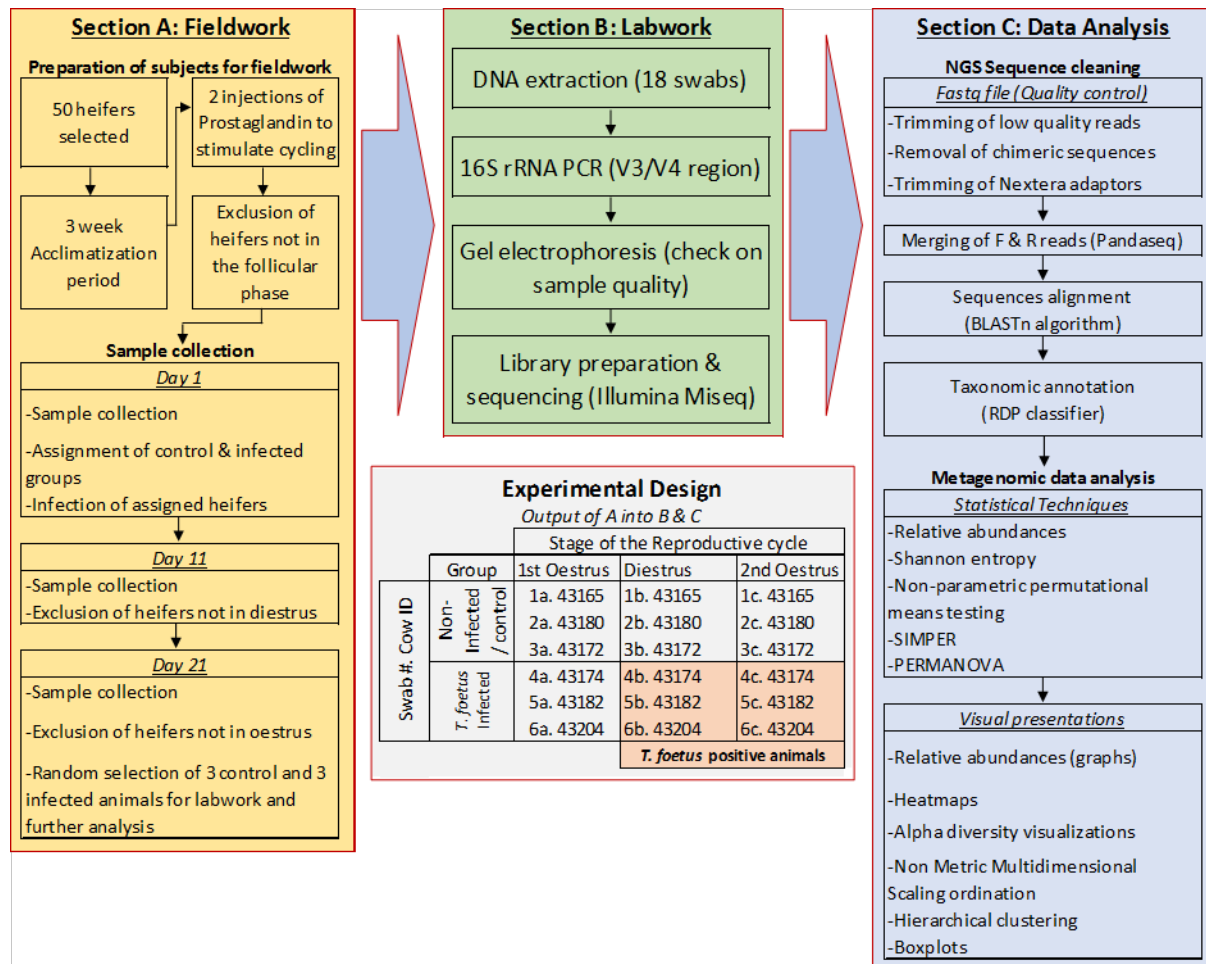


Figure 7: Flow diagram indicating the experimental design and the actions taken during the fieldwork, laboratory work and data analyses sections of the project.

4.5 Section B: Laboratory activities and next generation sequencing

The vaginal swabs collected in the field were used in a number of laboratory procedures to prepare the DNA of the bacteria prior to sequencing. The laboratory procedures performed were as follows:

4.5.1 DNA extraction from vaginal swabs

DNA was extracted from the raw sample without centrifugation, which was possible because the samples were collected with dry swabs. The metagenomic DNA was extracted from the 18 swabs (Figure 7 - Section B) with the *QIAamp DNA mini kit* (QIAGEN, 2016) as stipulated by the manufacturer's protocol for DNA purification from tissues. However, some modifications were required to deal with the thick mucoid nature of samples. In short, the following protocol and modifications were followed: Samples were allowed to thaw at room temperature for 20 minutes prior to extraction. The swabs were placed in a collection tube and soaked with 180µl of lysozyme at 20mg.ml⁻¹ (Fluka lysozyme L6876, Sigma Aldrich, Belgium) for 30 minutes at 37°C. Subsequently, 20µl of proteinase K (QIAGEN) and 200µl of Buffer AL were added, mixed by vortexing, and incubated for 1 hour at 56°C, followed by 5 minutes at 95°C. The extraction protocol was then followed by an addition of 200µl of ethanol (96 to 10 percent) and mixing by pulse vortexing. The mixture was placed into a mini spin column and centrifuged at 6000 × g for 1 minute. After centrifugation, the spin column was placed in a 2ml collection tube and 500µl of Buffer AW1 was added which was then followed by another centrifugation step of 6000 × g for 1 minute. Subsequently, 500µl of Buffer AW2 was added and centrifugation repeated at full speed for 3 minutes. The spin column was placed in a new micro centrifuge tube to elute the DNA with 100µl of Buffer AE. The buffer was added and left for incubation for 5 minutes prior to a 1 minute centrifugation step at 6000 × g. Nucleic acid yields were quantified in each sample to assess DNA concentration and purity, using a spectrophotometer (NanoDrop 2000 full-spectrum, UV-Vis micro-volume spectrophotometer, Thermo Scientific™).

4.5.2 16S rRNA amplification

The bacterial diversity was determined in each sample by targeting a region of the 16S ribosomal RNA gene with a polymerase chain reaction (PCR). The *Illumina Metabarcoding Protocol* was followed for 16S rRNA metabarcoding (Illumina). In short, the forward primer (TCG TCG GCA GCG TCA GAT GTG TAT AAG AGA CAG CCT ACG GGN GGC WGC) and the reverse primer (GTC TCG TGG GCT CGG AGA TGT GTA TAA GAG ACA GGA CTA CHV GGG TAT CTA ATC C AG) were chosen to amplify the V3 and V4 hypervariable regions of the 16S rRNA gene. A targeted PCR was performed according to the protocol, namely with primers at a final concentration of 1µM, 1× KAPA Hifi Hotstart Ready Mix (KAPA, Roche), and 50ng DNA in a 25µl final volume. The PCRs were performed in a thermal cycler (Verti™ Thermal Cyclers, Thermo Fisher Scientific) with an initial denaturation step at 95°C for 3 min, followed by 25 cycles of denaturation at 95°C for 30 seconds,

annealing at 55°C for 30 seconds and extension at 72°C for 30 seconds, with a final extension at 72°C for 5 min before samples were kept at 4°C until removed.

Amplicons, with an expected size of approximately 460 base pairs, were visualized by electrophoresis using a 2 percent agarose in TAE buffer (Tris base 242g, acetic acid 57.1ml and 100ml of 500mM EDTA) gel stained with ethidium bromide. All PCRs were run with a negative control to which no DNA was added and a positive control containing pure cultured bacteria (*E. coli*). This was done to ensure that there was no contamination in the PCR reaction mixes used that may have resulted in false positives, i.e. the negative controls have no bands when uncontaminated, and that the PCR reagents worked, i.e. the expected band size in the positive control reactions was observed. The positive control ensured that any PCR related problems could be identified, for example, missing components in the reaction mixture, PCR machine cycling problems, primer dimers, etc. These measures ensure that sample-specific problems could also be identified.

4.5.3 Metagenomic libraries construction and next generation sequencing

The PCR products free from contamination and of the appropriate size were submitted for next generation sequencing (NGS) at the ARC's (Agriculture Research Council's) Biotechnology Platform (Onderstepoort, Pretoria). Prior to library construction, a PCR clean-up (Agencourt Ampure magnetic beads, Beckman Coulter) was performed to purify the 16S V3/V4 amplicons by removing the free primers, any primers dimers, remaining enzymes and other impurities. The NGS facility performed the library indexing and sample preparation prior to sequencing on an Illumina MiSeq. In short, cleaned amplicons were PCR-indexed with Illumina-specific sequencing adapter indexes from the Nextera XT Index kit (Illumina). The procedure was in accordance with the manufacturer's recommendations. Samples were quality checked (Qubit, Life Technologies), normalised and pooled prior to NGS. The NGS reactions were performed on the Illumina MiSeq platform using the 300 bp paired-end (PE) sequencing kit v3 (Illumina).

4.6 Section C: Sequence and data analyses

4.6.1 NGS sequence cleaning prior to analyses

The MiSeq run was demultiplexed automatically using the MiSeq Reporter software on the instrument. The ARC's Biotechnology Platform Sequencing Core facility provided both the paired-end reads from the Illumina MiSeq runs as two fastq files (forward and reverse reads). The sequences were uploaded into CLC Genomic Workbench version 8.5.1 (Inqaba Biotech) as paired-end reads for sequence summary and removal of low quality reads. The *Trimmomatic* (Bolger et al., 2014) tool was also used for quality assessment of the datasets, with all datasets and reads assessed and purified according to the following criteria: All low quality reads with a Phred score of ≤ 20 were removed; Any Nextera adapters sets added during library preparation were removed; Any chimeric sequences were removed. All the reads were subsequently merged using *PANDAseq* (Masella et al., 2012) prior to further analysis. The merged reads were aligned against the sequences database using the BLASTn algorithm NCBI (1988). The resulting alignments were clustered to generate operational taxonomic units (OTUs) using the RDP cluster algorithm (Cole et al., 2014). Genus-level taxonomic assignments were performed using the RDP classifier (Wang, 2007). Sequences were also uploaded in the MG RAST server (Meyer et al., 2008) for analyses and to demonstrate the consistency of the analysis, i.e. no bias was introduced by the analysis pipeline (Meyer et al., 2008). For each sequence annotation, a confidence score was assigned. The output tab-delimited files were processed using Microsoft Excel (Microsoft, 2013).

4.6.2 Metagenomic data analyses of 16S rRNA samples

The methodology used in this work follows the methodologies adopted in similar metagenomic studies (Swartz et al., 2014, Laguardia-Nascimento et al., 2015, Moore et al., 2017). Analyses of the cleaned metagenomic sequencing data was separated into two sections: the first being concerned with the analysis of the bacterial microbiota of healthy oestrous versus dioestrous animals, and the second focussing on the healthy versus *T. foetus*-infected animals.

The first section compares the samples collected from animals in a healthy status, grouped by reproductive cycle stage of the animals from which the samples were drawn. In total, 12 samples from such animals were collected, with 3 being dioestrus samples that were grouped and compared to the remaining 9, oestrus samples.

The second section compares the samples taken from un-infected animals with those of *T. foetus*-infected animals. Briefly, the 12 samples from the first section of the experiment are grouped and compared with the remaining 6 samples from the *T. foetus*-infected animals.

All analyses were based on the abundances gleaned from the sequencing data, which were normalised to a percentage scale to allow direct comparisons and confined to taxonomic classifications which were

obtained at confidence scores of 80 percent or above. Both sections are explored in the same fashion via the following methodology (unless otherwise indicated):

4.6.2.1 Relative abundance

Simple graphical presentation of the percentage abundances at the phylum and family taxonomic level were first provided as a means of visually appraising the data via Microsoft Excel (Microsoft, 2013). These presentations were further supported by a heatmap of the most prevalent families observed in each sample, at the family level.

4.6.2.2 Alpha diversity

Alpha diversity is defined as the diversity inherent to a particular sample (within-sample diversity) and can be expressed with a number of metrics, the most popular being Shannon entropy (also known as the Shannon-Weaver index), which was developed in the sphere of information theory during the latter half of the 20th century and has since been applied to many fields, including ecology and microbiology (Jost, 2006).

Shannon Entropy is expressed as follows:

$$H' = - \sum_1^i p_i \ln(p_i)$$

Where:

H' is the Shannon Entropy

p_i is the i^{th} proportional abundance of the Operational Taxonomic Unit (OTU) within the sample.

$\ln(p_i)$ is the natural logarithm of the i^{th} proportional abundance of the OTU within the sample.

H' thus weights the proportional abundances of each OTU according to their respective proportional contribution to the sample's composition as a whole and is generally preferred as the weightings are intuitively unbiased. The resulting statistic generally ranges from between 1 and 4, with higher figures indicating greater diversity and lower ones indicating less diversity.

Alpha diversity was thus calculated using the Shannon Entropy and graphically contrasted to the family-level richness (number of families counted) as well as the count of the number of families observed per sample with abundances of greater than 1 percent. The statistics were ranked and presented together in tabular format for visual appraisal. The relationship between the calculated Shannon Entropies and the factor being analysed was then statistically evaluated using a non-parametric permutational pseudo t-test with p-values being evaluated at the 0.05 level.

A non-parametric permutational t-test negates many issues related to the parametric t-test. Put simply, p-values are derived from the comparison of the given test statistic (arising from the means of the grouped samples) with a null distribution which is generated by a process of repeated randomization (N times) of the underlying data across the grouping categories (for example, dioestrus vs. oestrus), each time calculating the means of each new randomized grouping of samples. These repeated randomizations enable the construction of a distribution of all possible test statistics derived from N combinations of the underlying data. These test statistics are then ordered from smallest to largest. In a one-tailed test, if the original test statistic falls in the top 5 percent of the distribution (when statistical significance is assessed at that level), then it is indicative of a statistically significant difference between the means of the two original groupings. Thus, distributional issues encountered in parametric statistical tests are largely obviated, and p-values derived are significantly more robust. However, it must be noted that the reliability of such values is enhanced by three factors, namely the size of the sample, the number of permutations performed (10 000 or more is recommended) and the variance of the sample groupings, which should be close to equal.

4.6.2.3 Beta diversity

Beta diversity in microbiology is defined as the between-sample (or group) difference in diversity that exists in a bacterial population, taking into consideration shared taxa across all samples as well as their relative abundances. Statistical analysis of beta diversity enables the determination of the true distinctness of samples or groups of samples (Jost, 2007).

This work relies on the Bray-Curtis similarity statistic (Bray and Curtis, 1957) to assess the distinctness of the bacterial microbiota of the heifers grouped by factor using firstly ordination and clustering techniques to visualise the relatedness/similarity of the individual heifers before testing the *a priori* groupings via means of the non-parametric test PERmutational Multiple ANalysis Of VAriance (PERMANOVA).

For a two-sample comparison, the Bray-Curtis (BC_{ij}) similarity is defined as:

$$BC_{ij} = \frac{2C_{ij}}{S_i + S_j}$$

Where:

$2C_{ij}$ is the sum of the lesser abundances of shared members of a specific OTU, multiplied by 2;

S_i is the sum of abundances for sample i while S_j is the sum of abundances for sample j;

BC_{ij} thus ranges from 0 to 1 with values closer to 1 indicating similarity and values closer to 0 indicating dissimilarity.

The choice of the Bray-Curtis similarity statistic takes into consideration the abundance of each particular member of a specific OTU level in an unbiased and simple-to-understand manner and is therefore one of the most widely-used statistics in the analysis of beta diversity.

4.6.2.3.1 Visual evaluation (clustering and ordination)

In order to explore the relatedness of the samples, percentage abundance data of all samples at the family-level designation were fed into PAST 3.16 (Hammer et al., 2001). Non-metric Multi-Dimensional Scaling (NMDS) was then performed on the Bray-Curtis similarities inherent to each pair of samples with the programme automatically detecting and representing these in a two-dimensional space, relative to each other. Following NMDS, clustering of the Bray-Curtis similarities was performed using average linkage hierarchical clustering to clarify or enhance the results seen in the NMDS.

The NMDS procedure ranks each set of similarities and groups samples with similar ranked similarities relative to all other samples together while those which are more dissimilar are separate. The axes on which the groupings are plotted are not meaningful for interpretation purposes as they are derived from the ranks of each similarity calculated relative to others and not their actual values. However, the groupings obtained are a very useful and visual means of determining the similarity of each sample relative to the others.

The clustering average linkage procedure groups samples in pairs by their Bray-Curtis similarities. It then performs the procedure in iterations, re-computing similarities between paired groups, and in the process creating clusters of pairs, until all possible groupings have been assessed (Buttigieg and Ramette, 2014). This procedure is supported by Bootstrapping and was duly performed at N=9999. Both procedures enable a robust visual comparison of similarity-based groupings with *a priori* groupings.

4.6.2.3.2 Non-parametric statistical evaluation

Following the visual presentation, differences in the bacterial composition of the grouped samples were assessed through a non-parametric permutational statistical procedure. PERmutational Multivariate ANalysis Of VAriance (PERMANOVA) analyses the raw similarities calculated and calculates a test statistic based on the variances of the similarities within and between groups of samples. The procedure was assessed against a null distribution permuted 9999 times. The p-values derived from the procedures are exact permutational p-values and were all assessed at the 0.05 level of significance.

4.6.2.3.2.1 Beta diversity statistical procedures specific to the oestrus vs. dioestrus section

The samples were grouped according to when they were taken, by group of heifers (those which were to be infected with *T. foetus* and those which would be controls in that section of the project) and were

assessed using PERMANOVA under the null hypothesis that there are no differences in the mean relative abundances of OTUs at the family level between the four sample groups. Post-hoc pairwise analysis of the PERMANOVA results was also performed to identify from where any significant differences arose.

Following these assessments by sample group, the PERMANOVA procedure was applied to compare the three dioestrus samples with the nine oestrus samples, under the null hypothesis that there are no differences in the mean relative abundances of OTUs at the family level between the two sample groups.

4.6.2.3.2.2 Beta diversity statistical procedures specific to the healthy versus *T. foetus*-infected section PERMANOVA was used to compare the 12 non-infected samples with the six *T. foetus*-infected samples under the null hypothesis that there are no differences in the mean relative abundances of OTUs at the family level between the two sample groups.

4.6.2.4 Treatment effects at the bacterial family level

Families which contribute the most to the dissimilarity observed between the *a priori* sample groupings were identified via the SIMilarity PERcentage (SIMPER) procedure on the basis of their Bray-Curtis dissimilarities². SIMPER determines the relative contribution of each taxon to the overall dissimilarity observed between groups of samples, ordered from the taxon with the greatest contribution to that with the smallest contribution (Buttigieg and Ramette, 2014). Those which contributed 1 percent or more to the total dissimilarity observed were assessed for statistically identifiable relationships with the factor concerned, via the same method used to assess alpha diversity (a non-parametric permutational pseudo t-test). The inputs of the test are the relative abundances observed for each family across the sample groups.

Statistical analyses, as well as the provision of the heatmaps and the visual appraisals of similarity, were conducted using PAST (Hammer et al., 2001).

² The converse of B-C similarity (1 – B-C similarity)

5 Results arising from laboratory procedures

This section documents the outcomes of the processes described in Section B of Materials and Methods (Figure 7), which includes the results of the DNA extraction, PCR gel electrophoresis, data cleaning and preparation before analysis.

5.1 DNA extraction

The DNA concentration was determined by measuring the absorbance at 260nm (A_{260}). DNA purity was determined by calculating the ratio of absorbance at 260nm (A_{260}) to that at 280nm (A_{280}). A ratio of ~1.8 is generally accepted as “pure” for DNA (ThermoScientific, 2012). The samples with their DNA concentrations and purity are given in the Table 1.

Table 1: Samples used in this work, with their DNA concentration and purity indicated.

#	Heifer ID	Nucleic acid		A_{260}	A_{280}	A_{260}/A_{280}
		concentration	Unit			
1a	43165 sw1	105.6	ng/ μ l	na ¹	na ¹	na ¹
1b	43165 sw2	41.7	ng/ μ l	0.834	0.45	1.86
1c	43165 sw3	14.2	ng/ μ l	0.284	0.17	1.63
2a	43172 sw1	109.7	ng/ μ l	2.195	1.15	1.92
2b	43172 sw2	50.5	ng/ μ l	1.011	0.6	1.7
2c	43172 sw3	39.1	ng/ μ l	0.782	0.42	1.84
3a	43180 sw1	129.8	ng/ μ l	2.596	1.39	1.87
3b	43180 sw2	44.8	ng/ μ l	0.896	0.48	1.86
3c	43180 sw3	293.5	ng/ μ l	5.87	3.09	1.9
4a	43182 sw1	44	ng/ μ l	na ¹	na ¹	na ¹
4b	43182 sw2	145.4	ng/ μ l	2.909	1.58	1.84
4c	43182 sw3	266.4	ng/ μ l	5.327	2.87	1.85
5a	43204 sw1	60	ng/ μ l	na ¹	na ¹	na ¹
5b	43204 sw2	63.1	ng/ μ l	1.262	0.67	1.9
5c	43204 sw3	36.2	ng/ μ l	0.724	0.41	1.77
6a	43172 sw1	32	ng/ μ l	0.799	0.46	1.74
6b	43172 sw2	118.5	ng/ μ l	2.37	1.2	1.97
6c	43172 sw3	56	ng/ μ l	1.119	0.66	1.7

na¹–“Not available” due to a mechanical failure of the instrument.

5.2 PCR gel electrophoresis

Gel electrophoresis were performed to visualize the PCR amplicons produced from samples for the V3 and V4 region of the 16S rRNA gene. Figure 8 illustrates a typical PCR reaction, with negative controls (NC), positive control (PC) and nine samples on the gel image. A marker (100bp maker) was run in lane 1. The amplicon produced had an expected size of approximately 460 base pairs.

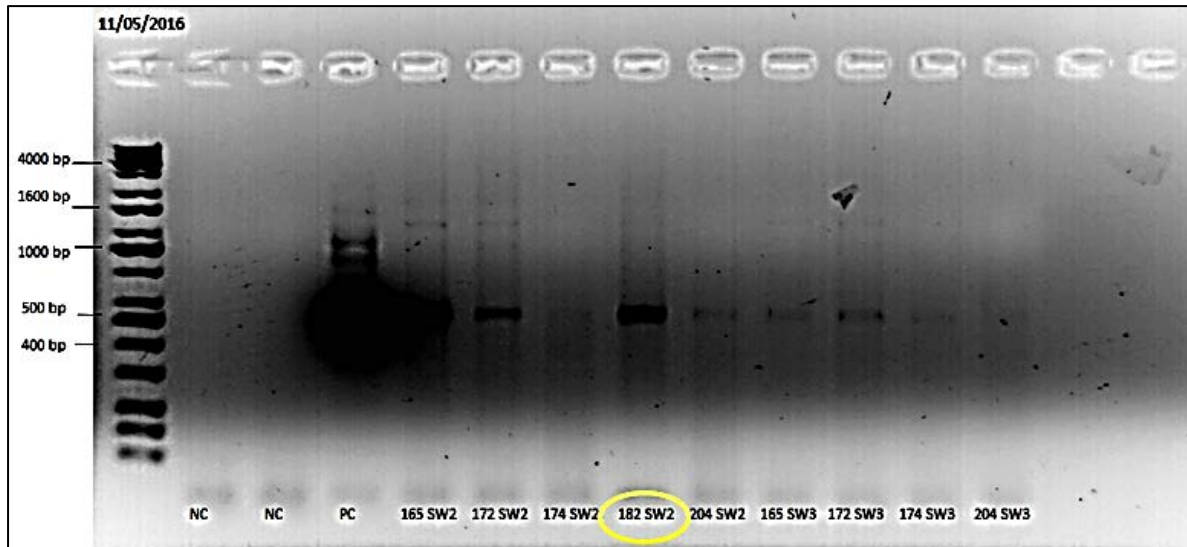


Figure 8: Gel electrophoresis image of nine different samples and their designations.

Using a 100bp marker, also with negative controls (NC) and positive control (PC) indicated. The expected V3-V4 amplicon is clearly visible as a band in heifer 182SW2 which represents the sample 4b as indicated in (Table 1).

The strong reaction produced by the positive control was due to it being a bacterial-pure culture. Although some of the bands were weak, the PCR products were used for library preparation and Illumina sequencing.

5.3 Sequencing results

Fastq files produced by the Illumina sequencer were used for generating general sequence statistics prior to being trimmed by CLC Genomics Workbench (version 8.5.1) and Trimmomatic to remove low quality sequences and bases. The number of reads produced in each sample, as well as the reads trimmed at each step of the process and the final number of reads left for further analysis are presented in Table 2.

The total number of sequences obtained from the MiSeq sequencer for all the samples was 5 714 732 (Table 2). Initial trimming left 4 546 030 reads that were further trimmed to improve quality parameters resulting in a final total of 1 237 627 reads for further analysis (Table 2). From that dataset, bacterial and archaeal organisms were identified. The bacterial domain contributed 1 121 216 of the total number of reads, while the archaeal domain contributed 116 411 reads. Figure 9 represents the percentage proportions of organisms of the archaea and bacteria domains across all the samples analyzed.

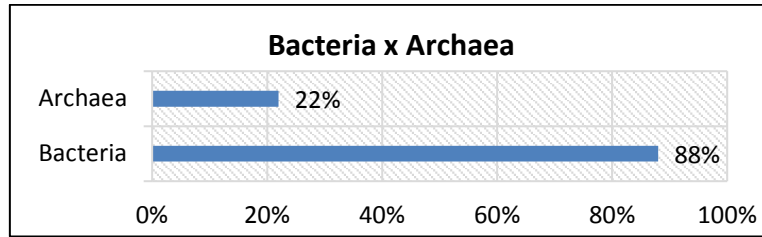


Figure 9: Relative percentage of archaeal and bacterial reads across all the samples.

Most of the archaeal organisms identified by the RDP classifier presented with a confidence score below the cut-off point of 0.8, at the phylum-level designation. Therefore, it was decided not to explore the archaea organisms identified further. The initial 1 121 216 bacterial reads were further reduced to 848 754 reads after the removal of sequences with a confidence score of below 80 percent.

Table 2: Next generation sequencing results presenting the total reads obtained for each sample and how these changed during the course of the trimming and quality check procedures.

<i>Sample ID</i>	Initial trimming (CLC Genomics Workbench)			Secondary trimming (Trimmomatic)¹		Removal of archaea		Removal of reads with confidence score < 0.8²	
	Total reads	Reads trimmed	After trim	Reads trimmed	After trim	Archaea trimmed	Bacteria	Reads trimmed	After trim
<i>1a</i>	290 908	229 704	61 204	48 061	13 143	2 280	10 863	5 196	5 667
<i>2a</i>	684 420	522 866	161 554	137 604	23 950	6 042	17 908	12 860	5 048
<i>3a</i>	272 856	7 576	265 280	176 480	88 800	214	88 586	8 285	80 301
<i>4a</i>	513 684	134 132	379 552	217 855	161 697	3 700	157 997	11 875	146 122
<i>5a</i>	431 446	8 208	423 238	255 233	168 005	4 537	163 468	12 637	150 831
<i>6a</i>	692 308	12 278	680 030	410 423	269 607	5 344	264 263	18 109	246 154
<i>1b</i>	257 670	10 106	247 564	171 509	76 055	3 308	72 747	8 070	64 677
<i>2b</i>	141 100	5 412	135 688	101 223	34 465	2 762	31 703	5 825	25 878
<i>3b</i>	135 506	9 220	126 286	106 073	20 213	3 496	16 717	6 490	10 227
<i>4b</i>	175 400	4 960	170 440	155 126	15 314	3 398	11 916	6 169	5 747
<i>5b</i>	667 862	14 266	653 596	544 745	108 851	32 816	76 035	56 659	19 376
<i>6b</i>	186 824	13 050	173 774	142 992	30 782	5 268	25 514	12 017	13 497
<i>1c</i>	211 016	73 750	137 266	115 049	22 217	4 349	17 868	12 841	5 027
<i>2c</i>	120 998	2 480	118 518	91 397	27 121	594	26 527	3 587	22 940
<i>3c</i>	153 860	35 620	118 240	97 686	20 554	3 814	16 740	8 613	8 127
<i>4c</i>	126 126	12 940	113 186	92 298	20 888	3 498	17 390	10 026	7 364
<i>5c</i>	398 600	10 738	387 862	319 056	68 806	12 634	56 172	31 157	25 015
<i>6c</i>	254 148	61 396	192 752	125 553	67 199	18 357	48 842	42 086	6 756
TOTAL	5 714 732	1 168 702	4 546 030	3 308 363	1 237 667	116 411	1 121 256	272 502	848 754

¹ Performed by the ARC's Biotechnology Platform.

² Confidence scores on the basis of the bacterial family OTU level.

6 Results and discussion

The results and discussion of the two main aims of the project – namely the impact of the sex hormones (i.e. during oestrus and dioestrus) and the impact of *T. foetus* infection on the vaginal microbiota of the heifers in the experiment – are delineated into two separate sections below. The rationale behind this arrangement of the information is that the factors are arguably independent of one another and therefore should be examined and discussed separately.

6.1 Section 1: The healthy microbiota of oestrus and dioestrus and the possible impact of sex hormones

This section describes the microbiota of healthy heifers in oestrus and in dioestrus with the aim of documenting the normal vaginal microbiota in the studied heifers. Differences in the microbiota between oestrus and dioestrus could indicate possible hormonal influences on the composition of the microbiota. The existence of such differences was evaluated statistically.

The average abundances of bacterial phyla observed in the nine samples of healthy heifers in oestrus in contrast with that of the three samples of healthy heifers in dioestrus, are presented in Figure 10. Both groups are dominated by five phyla: *Tenericutes*, *Proteobacteria*, *Firmicutes*, *Actinobacteria* and *Bacteroidetes* with minor abundances of *Cyanobacteria*, *Fusobacteria* and *Chloroflexi* at above 1% abundance in at least one sample also observed. A further nine phyla were observed at trace levels. Note the dominance of *Tenericutes* in both samples, but acutely so in the dioestrus samples.

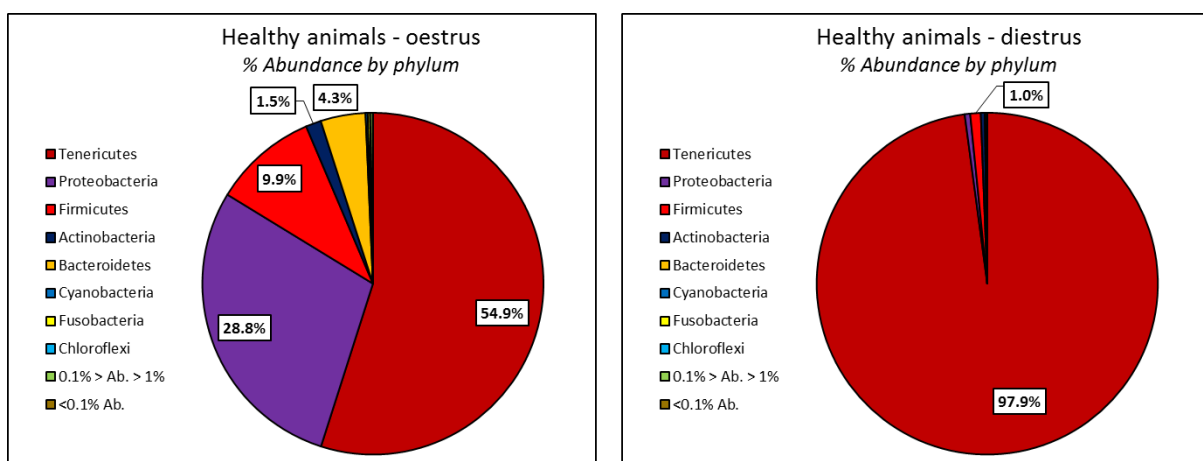


Figure 10: Phylum-level bacterial abundances presented by cycle stage for healthy heifers.

Note: The relative abundances at the phylum level from nine oestrus samples and three dioestrus samples were averaged to construct the figures.

The family-level abundances observed in the 12 samples analysed for the two groups of healthy heifers during the different reproductive cycle stages are presented in Figure 11.

A great deal of variation in the composition of the bacterial microbiota between samples is apparent, especially with regard to the samples which were recorded for heifers in the oestrus stage of their reproductive cycle (Figure 11). Furthermore, while Figure 10 showed a relatively straightforward dominance of the oestrus stage by the phylum *Tenericutes* (in which the family *Mycoplasmataceae* reside), with the phylum *Proteobacteria* (in which the family *Pasteurellaceae* reside) coming in second, this general interpretation is not true for each individual sample. Samples 1a, 2a, 3c and 6a show a total domination by *Mycoplasmataceae* while 4a and 5a are dominated by *Pasteurellaceae*. Only samples 3a and 2c could be said to be characterised by the relationship observed in Figure 10.

Samples 1c and 2c in particular exhibited considerable bacterial diversity compared to other samples. Both these samples, in addition to having greater abundances of families found in small quantities, had a substantial abundance of unique families in the data set, as shown by the large proportion of each sample given to the “1 sample Ab. > 1%” grouping.

In contrast, the relationships characterised in Figure 10 regarding the dioestrus set of samples appears to hold, with all three samples being largely indistinguishable.

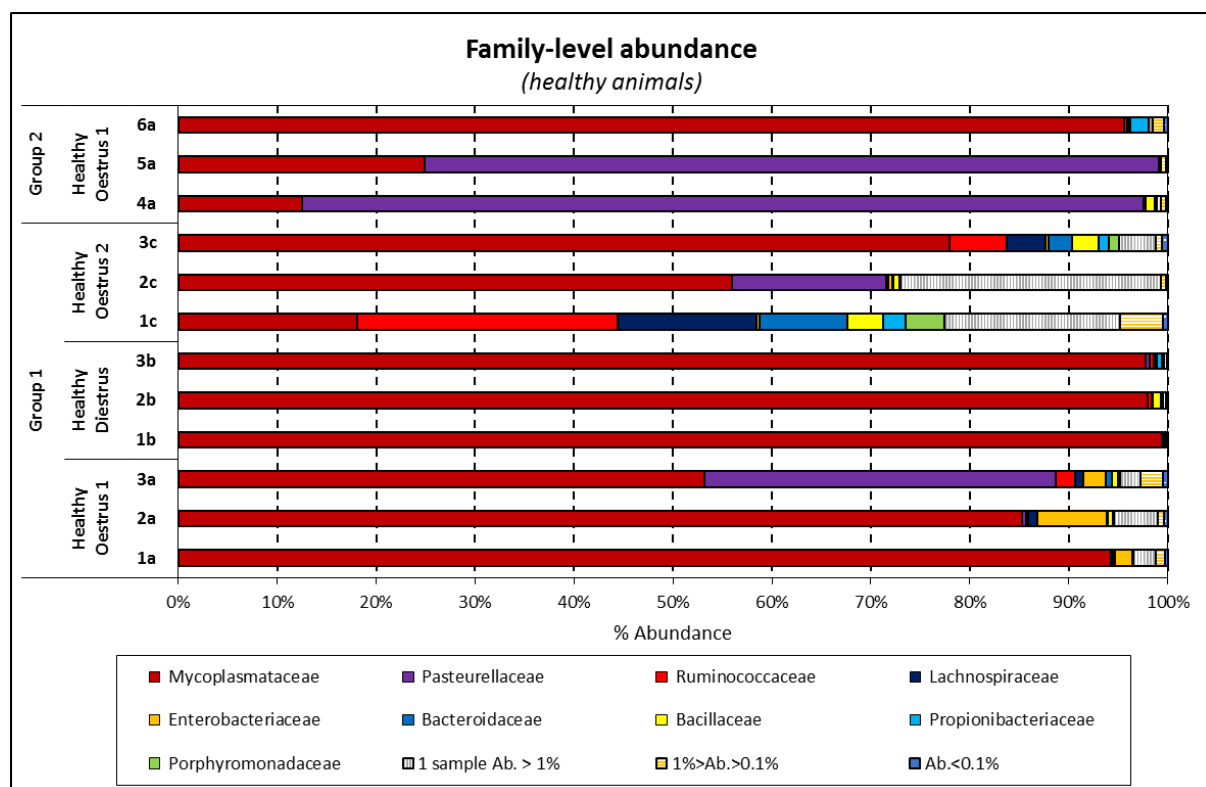


Figure 11: Family-level bacterial abundances in all healthy heifers investigated.

Note: The relative abundances of each family observed were grouped as follows: Those families which presented with more than 1% abundance in more than one sample are depicted individually on the figure and were ordered by their relative contribution to abundance across the entire sample; Those families which presented with more than 1% abundance but were characteristic of only one sample were grouped together in “1 sample Ab. > 1%”; Those families which presented with abundances of between 0.1% and 1% across all samples were grouped in “1%>Ab.>0.1%”; Those families which presented with less than 0.1% abundance across all samples were grouped in “Ab. < 0.1%”.

Figure 12 provides a heatmap of the abundances of the bacterial families depicted in Figure 11, further highlighting the dynamics illustrated. Each cell in the matrix is attributed a colour scale with lighter colours showing abundances closer to 100% for each particular family and darker colours for those closer to 0%. The heat map represents an easy method to visualize the relevance of *Mycoplasmataceae* and *Pasteurellaceae* in each sample, as well as the increased diversity found in samples 2c and 1c. From Figure 12, samples 1a, 2a, 3c and 6a are relatively similar while 4a and 5a are also relatively similar to each other. Samples 3a and 2c are somewhat similar, while 1c appears to be unique. Again, the dioestrus samples are indistinguishable.

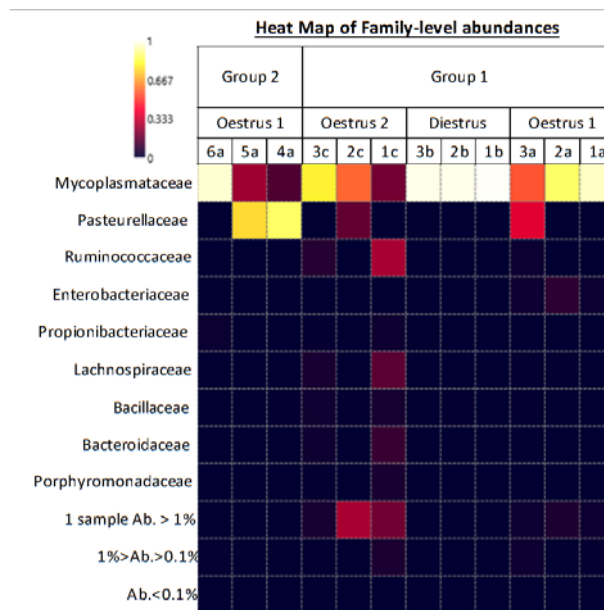


Figure 12: Heatmap of bacterial abundances for the 12 samples from healthy heifers. Lighter colours indicate higher levels of bacterial abundance (scale in top-left corner of the figure).

6.1.1 Alpha diversity analysis

The Shannon Entropies of the 12 samples under consideration with the total number of families observed per sample, as well as the number of families observed per sample with an abundance of greater than 1%, are presented in Figure 13. Note how Shannon Entropy largely follows the count of families with Ab. >1% function and not the richness function.

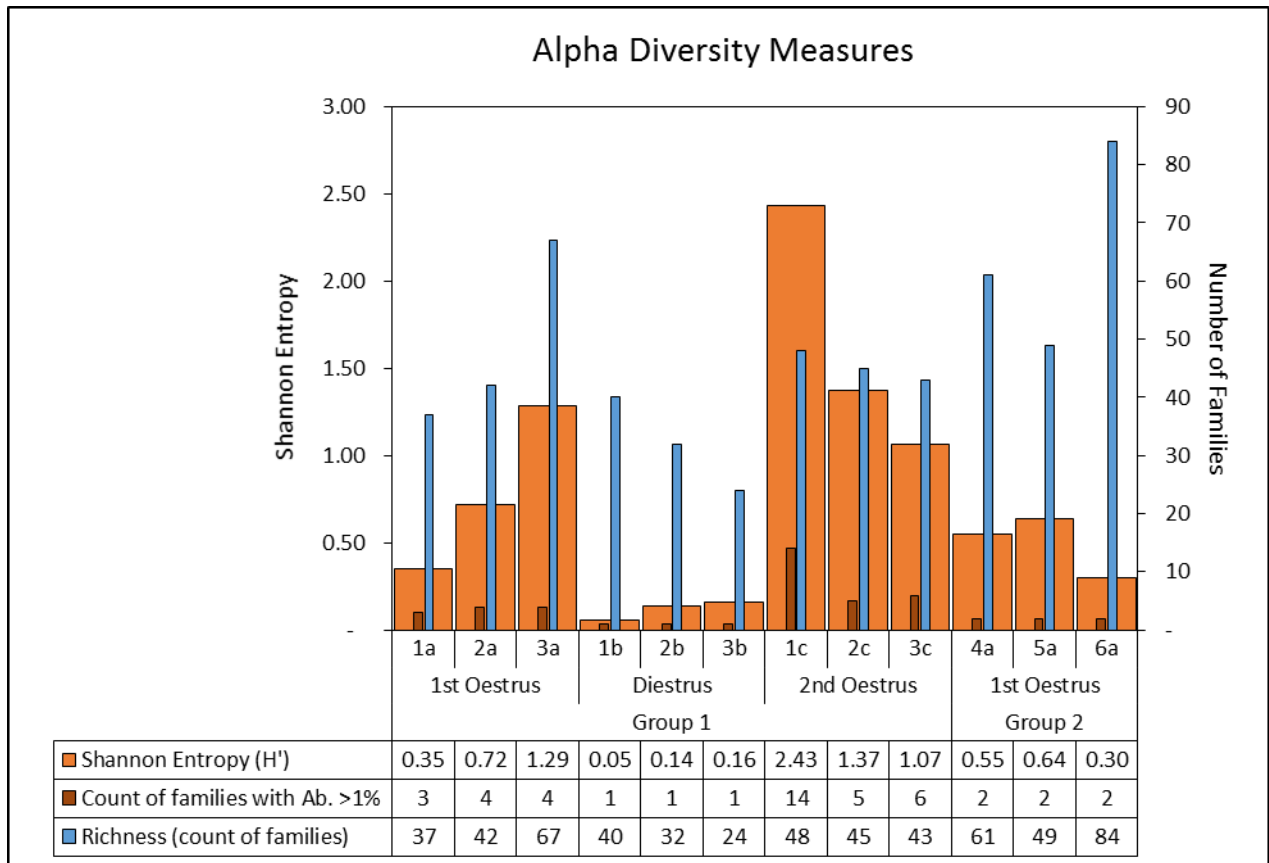


Figure 13: Alpha diversity in the healthy heifers sampled during oestrus and dioestrus.
 Note: Shannon Entropy measured scale on left axis, and number of families on the right.

Table 3 portrays the data used to generate Figure 13, and ranks each sample according to the three alpha diversity measures relative to the alpha diversity scores of the other samples on a scale of 1 to 12, with 1 being the least diverse and 12 being the most diverse.

Table 3: Tabular representation of ranked alpha diversities calculated for illustration in Figure 13.

		Shannon Entropy (H')		Count of families with Ab. >1%		Richness (count of families)			
		Stat	Rank	Stat	Rank	Stat	Rank		
Group 1	1st Oestrus	1a	0.35	5	3	7	37	3	
		2a	0.72	8	4	8	42	5	
		3a	1.29	10	4	8	67	11	
	Dioestrus	1b	0.05	1	1	1	40	4	
		2b	0.14	2	1	1	32	2	
		3b	0.16	3	1	1	24	1	
	2nd Oestrus	1c	2.43	12	14	12	48	8	
		2c	1.37	11	5	10	45	7	
		3c	1.07	9	6	11	43	6	
	Group 2	1st Oestrus	4a	0.55	6	2	4	61	10
			5a	0.64	7	2	4	49	9
			6a	0.30	4	2	4	84	12

6.1.1.1 Hypothesis testing

Using a non-parametric permutational pseudo t-test, based on all possible permutations of the differences between the two sample means, enables the calculation of an exact p-value. The null hypothesis of the test is that the means of both groups are equal.

Table 4 reveals the results of the non-parametric permutational pseudo t-test. The p-value derived from this test allows the null hypothesis only to be rejected at the 10% level of significance (exact permutational p-value of 0.059091). The inference of the test is that there is a tendency to increased alpha diversity in heifers in oestrus as measured by the Shannon Entropy statistics (Figure 13).

Table 4: Statistical tests of significance of reproductive cycle on Alpha Diversity.

Test for equal means			
Oestrus		Dioestrus	
N:	9	N:	3
Mean	0.96835	Mean	0.076848
95% conf.:	(0.4633; 1.4734)	95% conf.:	(-0.019848; 0.17354)
Variance	0.43173	Variance	0.0015152
Difference between means:		0.89151	
95% conf. interval (parametric):		(0.018153; 1.7649)	
95% conf. interval (bootstrap):		(0.43595; 1.2111)	
t:	2.2744	p (same mean):	0.04622
Uneq. var t:	4.0492	p (same mean):	0.0035342
Monte Carlo permutation:		p (same mean):	0.5642
Exact permutation:		p (same mean):	0.059091

6.1.2 Beta diversity analysis

Figure 14 displays the output provided by PAST 3.12 both for the NMDS and clustering procedures described.

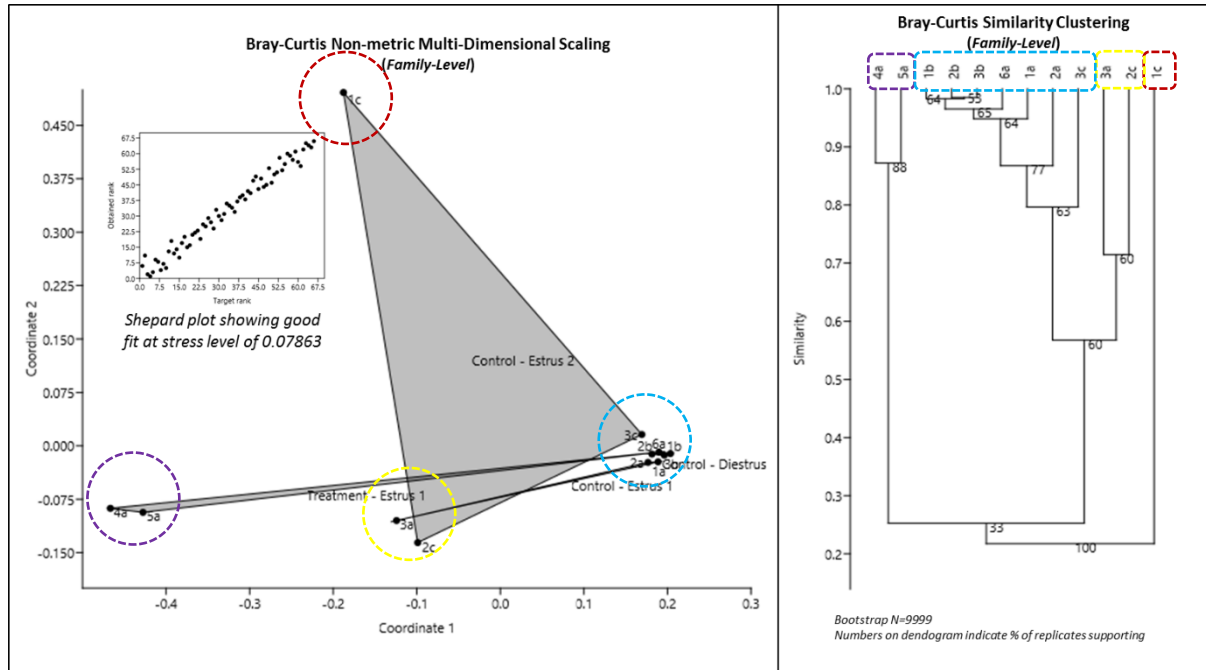


Figure 14: NMDS ordination and hierarchical clustering of the healthy heifer samples.

The NMDS shows four distinct groupings, which is supported by the hierarchical clustering procedure. *A priori* sample groupings are indicated by the shaded, bounded areas on the NMDS output. All the dioestrus samples are clustered together with four of the oestrus samples (light blue). Two further clusters of oestrus samples are evident (purple and yellow) while another oestrus sample (red) appears to be distinct from the others.

6.1.2.1 Hypothesis testing

In order to ascertain whether the *a priori* groupings by cycle stage are necessarily distinct from one another, the Bray-Curtis similarities were assessed using the non-parametric permutational statistical technique, PERMANOVA.

6.1.2.1.1 Between sample groups

Table 5 shows the results of the PERMANOVA procedure applied to the dataset under the null hypothesis that there are no differences in the relative abundances of families between the four sample groups. As can be noted, the p-value of 0.0854 is not supportive of a rejection of the null hypothesis at the 5% level, but indicates a tendency towards differences existing between the groups.

Table 5: PERMANOVA test of significant differences between the four groups of samples.

PERMANOVA

<i>Permutation N:</i>	9999
<i>Total sum of squares:</i>	1.685
<i>Within-group sum of squares:</i>	0.9522
<i>F:</i>	2.051
<i>p (same):</i>	0.0854

Table 6 shows the post-hoc tests performed via PERMANOVA on the four groups which show insignificant p-values at the 5% level, although the differences between the first group of heifers in their first oestrus (1a, 2a and 3a) and their dioestrus samples (1b, 2b, and 3b) show a p-level indicative of a possible tendency towards differences existing between those groupings.

Table 6: PERMANOVA Post-hoc pairwise tests between groups.

Post-hoc pairwise tests for significant differences

	Group 1 - Oestrus 1	Group 1 - Dioestrus	Group 1 - Oestrus 2	Group 2 - Oestrus 1
<i>Group 1 - Oestrus 1</i>		0.0952	0.4991	0.1966
<i>Group 1 - Dioestrus</i>	0.0952		0.1074	0.1012
<i>Group 1 - Oestrus 2</i>	0.4991	0.1074		0.3042
<i>Group 2 - Oestrus 1</i>	0.1966	0.1012	0.3042	

6.1.2.1.2 Between factors (oestrus versus dioestrus)

Table 7 shows the results of the PERMANOVA test on the null hypothesis that there are no differences in the relative abundances of microbial families between the samples of heifers in oestrus versus those in dioestrus (9 by 3 experiment). The results of the test indicate an insignificant p-value of 0.1318, which renders it impossible to reject the null hypothesis.

Table 7: PERMANOVA test for significant differences between oestrus and dioestrus factors.

PERMANOVA

<i>Permutation N:</i>	9999
<i>Total sum of squares:</i>	1.685
<i>Within-group sum of squares:</i>	1.441
<i>F:</i>	1.691
<i>p (same):</i>	0.1318

6.1.3 Treatment effects at the bacterial family level

For the purposes of this paper, the SIMPER function was applied to the converse of the Bray-Curtis similarities determined in PAST 3.16 between the 9 oestrus samples and the 3 dioestrus samples.

6.1.3.1 SIMPER analysis results

Table 8 depicts the results of the SIMPER procedure between the grouped oestrus and dioestrus samples. From this, 9 specific families contributed 92.39% of the total dissimilarity observed between the two sample groups, with each family contributing 1% or more to the dissimilarity. The other 112 families only contributing a total of 7.61% to the total dissimilarity between the 2 groupings. It should be noted that 7 out of the 9 families identified via SIMPER were the same families identified earlier during the family abundance analysis (Figure 11).

Table 8: SIMPER analysis results indicating the family-level contributors to dissimilarity.

<i>Taxon</i>	Av. dissim	Contrib. %	Cumulative %	Mean OESTRUS	Mean DIOESTRUS
<i>Mycoplasmataceae</i>	20.44	49.23	49.23	0.575	0.984
<i>Pasteurellaceae</i>	11.7	28.19	77.42	0.234	0.00161
<i>Ruminococcaceae</i>	1.914	4.61	82.03	0.0387	0.00261
<i>Lachnospiraceae</i>	1.096	2.64	84.67	0.0224	0.00112
<i>Pseudomonadaceae</i>	0.7475	1.8	86.47	0.015	0.00017
<i>Bacteroidaceae</i>	0.6738	1.623	88.09	0.0136	0.000245
<i>Enterobacteriaceae</i>	0.6646	1.601	89.69	0.0137	0.000464
<i>Moraxellaceae</i>	0.6352	1.53	91.22	0.0127	0.0000722
<i>Bacillaceae</i>	0.4842	1.166	92.39	0.0102	0.0028

6.1.3.2 Statistical assessment of individual families

Each family was assessed via a permutational pseudo t-test to determine any significant differences between the means of the proportional abundances for each family between the oestrus and dioestrus reproductive cycle stages under the null hypothesis that no difference exists between the means of the proportional abundances observed per group, per family (Table 9). While 8 of the 9 families analysed showed no significant differences in their mean proportional abundances, *Mycoplasmataceae* showed a difference at the 5% (*) level of significance, with the family appearing to be negatively associated with the oestrus stage of the reproductive cycle.

Table 9: Results of pseudo t-test to identify family-level changes to abundance due to cycle stage.

<i>Taxon</i>	Exact	
	Oestrus	Perm. P
<i>Mycoplasmataceae</i> *	-	0.036364
<i>Pasteurellaceae</i>	+	0.29091
<i>Ruminococcaceae</i>	+	0.60455
<i>Lachnospiraceae</i>	+	0.44545
<i>Pseudomonadaceae</i>	+	0.35455
<i>Bacteroidaceae</i>	+	0.30909
<i>Enterobacteriaceae</i>	+	0.27727
<i>Moraxellaceae</i>	+	0.39545
<i>Bacillaceae</i>	+	0.38636

* Indicates significance at the 5% level.

6.1.4 Discussion

The vaginal bacterial microbiota of healthy heifers was dominated by the phyla *Tenericutes* and *Proteobacteria*, belonging to the families *Mycoplasmataceae* and *Pasteurellaceae*, respectively (Figure 10, Figure 11 and Figure 12). Further analysis at the genus level (data not shown) indicated that *Mycoplasmataceae* was represented by *Ureaplasma* and *Mycoplasma*, and *Pasteurellaceae* by the genus *Histophilus*. The phylum *Firmicutes* (3%) was also present, although in small numbers. These findings are consistent with the research presented by Santos et al. (2011).

Many studies that investigated the bovine vaginal bacterial microbiota using culture-based methods failed to isolate *Mycoplasma*, most likely due to its fastidious nature which challenges plate growth. The augmented costs involved with its isolation and the prolonged culturing time also weigh against culturing for it (Husted, 2003, Taubrich, 1958, Otero et al., 2000, Zambrano-Nava et al., 2011). Even with these constraints, *Mycoplasma spp.* has been isolated from the bovine genital tract and is known to be a part of the cervico-vaginal microbiota of cows (Nakamura et al., 1977, Langford, 1975, Panangala et al., 1978).

Furthermore, *Mycoplasmas* has been extensively associated with a variety of diseases in both humans and animals. In bovines, it is known as a surface inhabitant of the epithelial lining of the urogenital and respiratory tract (Griffin et al., 2010, Afshar, 1967). *Mycoplasma spp.* has been isolated in the genital tract of female cattle and associated with reproductive disorders including vaginitis, cervicitis, endometritis, salpingitis and abortion, as reviewed by Afshar (1967) and Givens and Marley (2008).

Similar to the *Mycoplasma spp.*, the genera *Ureaplasma* and *Histophilus* have also been described as normal inhabitants of the vagina in bovines. Unfortunately, this study did not analyse the organisms to the species level, but sequences uploaded to the MG-RAST server for analysis consistency revealed that the two species represented were *Ureaplasma diversum* and *Histophilus somni*, which are of major importance in bovines and are related to reproductive disease causing vaginitis, endometritis, abortion and infertility (Givens and Marley, 2008, Yaeger and Holler, 2007).

In spite of the difficulties encountered with *Mycoplasma* culturing, recent studies based on the genomes of bacterial communities have uncovered a great abundance of organisms belonging to the phyla *Tenericutes* and *Proteobacteria* as part of a healthy uterine bacterial microbiota of post-partum cows. (Santos and Bicalho, 2012, Santos et al., 2011, Machado et al., 2012). These studies provide support to the notion that the discovery of such potentially destructive bacteria in the heifers' vaginal microbiota is to be considered normal, as they are part of the naturally occurring commensal bacterial community.

6.1.4.1 Alpha diversity

Alpha diversity can be thought of as a concept which combines both the number of different OTUs (in the present case, bacterial families) present in a single sample and their abundance. Samples with a more evenly-spread microbiota – in other words, those with many families, each with notable abundances – are considered to be more diverse than those with abundances concentrated in a small number of families, with other families being represented at trace abundance levels.

For illustration purposes, consider two samples each containing 10 different bacterial families. In the first sample, each family is represented evenly with an abundance of 10%. In the second sample, two families represent 92% of the total abundance while the other 8 families represent 1% abundance each. Intuitively, although both samples are each represented by 10 bacterial families, the first sample is more diverse than the second. This is the essence of alpha diversity and any measures of such should reflect this dynamic.

Referring back to Figure 13, note how the Shannon Entropy largely tracks the count of families with abundance above 1% function, while the richness of each sample is much more variable. This illustrates the utility of the Shannon Entropy in capturing the alpha diversity of a sample as it appropriately takes into account the abundances of each family, relative to all others. Furthermore, it appears that the alpha diversity of the dioestrus animals is substantially lower than that seen in all the oestrus samples. Additionally, within the set of oestrus samples themselves, considerable variation in alpha diversity is observed, in which the second oestrus (1c, 2c and 3c) appears to be more diverse than that of the first oestrus (1a to 3a and 4a to 6a). Ranking the alpha diversity statistics, as was done in Table 3, highlights these dynamics. As can be noted, the ranks related to the dioestrus heifers were consistently lower than those observed in the oestrus heifers.

Given the small sample size and unbalanced nature of the experiment, it was somewhat difficult to attempt statistical analysis beyond the simple comparative observations detailed above. However, even with such constraints, it is still possible to detect statistical significance given a large enough effect. The means of the two groupings can still be evaluated for differences using a non-parametric permutational pseudo t-test.

The p-value derived in the analysis of 0.059091 under the null hypothesis that the means of the two groupings are equal is indicative of a tendency towards increased alpha diversity in heifers in oestrus when compared to those in dioestrus. While this figure is not conclusive, it provides an indication that such a relationship may indeed exist. Given the small and unbalanced sample sizes and the fact that the variances of the two groupings are very dissimilar, it is arguable that the experiment, if conducted on a larger scale in a balanced setup, would produce more conclusive results which would be a valuable contribution to science, as similar studies such as Otero et al. (1999) did not identify this dynamic.

6.1.4.2 *Beta diversity*

The quantification of beta diversity is fundamental to determining whether *a priori* groupings of samples are distinct from each other (Jost, 2007). While alpha diversity measures are useful in identifying the bacterial diversity within a sample, their comparison does not reveal whether samples or groups of samples are truly distinct from each other, as information regarding the extent of shared species is not included in the statistics produced.

In order to illustrate this, consider a case where two samples each are composed of 10 bacterial families and that each bacterial family is represented evenly at 10% abundance. Any alpha diversity statistics derived from such samples will yield the same value regardless of whether the bacterial families within the samples are shared or completely different. Relying on such statistics to conclude that the samples are the same is inherently erroneous, with the only positive conclusion that can be made in such a case being that both samples have the same alpha diversity.

It is therefore necessary, when comparing groups of samples, to assess beta diversity which accounts for the relative abundances of both shared and unshared bacterial OTUs between the groups. Groups showing similar abundances of the same OTUs are deemed to be more similar, while those showing very dissimilar abundances of shared OTUs as well as completely different OTU's, are deemed to be distinct, as logic dictates.

Beta diversity was first assessed in this work via means of Non-metric Multi-Dimensional Scaling (NMDS), followed by hierarchical clustering (Figure 14). The assessments were based on the Bray-Curtis similarity statistic, which provides an unbiased measure of similarity between samples on the basis of the shared bacterial families in each sample and their respective abundances (see Materials and Methods for further detail). Both methods offer a complementary visual appraisal of the degree of similarity between samples: the NMDS on an ordinal two-dimensional plain and the hierarchical clustering as a dendrogram.

The NMDS procedure produced what appears to be 4 distinct groupings of samples, with 7 of the 12 samples grouped closely together, 2 groupings of 2 samples each and 1 sample completely separate from the others (Figure 14). The *a priori* groupings of samples into 4 groups (by group and cycle stage) were also plotted in the shaded areas of Figure 14. All the dioestrus animals are located in the tight cluster of 7 samples, while the oestrus samples are more dispersed. Interestingly, the hierarchal clustering technique produces the same groupings, but provides information as to the relative similarity of each cluster (read off from the vertical axis). Furthermore, the procedure is supported by bootstrapping, which was duly performed at N=9999 to ascertain the veracity of the clustering results. In most cases, the procedure showed robust results. What these visualisations suggest is that the dioestrus samples are remarkably similar, being clustered tightly together in both analyses, whereas a

substantial degree of variation in the microbial biota of heifers in oestrus can possibly be expected. However, a number of oestrus samples (four out of nine) are also tightly clustered around the dioestrus samples, with the other five samples being apparently distinct. As two of the five separate samples (1c and 2c) were taken from heifers in their second oestrus, the other three being from heifers in their first oestrus, it is unlikely that these differences in similarity between the samples are a result of temporal factors, but are rather more likely to represent differences in the dynamics of the vaginal microbial community of healthy heifers in oestrus. These results could be taken as being suggestive of possible competition in the community between different commensal bacteria, but such theorizing should be the focus of further research.

Statistical evaluation of beta diversity was conducted using PERMANOVA. While most studies apply the ANalysis Of SIMilarities (ANOSIM) technique in assessing beta diversity, this technique performs poorly in cases where experiments are unbalanced, such as the current work comparing three against nine samples. PERMANOVA was therefore applied as it is more robust in such cases (Buttigieg and Ramette, 2014).

Statistical testing was first conducted to determine whether significant differences existed between the samples as they were grouped in the initial experiment. In other words, the twelve samples were grouped according to heifer and when they were sampled yielding four groups of three samples each (the first being the healthy heifers in their first oestrus; the second being the healthy heifers in dioestrus and so on). This procedure revealed that a tendency indicating differences between the means of the groups existed but the null hypothesis could not be rejected at the 5 percent level. Further post-hoc analysis showed a tendency indicating differences between the first group of heifers in oestrus and the same group in dioestrus and p-values close to the 10 percent level in comparisons between the other two sample groups and the dioestrus group. However, none of these results enabled a rejection of the null hypothesis that there were no differences between the relative abundances existing between the sample groups.

Following the first batch of statistical tests, the estrus samples were grouped together and compared with the dioestrus samples, also using PERMANOVA. The test revealed that the differences between the groupings by the cycle stage factor were not significant.

6.1.4.2.1 Bacterial families which differed according to reproductive status

Following on from the discussion above, it cannot be concluded conclusively that there are significant differences in the abundance compositions of the microbial communities at the family taxonomic level between heifers in oestrus and those in dioestrus. However, this result does not preclude the thorough investigation of associations between the abundances of specific taxa and the reproductive cycle stage

of the heifers. In order to aid this endeavour, it is appropriate to exclude taxa which are likely to be irrelevant to the analysis.

For this purpose, SIMPER was applied to the Bray-Curtis dissimilarities³ of the dataset. From this procedure, 9 families were identified which contributed more than 1% each to the total dissimilarity. Assessment of these families via the same method used to assess alpha diversity revealed that *Mycoplasmataceae* appeared to show a significant relationship with the reproductive status of the sampled heifers. The relationship was characterised by a negative association of the family with the oestrus stage of the cycle.

While only the last relationship between *Mycoplasmataceae* and the reproductive cycle stage of the heifers sampled was significant, the statistical tests performed on alpha diversity and beta diversity showed tendencies indicating possible differences between the samples, delineated by reproductive cycle stage. It is therefore pertinent to discuss possible reasons behind these possible relationships, supported by other related studies.

During oestrus, the dominant follicle secretes estrogen. Extrapolating from human research, Brotman et al. (2014) mentioned that most of the cells in the reproductive tract express estradiol receptors (epithelial cells, macrophages, stromal cells and lymphocytes) and there appears to exist a reduction in the immune-response of the cervico-vaginal region during high estrogen levels. This period, in the woman's cycle, has been termed as the "window of vulnerability".

In bovines, the "window of vulnerability" could also resemble that observed in women. The binding of estradiol to its receptors in the reproductive tract causes secretion of mucus of low viscosity, the relaxation of the cervix, proliferation of the epithelial cells of the vagina and possibly immune-suppression. This cascade of events, driven by oestradiol, also represent a mechanism of "self-cleansing" of the reproductive tract to eliminate any existing pathogen. This "self-cleansing" mechanism observed during oestrus in conjunction with *Mycoplasmataceae* being a surface inhabitant organism could be a plausible explanation of the family's decreased relative abundance over oestrus when compared to dioestrus, and potentially be driving the tendency towards augmented bacterial diversity observed in oestrus. The impact of the sex hormones on the vaginal microbiome is a complicated topic. Studies detailing how the sex hormones were found to influence the vaginal microbiota in women are well documented, as most of the reports identified were from human medicine and the experiments addressed the changes in the bacterial microbiota throughout the women's

³ Dissimilarity being the converse of a similarity (e.g. 1 – similarity)

menstrual cycle in health and diseased status. Even though a direct comparison between human and bovine reproductive cycles may be criticised as inadequate due to each species' cyclical particularities, the extensive number and depth of research papers available in human medicine is worthy of review, especially in comparison to that conducted on other species.

The human vaginal bacterial microbiota is mostly populated by *Lactobacillus* species throughout the cycle; however, during menses, a stage in which the uterine endometrium sloughs due to non-pregnant status, the bacterial community changes and an increase in non-*Lactobacillus* species is observed. The menstrual blood provides an additional substrate for bacterial proliferation (Gajer et al., 2012)

Contrastingly, a study conducted with the use of oral hormonal contraception (HC) and the oral microbial community with samples taken before HC initiation, 10 days and 3 weeks post initiation, observed a striking increase in the number of *Prevotella* species in samples taken at 3 weeks post-HC initiation. This study suggests that sex hormones influenced the oral microbiota; however, the duration of exposure was crucial to observe the changes and the time/duration may be a critical variable (Klinger et al., 1998)

In terms of microbiota stability, Gajer et al. (2012), studying the temporal dynamics of the vaginal bacterial community, found that the microbiota tended to be more constant, in other words, more stable, during peak estradiol concentration when compared to the progestogenic phase. It is believed that estrogen stimulates accumulation of glycogen in vaginal epithelial cells, serving as a carbohydrate source for *Lactobacillus* species (Brotman et al., 2014).

Given the above studies, it is therefore possible to infer that sex hormones could very well have an influence on the vaginal microbiota of heifers; however, the present study could not find conclusive proof of this conjecture. Hints of such a relationship's existence were nevertheless evident in the results obtained and a further experiment with a larger sample size and balanced setup should be undertaken to provide a more conclusive answer.

6.2 Section 2: Impact of *T. foetus* infection on the vaginal bacterial microbiota

This section reports the results of analyses comparing the microbiota of healthy heifers to those infected with *Trichostrongylus axei*, with the aim of establishing whether differences exist and the statistical significance thereof.

All the infected animals were confirmed positive for *T. foetus* using wet preparation and microscopy on both days of sampling. Similarly, controls were confirmed negative to exclude cross-contamination.

The average abundance of bacterial phyla observed in the 12 samples taken from healthy heifers (Control and Treatment Groups) with that of the 6 samples of heifers infected with *Trichostrongylus axei* (Treatment Group) are presented in Figure 15. Similarly to before, both groups are dominated by 5 phyla: *Tenericutes*, *Proteobacteria*, *Firmicutes*, *Actinobacteria* and *Bacteroidetes*, but with minor abundances of *Cyanobacteria*, *Fusobacteria* and *Chloroflexi* at above 1 percent abundance in at least one sample also observed. A further 12 phyla were observed at trace abundance levels.

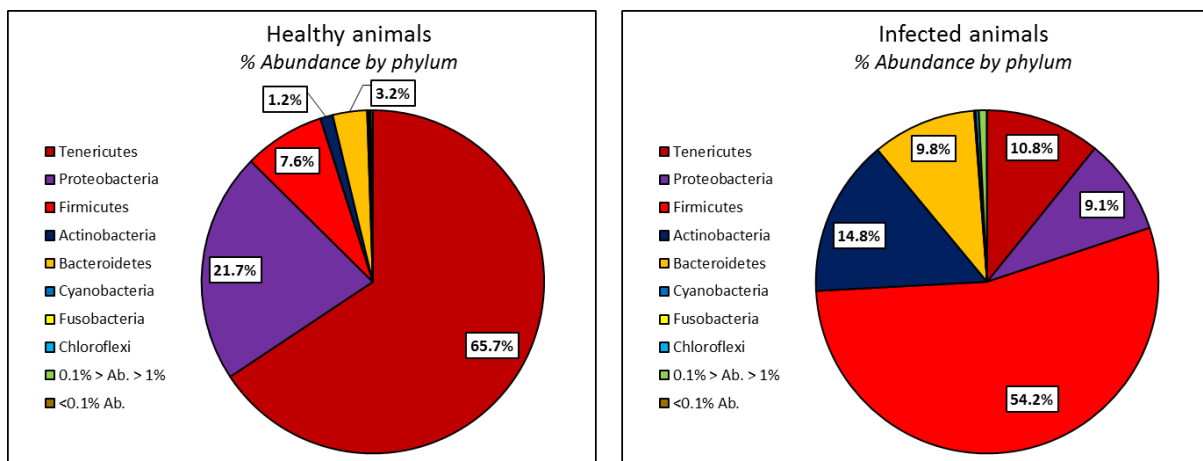


Figure 15: Phylum-level bacterial abundances for healthy versus infected heifers.

The mean relative abundance data at the phylum level from 12 healthy samples are presented in the first pie chart while that of six infected samples is presented in the second chart. The bacterial profile of the infected samples, though sharing the same phyla as that of the healthy animals, is distinct in terms of its relative abundance composition.

In keeping with the methodology adopted in the first section, Figure 16 depicts the family-level relative abundances of the 18 samples. The same grouping regime is employed as in the first results section, i.e. the same grouping used to generate Figure 11.

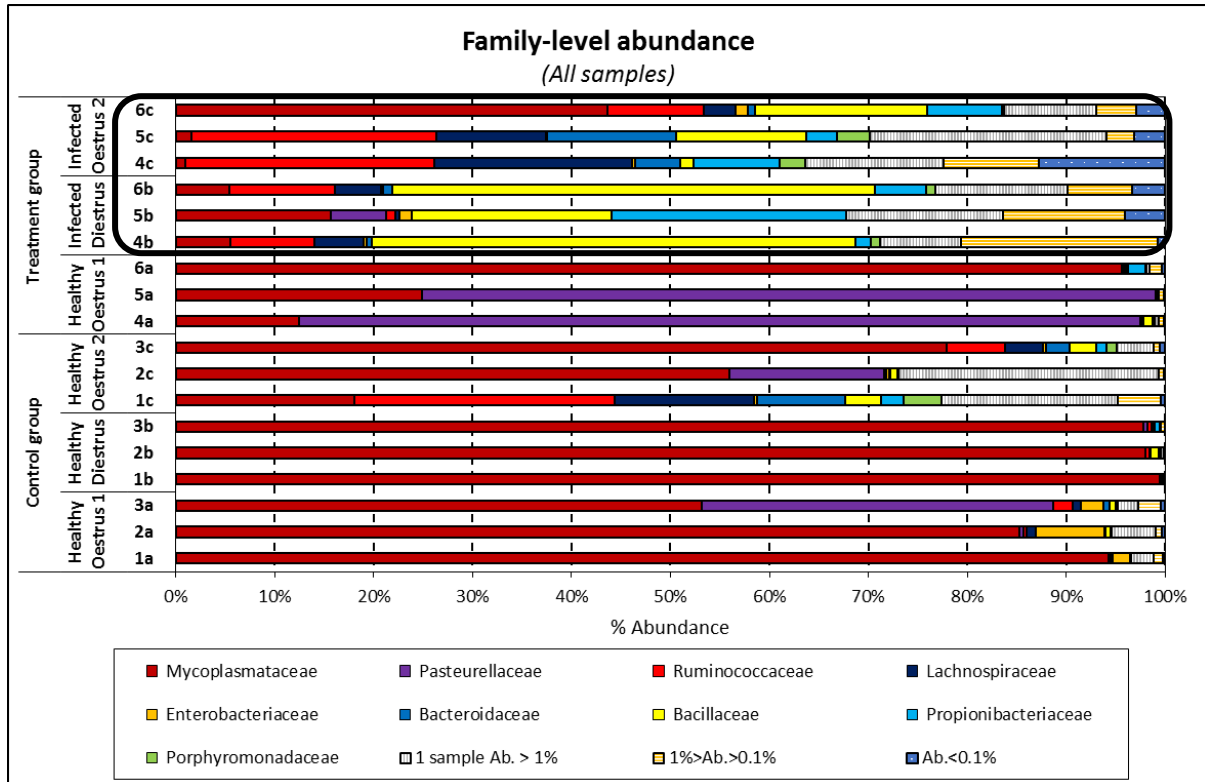


Figure 16: Family-level bacterial abundances for all 18 samples
 The figure includes both infected and uninfected animals in both oestrus and dioestrus. The bacterial family relative abundances of each sample are represented according to the same grouping regime used for Figure 11 (Section 1) and as indicated in the text preceding that figure (see the text below the figure).

Figure 17 provides a heatmap of the relative abundances of the bacterial families and groupings across all 18 samples, with higher abundances present in the healthy animals.

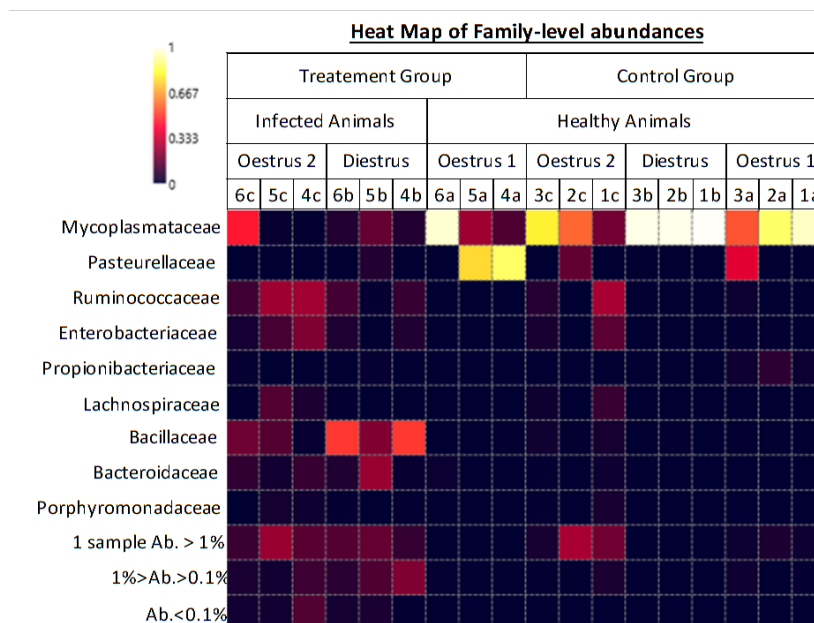


Figure 17: Heatmap showing bacterial abundances across all 18 samples.
 The map was derived from the bacterial family abundances, represented on a color scale (top left), with lighter colors indicating greater abundance and darker colors, lower abundance.

6.2.1 Alpha diversity analysis

Alpha diversity is presented as the Shannon Entropies of the 18 samples under consideration, contrasted with the total number of families observed per sample, as well as the number of families observed per sample with an abundance of greater than 1 percent each, and is depicted in Figure 18.

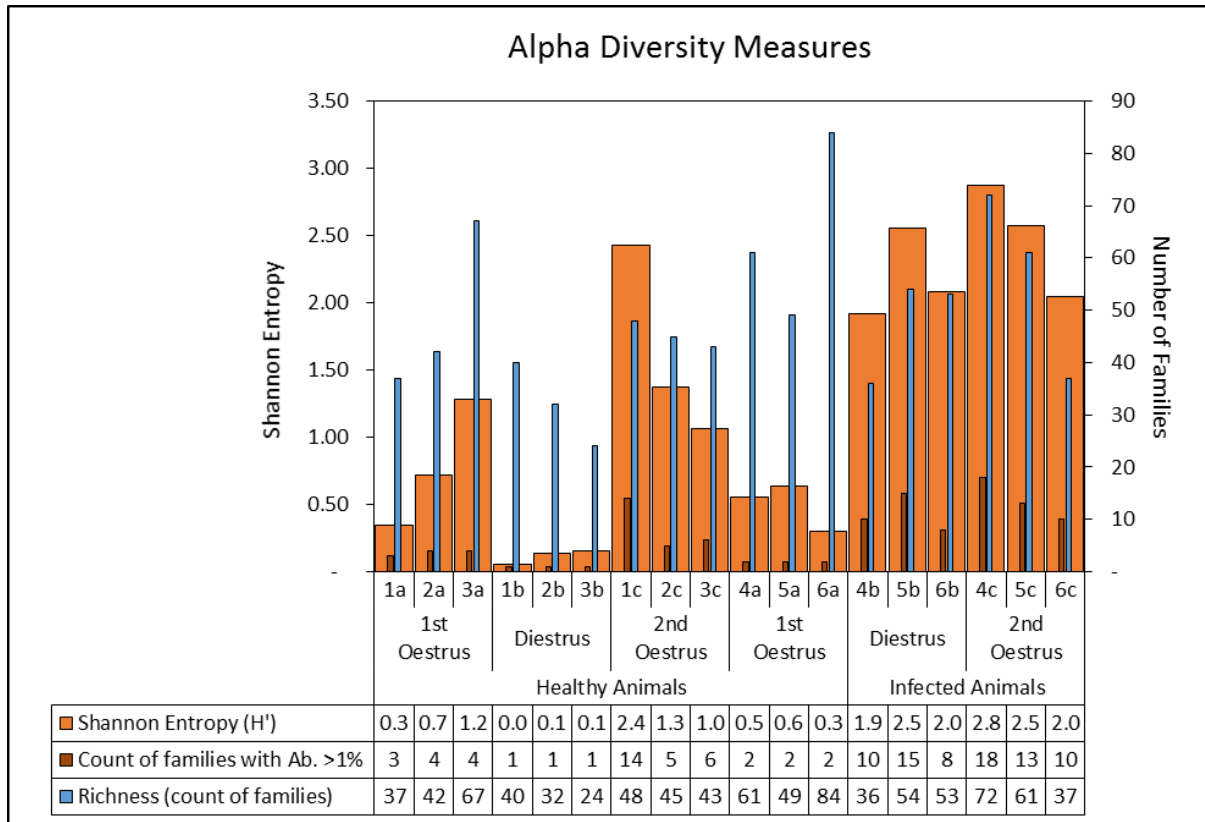


Figure 18: Alpha diversity across all heifers sampled during the study. The Shannon Entropy scale is indicated on the left axis, with the number of families meeting the required abundance levels, on the right.

The Shannon Entropy figures observed at a family level in the healthy samples were compared to those from the *T. foetus*-infected samples and are presented by a box-plot in Figure 19.

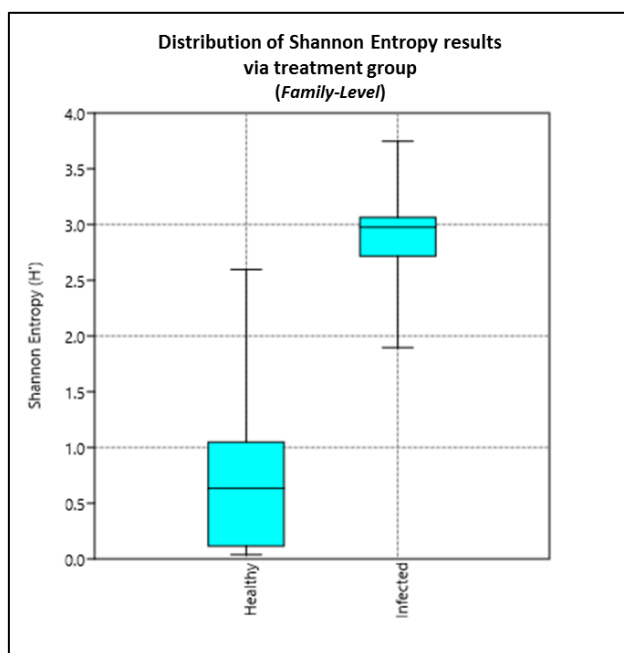


Figure 19: Box-plots contrasting Shannon Entropy by treatment group at a family level. Error bars indicate the upper and lower extremes of the samples, with the boxes bounded by 25% and 75% percentiles with means bisecting them.

Each sample was ranked according to the three alpha diversity measures relative to the alpha diversity scores of the other samples on a scale of 1 to 18, with 1 being the least diverse and 18 being the most diverse (Table 10). This dataset (Table 10) was used to generate Figure 18 and Figure 19.

Table 10: Tabular presentation of the ranked Alpha Diversity measures across all samples analysed in the study.

			Shannon Entropy (H')		Count of families with Ab. >1%		Richness (count of families)			
			Stat	Rank	Stat	Rank	Stat	Rank		
									1st Tercile	2nd Tercile
Control Group	Healthy Animals	1st Oestrus	1a	0.35	5	3	7	37	4	
			2a	0.72	8	4	8	42	7	
			3a	1.29	10	4	8	67	16	
		Diestrus	1b	0.05	1	1	1	40	6	
			2b	0.14	2	1	1	32	2	
			3b	0.16	3	1	1	24	1	
		2nd Oestrus	1c	2.43	15	14	16	48	10	
			2c	1.37	11	5	10	45	9	
			3c	1.07	9	6	11	43	8	
	Treatment Group	Infected Animals	1st Oestrus	4a	0.55	6	2	4	61	14
				5a	0.64	7	2	4	49	11
				6a	0.30	4	2	4	84	18
1st Oestrus			4b	1.92	12	10	13	36	3	
			5b	2.56	16	15	17	54	13	
			6b	2.08	14	8	12	53	12	
1st Oestrus		4c	2.87	18	18	18	72	17		
		5c	2.57	17	13	15	61	14		
		6c	2.05	13	10	13	37	4		

6.2.1.1 Hypothesis testing

The means of the two groupings were evaluated for differences using a non-parametric permutational pseudo t-test. The null hypothesis of the test was that the means of both groups were equal. The exact p-value derived from this test is 0.00010774, allowing the null hypothesis to be firmly rejected at the 1 percent level of significance. The inference of the test is that there is an increased alpha diversity of bacteria in heifers infected with *T. foetus* in comparison to healthy animals.

Table 11: Statistical tests of significance of *T. foetus*-infected vs. uninfected based on Alpha Diversity.

Test for equal means			
Oestrus		Dioestrus	
N:	12	N:	6
Mean	0.74548	Mean	2.8664
95% conf.:	(0.30674; 1.1842)	95% conf.:	(2.2377; 3.4951)
Variance	0.47683	Variance	0.035889
Difference between means:		2.1209	
95% conf. interval (parametric):		(1.4179; 2.824)	
95% conf. interval (bootstrap):		(1.5713; 2.7185)	
t:	-6.3951	p (same mean):	8.8719E-06
Uneq. var t:	-6.7221	p (same mean):	2.5901E-05
Monte Carlo permutation:		p (same mean):	0.00011
Exact permutation:		p (same mean):	0.00010774

As noted previously, the greater alpha diversity observed in the infected animals should not be misinterpreted as conclusive proof that the two groups are statistically distinct in terms of their bacterial composition.

6.2.2 Beta diversity analysis

The NMDS groupings of the Bray-Curtis similarities along with hierarchical clustering of the bacterial families identified for the samples are shown in Figure 20.

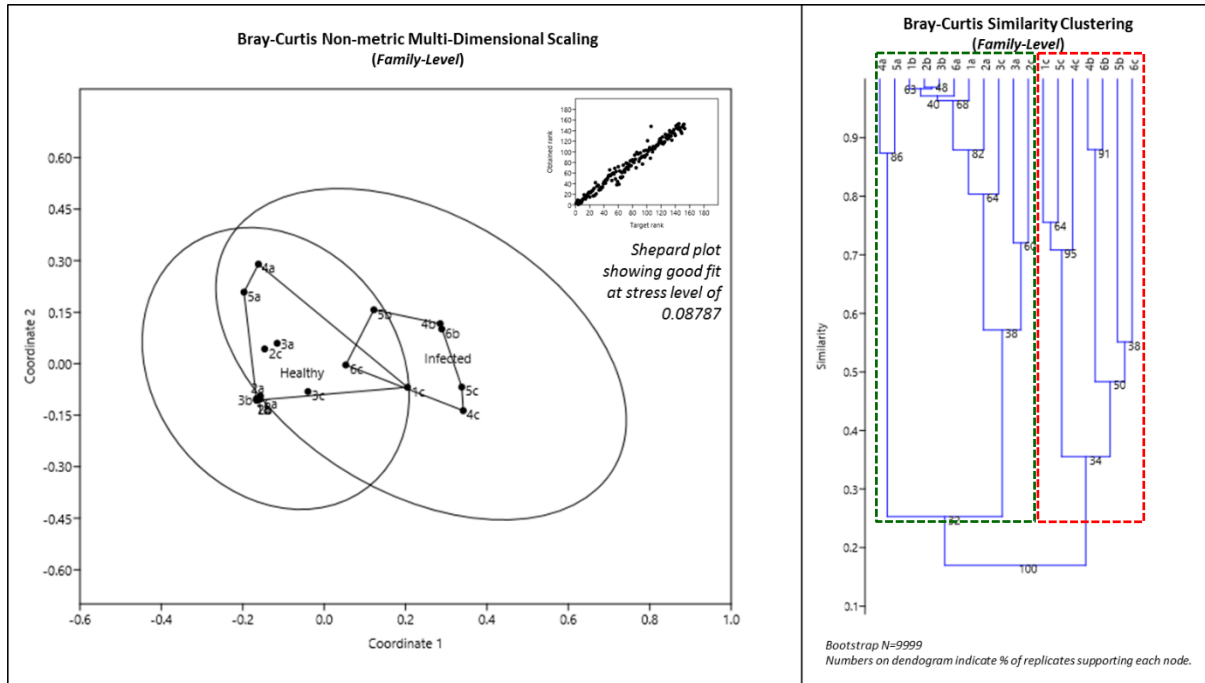


Figure 20: NMDS ordination and hierarchical clustering of all heifer samples.

Note: The NMDS grouping of the Bray-Curtis similarities of the samples is shown. The *a priori* groupings of healthy and infected animals are well supported by the relative similarities of the 18 samples, with only one sample from the healthy animals (1c) being more comparable to the infected samples and one sample from the infected animals (6c) being closer to the healthy samples. 95% confidence intervals around the *a priori* groupings are indicated by the ellipses. Applying hierarchical clustering provides a more detailed view of the similarity of the different samples. Healthy samples are grouped together, with the exception of sample 1c, which has been clustered with the infected sample 5c at approximately 75% similarity. The dendrogram strongly delineates the infected samples (+ sample 1c) and the healthy samples, as is indicated by the red box, vs. the green box showing the remaining healthy samples. Bootstrapping was performed at N=9999.

6.2.2.1 Hypothesis testing

The Bray-Curtis similarities were assessed using the non-parametric permutational statistical technique, PERMANOVA. Table 11 shows the results of the PERMANOVA procedure applied to the dataset under the null hypothesis that there are no differences in the relative abundances of bacterial families between the two sample groups. The p-value of 0.0002 indicates the null hypothesis is rejected at the 1 percent level of significance.

Table 11: PERMANOVA test of significant differences between healthy and infected heifer samples.

PERMANOVA

<i>Permutation N:</i>	9999
<i>Total sum of squares:</i>	4.199
<i>Within-group sum of squares:</i>	2.547
<i>F:</i>	10.38
<i>p (same):</i>	0.0002

6.2.3 Treatment effects at the bacterial family level

Families which contributed the most to dissimilarity between the grouped samples were identified via SIMPER and then analysed for statistical significance via a non-parametric permutational pseudo t-test.

6.2.3.1 The SIMPER analysis results

Table 12 depicts the results of the SIMPER analysis between the healthy and infected grouped samples. As can be noted, 11 specific families contributed to 86.92 percent of the total dissimilarity observed between the two sample groups (each family contributing 1 percent or more to the dissimilarity), with the other 112 families only contributing a total of 13.08 percent to the total dissimilarity between the two groupings.

Table 12: SIMPER results indicating the bacterial family-level contributors to dissimilarity of healthy vs. infected heifers.

Taxon	Av. dissim	Contrib. %	Cumulative %	Mean Healthy	Mean Infected
<i>Mycoplasmataceae</i>	28.9	35.4	35.4	0.677	0.121
<i>Bacillaceae</i>	12.1	14.82	50.22	0.00836	0.249
<i>Pasteurellaceae</i>	8.947	10.96	61.18	0.176	0.00932
<i>Ruminococcaceae</i>	6.338	7.762	68.94	0.0297	0.133
<i>Propionibacteriaceae</i>	3.902	4.779	73.72	0.00548	0.0832
<i>Lachnospiraceae</i>	3.548	4.346	78.06	0.0171	0.0738
<i>Paenibacillaceae</i>	2.346	2.874	80.94	0.00174	0.0484
<i>Bacteroidaceae</i>	1.778	2.177	83.12	0.0103	0.033
<i>Micromonosporaceae</i>	1.235	1.513	84.63	0.000162	0.0247
<i>Prevotellaceae</i>	1.013	1.241	85.87	0.00385	0.0206
<i>Pseudomonadaceae</i>	0.8594	1.053	86.92	0.0113	0.0094

6.2.3.2 Statistical assessment of individual bacterial families in healthy and infected heifers

Each family was assessed via a non-parametric permutational pseudo t-test to identify significant differences between the means of the proportional abundances between healthy and infected animals. This is under the null hypothesis that no difference exists between the means of the proportional abundances observed per group, per family. The results of the test are displayed in Table 13, with Figure 21 depicting the data as box plots of the eleven families identified, with the relative abundances plotted on the y-axis.

Table 13: Non-parametric permutational pseudo t-test results identifying family-level changes to abundance as a result of *T. foetus* infection.

Taxon	Healthy	Exact Perm. P
<i>Mycoplasmataceae</i> **	1	0.0029089
<i>Bacillaceae</i> **	0	0.00010774
<i>Pasteurellaceae</i>	1	0.19958
<i>Ruminococcaceae</i> *	0	0.017022
<i>Propionibacteriaceae</i> **	0	0.0001616
<i>Lachnospiraceae</i> *	0	0.029035
<i>Paenibacillaceae</i> **	0	0.00010774
<i>Bacteroidaceae</i>	0	0.22231
<i>Micromonosporaceae</i>	0	0.11043
<i>Prevotellaceae</i> *	0	0.025587
<i>Pseudomonadaceae</i>	1	0.99903

** indicates families which showed significant differences at the 5% level and *** indicates those at the 1% level.

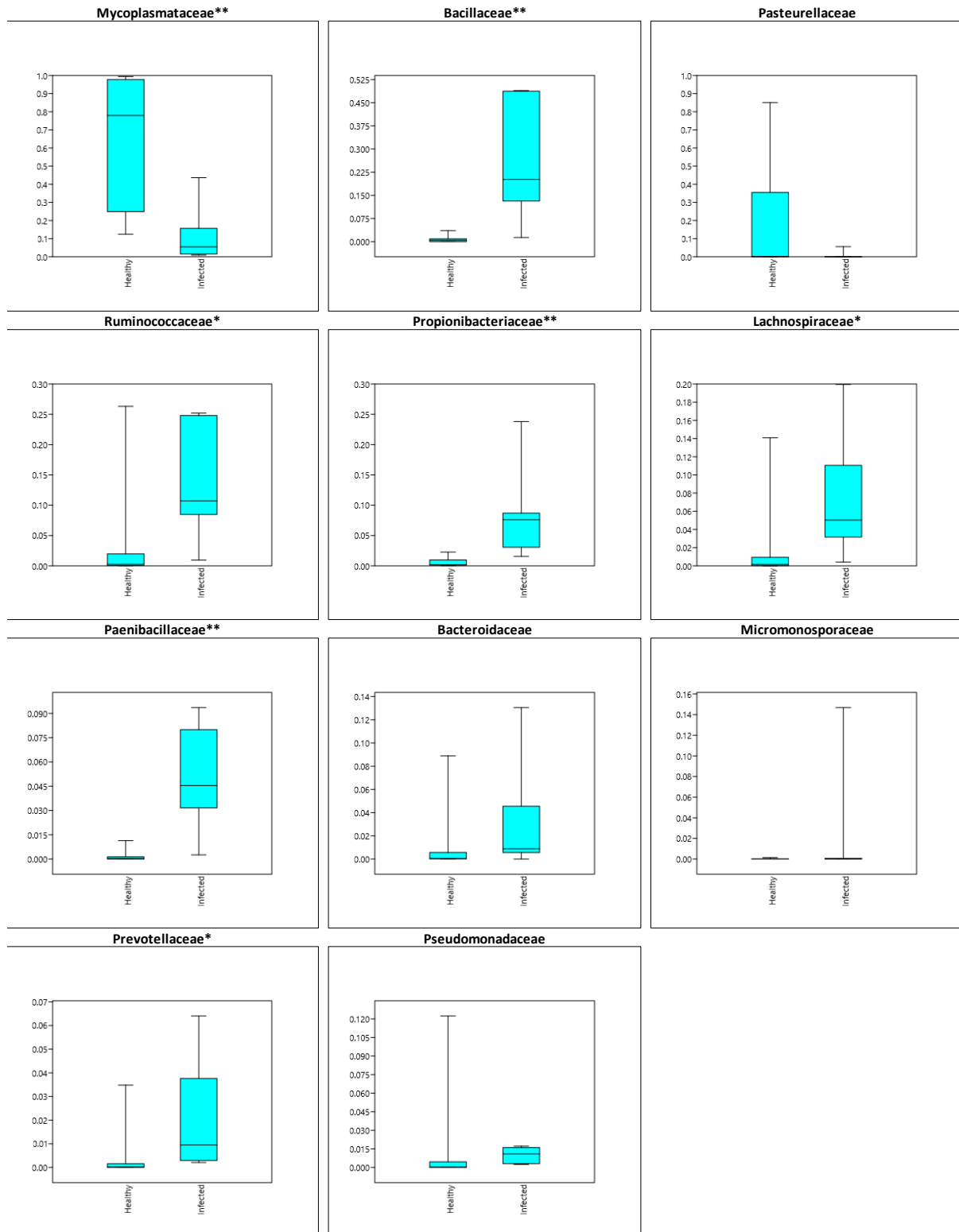


Figure 21: Box-plots of the eleven most important bacterial families' abundances for the healthy vs. infected samples. Seven of the eleven bacterial families identified by SIMPER showed significant changes in abundances due to *T. foetus* infection. Only *Mycoplasmataceae* showed a negative relationship with infection. "*" indicates significance at the 5% level and "**" at the 1% level.

6.2.4 Discussion

The bacterial phyla detected in the vagina of infected heifers during oestrus and dioestrus were similar to the phyla encountered in the healthy animals, namely: *Tenericutes*, *Proteobacteria*, *Firmicutes*, *Actinobacteria* and *Bacteroidetes*. These bacteria phyla have been described in the bovine reproductive tract by other authors (Swartz et al., 2014, Machado et al., 2012, Laguardia-Nascimento et al., 2015, Santos and Bicalho, 2012, Santos et al., 2011, Moore et al., 2017).

A distinct difference in their distribution was observed in the infected compared to the healthy groups. The healthy microbiota were dominated by the phyla *Tenericutes* (represented by the family *Mycoplasmataceae*) and *Proteobacteria* (family *Pasteurellaceae*), but changed dramatically in the infected group to a diverse composition of bacterial families belonging primarily to the phylum *Firmicutes* and *Actinobacteria*. As can be noted in Figure 16, only one of the infected samples could be said to be dominated by *Mycoplasmataceae*, and *Pasteurellaceae* barely shows a presence. *Bacillaceae*, *Propionibacteriaceae* and *Lachnospiraceae* appear to have proliferated with *Bacillaceae* dominating 2 of the 6 infected heifer samples (Figure 16). Furthermore, bacterial families which were found in low (even trace) abundances in the healthy animals have proliferated substantially in the infected animals (Figures 16 and 17).

From the results, it appears that the structure and dynamics of the vaginal microbiota in healthy samples is vastly different to that observed in the *T. foetus*-infected samples. This notion is reinforced by the heatmap provided in Figure 17. Interestingly, most bacterial families detected in the infected animals were also present in the control groups; however, mostly in low abundances. This finding demonstrates the community dynamics and the way that commensals can proliferate and behave opportunistically in an unstable environment caused by the host-parasite interaction during the early stages of colonisation of the vagina.

6.2.4.1 Alpha diversity

Figure 18 illustrates that the alpha diversity, as measured by the Shannon Entropy, is considerably higher (and apparently more stable) in the infected animals as opposed to the healthy animals, with only a single sample from a healthy animal (1c) being comparable to the figures seen in the infected animals. Figure 19 emphasizes this dynamic, depicting box-plots of the two groupings by Shannon Entropy. The box plots of the infected samples are more compact in nature than the figures of the healthy samples and the Shannon Entropies of the infected heifers appear higher when compared to the controls. Table 10 further highlights this relationship with five of the scores related to the infected samples being consistently within the upper tercile of the eighteen ranked results with only sample 1c of the healthy group achieving similar figures.

A non-parametric permutational t-test of the means of the two groupings revealed a significant difference in the alpha diversity between the two groups, confirming the previous observations and indicating that alpha diversity in *T. foetus*-infected heifers is considerably higher than that of healthy heifers. These results lend credence to the notion that *T. foetus* infection leads to a proliferation of bacteria in the heifer's vagina.

6.2.4.2 Beta diversity

Following on from the discussion in section 1 of the results, beta diversity was first examined visually, followed by statistical evaluation.

Examining the NMDS results as shown in Figure 20, it can be noted that most of the samples are grouped according to their *a priori* groupings of healthy and *T. foetus*-infected samples. The ellipses centred on the two groupings represent 95 percent confidence intervals of the respective groupings on the NMDS plain and show that the majority of the infected samples fall outside the 95 percent confidence interval of the healthy animals, while 5 of the 12 healthy samples fall outside the 95 percent confidence interval of the infected animals. Interestingly the healthy sample 1c barely resides within the 95 percent confidence interval for the healthy samples, a possible indication of an outlier. It is possible that this effect was as a result of another factor which was not observed during the project, or it is simply the natural dynamics of that particular heifer's vaginal bacterial microbiota. While this is impossible to establish, the peculiarity of that particular sample is notable.

Of further note is that the hierarchical clustering procedure grouped the samples in much the same way as the NMDS procedure (Figure 20). All the healthy samples, with the exception of 1c, were grouped together, while the infected samples were grouped with 1c. Even with the peculiarity noted in sample 1c, the groupings achieved by both procedures indicate that a difference between the vaginal bacterial microbiota of healthy vs. *T. foetus*-infected heifers is likely.

Statistical analysis of the beta diversities using the PERMANOVA (Table 11) procedure revealed this to be very much the case, with the null hypothesis that there are no differences between the relative abundances of bacterial families between healthy and *T. foetus*-infected samples being rejected at the 1 percent level. Taking all these indications together, as well as the effects seen on alpha diversity, it appears that *T. foetus* does indeed cause a profound change to the vaginal microbiota of heifers.

The results of the comparison of the healthy and infected groups revealed a substantial and statistically significant difference in bacterial community composition between the two groups, but did not reveal from where the observed differences arose. SIMPER analysis (Table 12) was therefore used to identify which bacterial families contributed the most to the difference. The procedure identified 11 families which each contributed more than 1 percent to the total dissimilarity observed between the samples, on

the basis of the converse of the Bray-Curtis similarity figure (Table 12). Non-parametric permutational pseudo t-tests revealed that seven of the bacterial families identified showed significant differences in abundance between the two sample groups at the 5 percent level, while five of those were statistically significant at the 1 percent level (Table 13, Figure 21).

This analysis revealed *Mycoplasmataceae* to be the bacterial family most affected in the transition from healthy to the diseased status. A significant drop in *Mycoplasmataceae* reads was observed in the infected group (p-value below 0.01). Conversely, the families (in order of abundance) *Bacillaceae*, *Ruminococcaceae*, *Propionibacteriaceae*, *Lachnospiraceae*, *Paenibacillaceae* and *Prevotellaceae* proliferated significantly (p-value below 0.05) in the infected microbiota. These families have been found as normal inhabitants of the bovine gastro-intestinal tract (Creevey et al., 2014, Malmuthuge et al., 2015).

Microorganisms belonging to the *Mycoplasmataceae* are surface organisms found mainly in the mucus membranes of the urogenital and respiratory tract of vertebrates. Adverse conditions causing immune suppression, or damage to the protective barriers of the mucosae, may allow proliferation and infection (Griffin et al., 2010, Afshar, 1967). At the time when the microbiota was studied, the bacterial dynamics suggests that *Mycoplasma* could possibly have competed with an outbreak of opportunistic commensal bacteria in the bovine vagina, or alternatively that it is possible that *Mycoplasma* is sensitive to environmental changes, for example changes in the pH, oxygen tension or chemical composition caused by *T. foetus*.

The dynamic of *Mycoplasmataceae* being the only bacterial family which saw a significant decline in bacterial abundance as a result of the infection, while exhibiting dominance in the healthy samples (see section 1), could indicate that the family has a stabilizing role, though further research with more sample collections over a prolonged period of time would be needed to investigate such a notion.

Copious numbers of *T. foetus* were detected in the vaginal exudate 14 days after initial infection, the period in which progesterone predominates. The survival of the parasite is related to its ability to adhere to the epithelial cell layer. Singh et al. (1999) conducted research on the interaction between *T. foetus* and bovine vaginal epithelial cells, and reported that *T. foetus* increases its adhesion significantly to progesterone-treated cells, which may also enhance protozoa multiplication and strengthen the infection.

The damage caused by the protozoa to the epithelial cell lining was not assessed in the current study. However, extensive damage of the epithelium was expected, as presented *in vitro* by Singh et al. (1999). Erosion of the epithelium, which represents a major physical barrier of the genital tract, and the change in the vaginal milieu was probably the major guide for the favourable growth of other bacteria types.

The damage caused by *Tritrichomonas foetus* on the epithelium lining and concomitant proliferation of opportunistic pathogenic bacteria present in the vagina triggers a local inflammatory immune response to control the infection. In women, sexually transmitted infections like trichomonosis and others cause disruption of genital cell membranes, mucosal immune response with an increased flow of inflammatory cells and, consequently, an increased likelihood of cells being invaded by the HIV virus (WHO, 2008). Due to the local inflammatory response produced in the vaginal tract of women, we speculate that bovine trichomonosis could enhance the acquisition of viral and bacterial pathogens transmitted venereally in different reproductive stages.

Further research of the commensal bacteria of the bovine genital tract is needed to broaden the existing knowledge of the vaginal microbiota. Cows in good general health and in an environment with sound biosecurity measures in place can be expected to display a healthy and balanced vaginal microbiota (dominated by commensals) which will not give rise to uterine infection, early embryonic losses, placentitis and pre-term birth, as shown in women. Given our observations of the association between *T. foetus* infection and the *Mycoplasmataceae* in the vaginal microbiota, a valuable contribution could be made through further study of the interaction between the organisms and the host's defences, as well as the dynamics existing between different commensal bacteria.

The commensal microbial community plays a vital competitive role and, by doing so, inhibits the growth of opportunistic organisms. The interaction that exists between the sex hormones, mucosal immune response and the microbes of the bovine genital tract and how they work in harmony to protect against infection, but simultaneously allow reproduction to continue, also requires further investigation.

7 Conclusion

The use of metagenomics provided an efficient and innovative means to uncover the healthy and *T. foetus*-infected bovine vaginal bacterial microbiota, independent of the need of bacterial media for culturing. While interpretation of the data proved to be a substantial task, the results of the exercise are satisfactory with the aims of the study being largely met.

To the authors' knowledge, this study is the first to explore the bovine vaginal bacterial microbiota over the oestrus and dioestrus stages of the cycle using metagenomics. Although certain results of this exploration were somewhat inconclusive, a tendency of a more diverse microbiota during oestrus compared to diestrus was observed. The family *Mycoplasmataceae* appeared to have played a significant role in the healthy vaginal bacterial microbiota during both stages of the cycle, with a significant increase during diestrus compared to oestrus being noteworthy. This highlights the need for further research in determining the roles of these organisms and how the sex hormones influence the microbiota.

Secondly, this study demonstrated the *in vivo* interaction between the protozoa *Trichostrongylus axei* and the bovine vaginal bacterial microbiota. It revealed that *T. foetus* infection caused significant changes in the vaginal microbiome. The microbiota of infected samples was substantially more diverse than that seen in the healthy samples. Analysis of beta diversity provided further evidence of the significant differences between the samples from healthy and infected animals. Specifically, *T. foetus* infection was associated with a reduction in relative abundance of the bacterial family *Mycoplasmataceae* while, conversely, increases to bacterial abundance were observed in the bacterial families *Bacillaceae*, *Ruminococcaceae*, *Propionibacteriaceae*, *Lachnospiraceae*, *Paenibacillaceae* and *Prevotellaceae*.

In both experiments, the role of the bacterial family *Mycoplasmataceae* appeared to be noteworthy, hinting at a possible important dynamic between the family and other commensal bacterial families in the healthy vaginal microbiota of the heifer. These dynamics, as well as the links between the proliferation of other bacteria and the pathogenesis of the disease, merit further study.

7.1 Suggestions for future research in this field

Most studies of the bovine vaginal and uterine microbiome to date have obtained samples from individuals at a single time point or with a sample interval of weeks or months. Although valid appreciation of the bacterial microbiota has been provided, more insights of the daily temporal dynamics would provide more detailed information on the community stability over a period of time and a better comprehension of the impact that sex hormones cause in the vaginal bacterial population.

Research intended to explore the temporal dynamics should ensure minimal vaginal manipulation and selection of a sampling method that avoids the infusion of agents (sterile phosphate buffered saline and/or sterile water). This is important, as these may contribute to changes in the bacterial/vaginal environment, and may consequently interfere with results.

Representative sampling of the population should take into consideration sexual maturity, copulation, parity and the environment, as these factors may impact on the diversity of the bacterial community (Panangala et al., 1978, Otero et al., 2000). Larger sample sizes should also be considered, as this will increase the confidence of the study, the sensitivity of the statistical analysis and improve the robustness of the results.

8 References

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