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And its effect on fertility in heifers

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Swedish University of Agricultural Sciences, SLU
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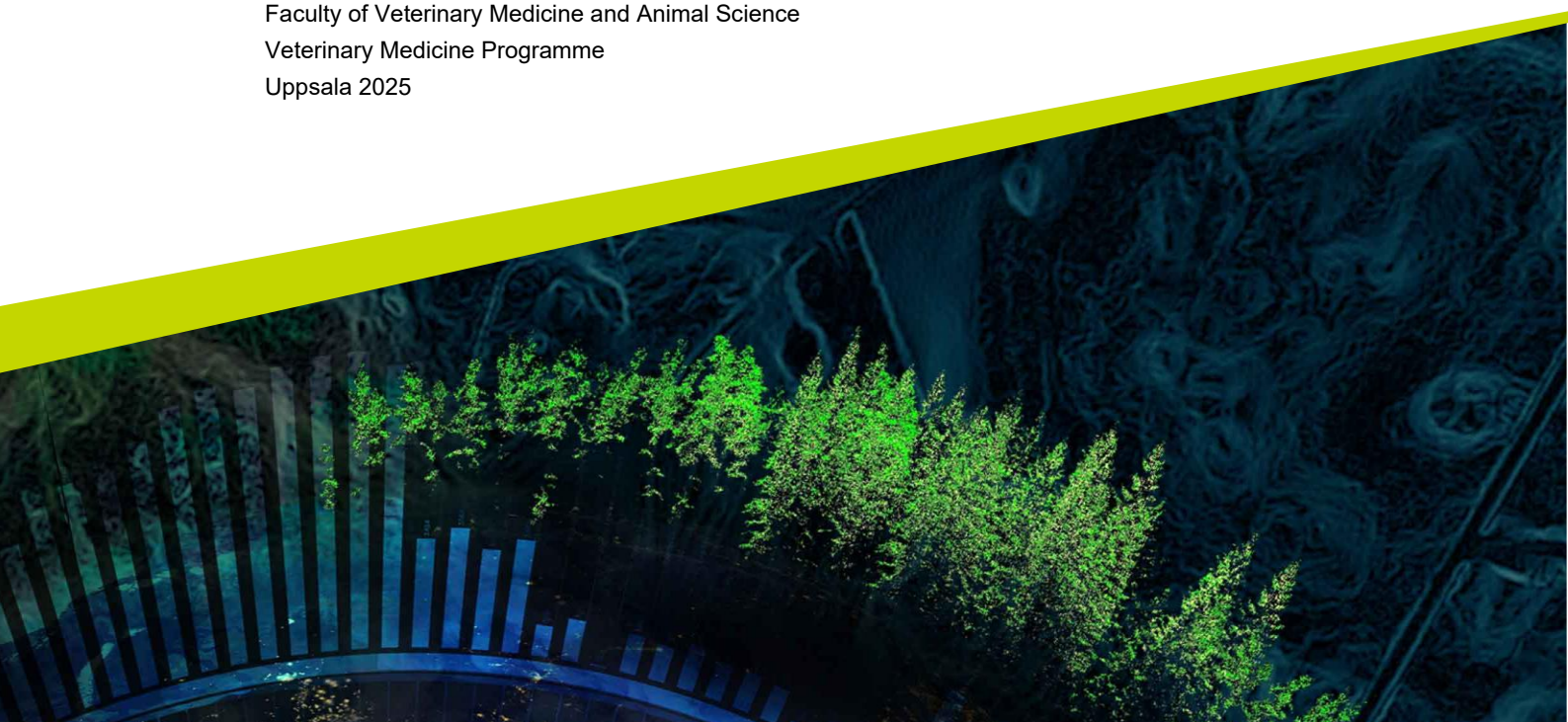

The normal vaginal microbiota in Holstein and Swedish Red cows - And its effect on fertility in heifers

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Abstract

The microorganisms found in the reproductive tract of dairy cows are essential for keeping the symbiosis balanced and healthy. They help protect against harmful bacteria and can influence overall fertility. Dysbiosis, the state when the balance of these microbes is disturbed, can lead to infections or other problems that might affect the cow's ability to reproduce successfully. Understanding how these microbial communities work together can provide insights into improving reproductive health and fertility in dairy cows.

This thesis aims to investigate what constitutes the normal vaginal flora in healthy heifers and examines whether any differences exist between the vaginal bacteria of those heifers that become pregnant and those that do not, the first time they are inseminated. Most currently published studies on the subject have investigated the bacterial flora when the cow is already experiencing sickness or fertility issues such as endometritis, retained placenta and metritis, whereas this study aims to show what is present before the heifer is first inseminated, as well as how the microbiota changes after insemination.

The study was conducted by sampling bacteria from the cranial vagina of healthy heifers prior to first insemination (D0) and within six days post-insemination (D6). Double guarded vaginal swabs were used for sampling and were plated out on four different agar media within 1-2 hours after sampling. Chocolate and blood agar plates were incubated at 37 °C with 5% CO2 for 24+24h. Lactose purple agar, and mannitol salt agar plates were incubated at 37 °C for 24+24h. Bacterial colonies were identified by Matrix-Assisted Laser Desorption / Ionization Time of Flight Mass Spectrometry (MALDI-TOF MS). Our findings showed that the microbiota in the vagina of heifers mainly consisted of *Actinobacillus seminis, Streptococcus* spp*., Histophilus somni* and *Bacillus* spp. both before and after artificial insemination. However, *Staphylococcus* spp. were more often isolated from the group of heifers that did not become pregnant at first insemination. This study provides valuable knowledge into the microbial species inhabiting the reproductive tract of dairy cows, underscoring the necessity of understanding the roles of these communities for enhanced reproductive outcomes and herd health. Further research is needed to explore the specific roles and interactions of these microbial species in the reproductive tract of cattle and the environment.

Keywords: AI, artificial insemination, cattle, bacteriology

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1. Introduction

The normal vaginal microbiota in cattle is made up of numerous different microbes, including different strains of bacteria. Normally there is balance among these bacteria, making the vaginal environment stable and able to function as protection against pathogenic microorganisms that could possibly cause disease or fertility issues. New microbes are constantly introduced into the vaginal environment, either by natural breeding, artificial insemination or contamination from the outer environment, and at the same time these microbes are also spread to the environment (Adnane *et al.* 2018; Appiah *et al.* 2020). Most currently published studies on the subject have focused on the bacteria present during uterine and vaginal disease, commonly in cows that have recently calved. This study is focused on investigating what bacteria are present in the vagina of healthy heifers, both before and after artificial insemination. Another question this thesis aims to answer is whether differences exist between the heifers that become pregnant and those that do not. This groundwork contributes to a solid base on which to explore other related subjects, such as investigating what effect the antibiotics present in semen samples for insemination have on the bacterial flora in the vagina of cattle.

2. Literature review

2.1 Risk factors for vaginal contamination

The anatomy of the vagina itself affects the microbiota in the vaginal canal in females of different species, including cattle.

2.1.1 Location on the body and defence mechanisms

Due to the anatomical location of the vagina and the closeness to the anus, the risk of faecal contamination of the reproductive tract through the vagina is high. The first line of defence to reduce the risk of bacterial contamination of the vagina is the vulva, consisting of the vulva lips, which are generally closed and therefore protect the vagina from contamination, as well as the cervix that also serves as a barrier between the vagina and the uterus, protecting the uterus from infections.

The second line of defence is the cervico-vaginal mucus (CVM) which mainly consists of water, mucins and epithelial cells from the lining of the uterus, cervix and vagina (Adnane *et al.* 2018). The flow of CVM is continuous, with varying properties depending on the time in the cycle. One of its functions is to trap bacteria and transport it out of the vagina(Adnane *et al.* 2018). This CVM also contains immunoglobin A (IgA), lysozyme and lactoferrin, which mediate microbial killing and block adhesion of bacteria in the vaginal tract. The characteristics of the CVM change depending on the stage of the oestrous cycle, being more liquid and having a higher pH during ovulation than during other times in the oestrous cycle (Adnane & Chapwanya 2022). The CVM aids the transport of spermatozoa and also help them stay viable for fertilization. During the luteal phase of the oestrous cycle, the CVM is more viscous and the pH is lower to protect against pathogens. This change is due to the influence of progesterone produced by the corpus luteum and oestrogens produced by the dominant follicle.

As well as the anatomical or structural defence against pathogens, the local immune system in the vaginal tract plays an important role (Sheldon & Owens 2017). Innate bacteria lower the pH in the vagina and may compete for nutrients with potentially pathogenic bacteria. The antimicrobial peptides, glycoproteins and mucins in the vagina, cervix and uterus also protect against pathogens by countering bacterial contamination and hinder growth of pathogenic bacteria. If a microbial invasion of the vaginal tract occurs, there are adaptive immune responses, with higher levels of antibodies present. The innate immunity in the female genital tract is dependent on the binding of pathogen-associated molecular patterns from microbes and pattern recognition receptors in the host cells. This starts an immunological response that triggers inflammation to fight infection.

Anatomical differences within the cattle population may affect the probability of faecal contamination of the vagina. Thus, if a greater portion of the vulva

opening is positioned above the level of the pelvic brim, or if the slope of the vulva is angled more horizontally, contamination is more likely in comparison to a vulva that is more vertical (Gautam & Nakao 2009). Having a more angled slope of the vulva also increases the risk of urovagina, compared to having a less angled slope, which could affect the defence mechanisms against microorganisms in the vagina.

Horizontal vulva

According to Gautam and Nakao (2009), a cow with a horizontal vulva, or a vulva with a higher slope, is defined as a cow with vulvar lips angled more than 45° from the vertical line. Having a horizontal vulva increases the risk of both urovagina and faecal contamination.

Urovagina

Urovagina is defined as the accumulation of urine in the vagina. Cases are classified from mild to severe (Gautam & Nakao 2009). Cows with moderate to severe urovagina had a lower success rate with artificial insemination and lower pregnancy rate compared to cows without urovagina, and therefore required a greater number of inseminations per pregnancy. Urovagina was associated with a greater risk of endometritis, leading to a negative effect on fertility. Risk factors for urovagina include low body condition score (BCS) postpartum, endometritis within 60 days postpartum and a more horizontal vulva.

Pneumovagina

Pneumovagina is when air is spontaneously sucked into the vagina (Goncagul *et al.* 2012). Pneumovagina leads to faecal contamination and bacteria being drawn into the vagina, which in turn can lead to infertility due to endometritis and uterine infections. A pathognomic sign of pneumovagina is foamy vaginal discharge during oestrous, and this can be seen even in mild cases.

2.1.2 Epithelium in different parts of the vaginal tract

The different parts of the vagina have different embryological origin, and hence differ in terms of epithelium (McGeady *et al.* 2017). When the vagina is formed, the tissue comes from two different structures: the paramesonephric duct and the urogenital sinus that fuse, forming a junction. This junction marks the border between the caudal and cranial vagina. The cranial vagina originates from the paramesonephric duct and is lined with simple ciliated columnar epithelium, while the caudal vagina originates from the urogenital sinus and is lined with stratified squamous epithelium (McGeady *et al.* 2017; Sjaastad *et al.* 2016). Clinically speaking, this difference is important since stratified epithelium can tolerate more irritation and abrasion than simple columnar epithelium (Sjaastad *et*

al. 2016). Some resident bacteria in the vagina produce lactic acid, which lowers the pH in the vagina and increases the resistance of the vaginal epithelium to bacterial infections (Sjaastad *et al.* 2016). Such bacteria include *Lactobacillus* spp. (Otero *et al.* 1999; Tachedjian *et al.* 2017)

2.1.3 Physiology of the oestrous cycle in cattle

Oestrus is defined as the period in which a female is receptive to mating, whereas an oestrous cycle is the time period from the beginning of one oestrus to the next (Sjaastad *et al.* 2016). Signs of a heifer entering oestrus include behavioural changes such as restlessness, changes in vocalisation and reduced appetite, and physical changes such as swelling of the vulvar lips and vaginal discharge. These changes start appearing prior to oestrus, during a period called pro-oestrus, to indicate that oestrus is coming. During proestrus the follicles produce increasing amounts of oestradiol leading up to oestrus.

The oestrous cycle can be divided into two different phases, the follicular phase and the luteal phase (Sjaastad *et al.* 2016). The follicular phase consists of the subphases pro-oestrus and oestrus. During pro-oestrus, a few primordial follicles are selected to grow and start secreting increasing amounts of oestradiol. It is yet unknown by what mechanism primordial follicles are selected to grow. When the levels of oestradiol in plasma reaches a certain concentration, ovulation is initiated, and oestrus begins. After oestrus, the luteal phase begins with the subphase metoestrus, during which granulosa and theca cells form the corpus hemorrhagicum, which later turns into the corpus luteum that develops in the ovaries, making progesterone the dominant hormone. Ovulation in cattle occurs during early metoestrus, differing from other animals where it occurs during oestrus. The following period is called dioestrus and is the time when the mature corpus luteum secretes large amounts of progesterone, ending when it undergoes luteolysis and the progesterone levels drop significantly.

During the luteal phase when the plasma progesterone concentration is high, the release of gonadotropin-releasing hormone (GnRH) is suppressed due to a negative feedback mechanism (Sjaastad *et al.* 2016). The rapid drop in progesterone levels following luteolysis results in the removal of this negative feedback signal, leading to the release of GnRH from the hypothalamus which, in turn, leads to increasing levels of both luteinizing hormone (LH) and follicle-stimulating hormone (FSH) from the anterior pituitary. In contrast, FSH causes the follicles to develop and mature, stimulating them to produce oestradiol, resulting in the start of the follicular phase with pro-oestrus and oestrus. The concentration of LH peaks during oestrus, causing the dominant follicle to ovulate.

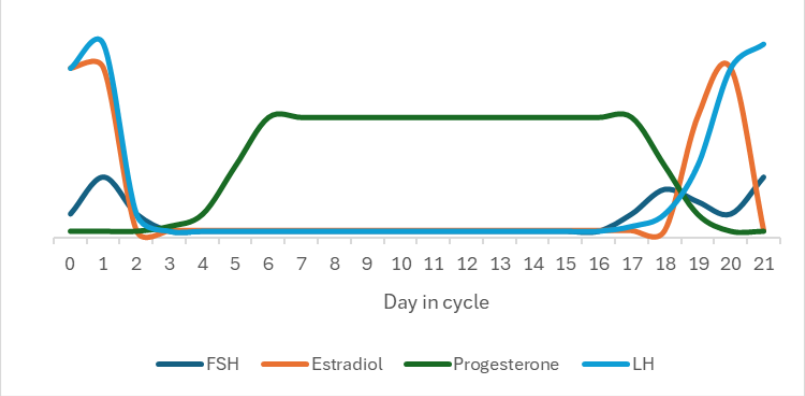

Figure 1. Schematic view of hormonal activity during the oestrous cycle. Illustration by the author.

Bovine epithelial cells in the oviduct are influenced by oestradiol, progesterone and LH prior to ovulation, which maintain immunologic homeostasis (Marey *et al.* 2016). These hormones inhibit proinflammatory responses that would kill sperm, while differentiating between sperm and microbes(Marey *et al.* 2016). Microbes are eliminated via phagocytosis caused by a proinflammatory response due to different mechanisms associated with the recognition of molecules linked to the microorganisms. During the preovulatory period, polymorphonuclear neutrophils are present in the oviduct fluid under physiological conditions. During oestrus, high levels of oestrogens affect the reproductive tract, resulting in the presence of large quantities of macrophages, granulocytes and dendritic cells, indicating that the local immune system in the uterus is at its peak (Kaushic *et al.* 1998). In the vagina, however, it was found that the opposite is true compared to the immune response in the uterus, meaning that more immune cells are present in the vagina during dioestrus than during oestrus (Kaushic *et al.* 1998).

Per farm protocol, heifers showing signs of heat at night are inseminated the following morning, and heifers showing signs of heat in the morning are inseminated in the evening. The optimal time interval to inseminate seems to be between 5-17 hours after the cow starts to increase her activity levels (Roelofs 2018). This is due to ovulation occurring during early metestrus in cattle, approximately 12 hours after the end of oestrus (Sjaastad *et al.* 2016).

A difference between natural mating and artificial insemination is where the semen is deposited (Sjaastad *et al.* 2016). During mating, the semen is deposited in the vagina, after which the spermatozoa are transported with the help of muscle contraction and highly viscous CVM through the cervix and into and through the uterus to the oviduct for fertilization. With the most common technique of AI the semen sample is deposited in the anterior cervical os (López-Gatius 2000; Hopper 2014).

2.1.4 Pregnancy

The immune system has a complex way of responding to mating, insemination and subsequent fertilization (Fair 2015). One response is initiated during the transmission of seminal fluid, when the female genital tract first comes into contact with paternal antigens. Pregnancy recognition is mainly signalled by type 1 interferon, IFNT, in cattle. This IFNT down regulates the expression of the endometrial epithelial cell oestrogen receptor and oxytocin receptor, which leads to an anti-luteolytic response that allows the corpus luteum to continue its progesterone production.

During pregnancy, all mammals depend on the hormone progesterone to maintain pregnancy (Sjaastad *et al.* 2016). In cattle, the plasma progesterone levels are highest in the beginning of pregnancy, lower toward the second half of pregnancy, before finally dropping significantly immediately prior to parturition. The progesterone in cattle is produced by functioning corpora lutea for the first two trimesters, before the placenta is able to secrete enough progesterone to maintain pregnancy (Hopper 2014). The placental production of progesterone is dependent on input from the corpus luteum (Hopper 2014). The plasma level of oestradiol is low during the entire pregnancy, until shortly before birth, when the levels increase greatly (Sjaastad *et al.* 2016).

When a cow is pregnant, the uterus is protected from microbial contamination by the CVM, which forms an impenetrable mucus plug (Adnane *et al.* 2018).

2.2 Normal bacterial flora in cattle

Bacteria are present in the vagina as a part of the normal microbiota in healthy cattle. In previous studies, species of bacteria commonly found in vaginal samples from cattle include coagulase-negative staphylococci, *Bacillus* and *Campylobacter* spp. (Petit *et al.* 2009; Zambrano-Nava *et al.* 2011; Rodrigues *et al.* 2015; Appiah *et al.* 2020). Other studies have not found an abundance of different bacteria in the vagina of healthy cattle, and *Lactobacillus* was considered to be the dominant bacteria in a healthy cow vagina (Chao *et al.* 2015). Some studies suggest that the bacterial community in the vagina varies depending on stage of the oestrous cycle, finding more *Streptococcus* spp. during the luteal phase and more *Lactobacillus* spp. during the follicular phase (Wang *et al.* 2018).

2.3 Artificial insemination

Artificial insemination (AI) is a common practice in animal husbandry and raising livestock (Mulu *et al.* 2018). Materials used are a straw containing the semen sample that is thawed in a warm water bath, an insemination gun that the straw is placed in that is later used to push the semen sample out of the straw, and a rectal glove for the hand used to palpate the cervix through the rectum.

The best and safest way of artificial insemination is the “recto-vaginal method”, meaning that the person inseminating uses a sterile catheter that is inserted into the vagina, after which the person uses their other, gloved hand to palpate the vagina and cervix through the rectum (Mulu *et al.* 2018). The hand inside the rectum is used to guide the catheter, or straw, into the uterus through the cervix, making sure not to go too far in to make sure that the uterus is not injured. Once the straw is in the anterior os of the cervix, part of the semen sample is carefully deposited inside the uterine body, after which the catheter is withdrawn, depositing the rest of the sample in the cervix on its way out. Another possible way to inseminate is to deposit the sperm deeper inside the uterus, closer to the uterine horn, either bilaterally or only in the horn with the ovulating follicle (Hopper 2014).

Most commercial insemination doses in Sweden contains antimicrobial substances (AMS), concentrations of which were previously regulated by law, as presented in Table 1. A legal alternative to these substances was a combination of antibiotics with the same effectiveness as these substances against *Campylobacter*, *Leptospira* and *Mycoplasma* (SJVFS 2004:41 Statens jordbruksverks föreskrifter om seminverksamhet med nötkreatur).

Table 1. Antimicrobial substances and their concentrations in semen samples (SJVFS 2004:41).

Antimicrobial substance	Concentration
Streptomycin	500 µg/mL
Penicillin	500 IE/mL
Lincomycin	150 µg/mL
Spectinomycin	300 µg/mL

The reason why most semen extenders contain antibiotics is to inhibit growth of possibly pathogenic bacteria present in the semen samples, stemming either from contamination from the bull, from semen collection or from the handling of the samples (Sannat *et al.* 2015). *Fusobacterium* spp. and *Histophilus* spp. are the bacteria most commonly found in semen samples in Sweden (Cojkic *et al.* 2024). Other bacteria commonly found were *Streptococcus* spp*., Staphylococcus* spp. and *Bacillus* spp. (Bielanski 2007; Cojkic *et al.* 2021).

Certain microbes can cause uterine infection, including *Escherichia coli, Fusobacterium necrophorum, Trueperella pyogenes, Prevotella and Bacterioides* (Sheldon & Owens 2017; Appiah *et al.* 2020). Diseases in the reproductive tract (vaginitis, cervicitis, endometritis etc.) often results in sub- and infertility, causing economic loss for the farmer, and are a common reason for culling of the affected animals (Sheldon & Owens 2017).

3. Method and materials

3.1 Literature review

The literature review was conducted using the keywords cattle, vaginal flora, environment, bacterial and microbiota in Google Scholar, PubMed and Web of Science and using a cut-off point at 20 years old, as well as searching for other articles within the reference lists of relevant articles.

3.2 Animals and sampling technique

Bacterial samples were collected from 26 heifers at the University Livestock Research Centre, Lövsta. Heifers had not been treated with antibiotics or inseminated previously. Samples were collected just before the first insemination (D0) of the heifer, with an additional sample taken within six days post-insemination (D6). The samples were taken between April and September 2023 by the same person. Oestrus was identified by the staff reports of heifer behaviour in the form of decreased appetite, increased vocalisation and mounting, in combination with analysis of movement using activity meters and was confirmed by examination of the external and internal genitalia as per farm routines.

The anatomical area for sampling was chosen after previous inspection of organs from the slaughterhouse. The samples were taken from an area of the cranial vagina, approximately 3 centimetres distal to the vaginal fornix.

The sampling was performed with the heifers confined in a stock at the time of insemination, as per the normal routine for the farm. The perineal area and vulva were cleaned with at least three washes with soap and clean lukewarm water until they were visibly free of dirt. The vulva and surrounding skin were then dried using bleached paper that was then inspected for cleanliness. If dirt was detected at this stage, the cleaning routine was performed again until visible cleanliness was achieved.

The heifers were sampled using a high hygienic standard with the hand in a clean rectal glove, sterile paraffin oil and a double-guarded occluded swab to avoid touching the lips of the vulva. One hand was used to palpate the sampling area rectally, while the other hand held and maneuvered the swab. The sampling area was identified by palpating the swab by the portio and then moving caudally a distance of approximately three centimetres. The sterile swab was then in contact with the ventral vaginal wall for about 15 seconds while being rotated to ensure that an adequate sample was collected.

The swab was then directly transferred into Amie’s medium and marked with the heifer’s number, date and time of sampling. The sample was then transported directly to the laboratory at SLU, within one hour of the sample being collected.

At the laboratory, the sample was stored at refrigerator temperature until plating out, which occurred within 30-60 minutes upon arrival.

3.3 Microbiology: Culture and identification of isolates

Using an inoculation loop containing approximately10 µL, the bacteria were cultured on bovine blood agar, chocolate agar, lactose purple agar (selective for *Streptococcus* spp.), and mannitol salt agar (selective for *Staphylococcus* spp.). The lactose purple agar, and mannitol salt agar plates were incubated at 37 °C for 24 + 24 hours, whereas chocolate agar and blood agar plates were incubated at 37 °C in 5% CO_2 for 24 + 24 hours. Individual colonies were selected for further plating out to obtain pure cultures. From the pure cultures, individual colonies were then identified to species level by Matrix-Assisted Laser Desorption/Ionization Time of Flight Mass Spectrometry (MALDI-TOF MS). Identification of some Gram-negative bacteria was facilitated by adding 0.1 µL formic acid to the MALDI-TOF plate before the matrix was applied. The MALDI-TOF MS was performed by supervisor Theodoros Ntallaris at the BVF laboratory at SLU.

4. Results

4.1 Normal vaginal flora in healthy heifers before AI

On the genus level, the majority of bacteria found in samples taken just before artificial insemination belonged to the genera *Actinobacillus*, *Streptococcus*, *Histophilus* and *Bacillus*. Other species found belonged to genera *Corynebacterium*, *Staphylococcus*, *Facklamia*, *Escherichia*, *Kocuria*, *Lysinobacillus* and *Micrococcus*, as presented in Figure 2. In total 11 genera were identified in the samples.

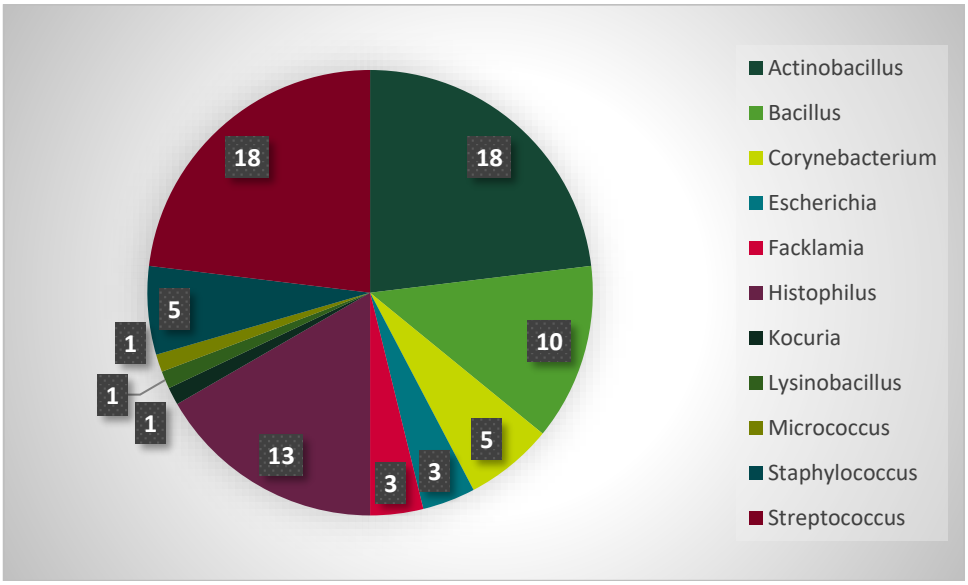

Figure 2. Results from sample 1 (collected before AI) on a genus level (n=26).

On the species level, the most diverse genus was *Staphylococcus* consisting of *S. borealis* (n=1), *S. chromogenes* (n=1), *S. epidermidis* (n=1), *S. haemolyticus* (n=1) and *S. hominis* (n=1). Other genera with more than one species identified within these samples were *Bacillus* and *Corynebacterium*. The most common species in the *Bacillus* genus was *Bacillus pumilus* (n=4) and *Bacillus licheniformis* (n=3), whereas the most common species of *Corynebacterium* was *Corynebacterium renale* (n=4), as presented in Figure 3. In total 16 species of bacteria were identified. In addition to the 16 identified species there were also 3 instances where only the genera could be identified.

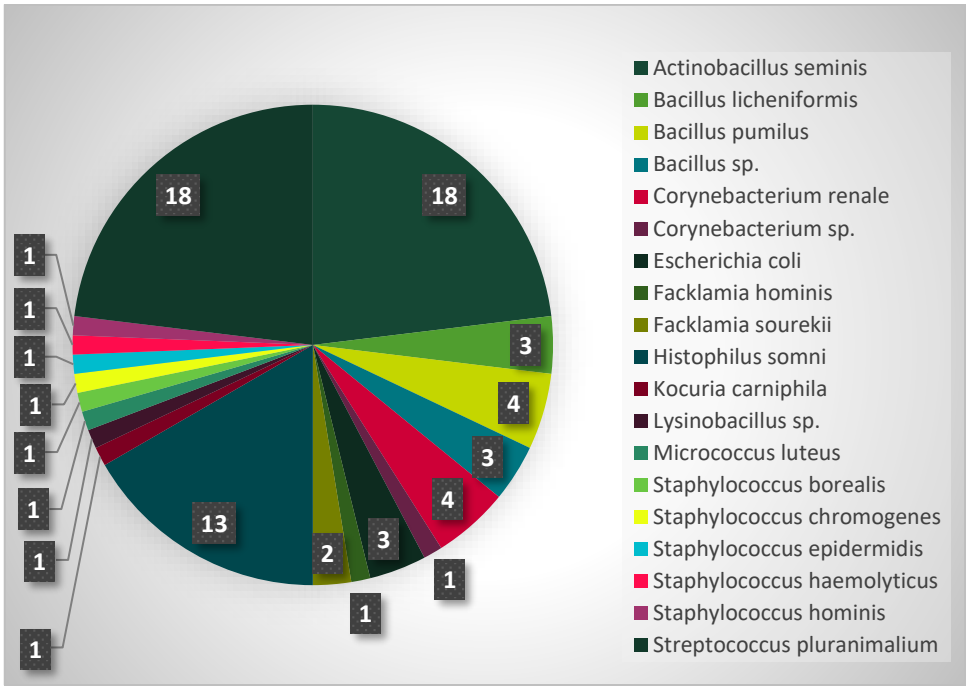

Figure 3. Result from sample 1 (collected before AI) on a species level (n=26)

4.2 Normal vaginal flora in healthy heifers after AI

The results from the second sample, collected within 6 days post-insemination, showed similar results to the first sample, but were more diverse at the species level. The four most common genera found in these samples were *Actinobacillus* (n=17), *Bacillus* (n=14), *Histophilus* (n=13) and *Streptococcus* (n=20), as shown in Figure 4. In total 13 genera were identified in the samples.

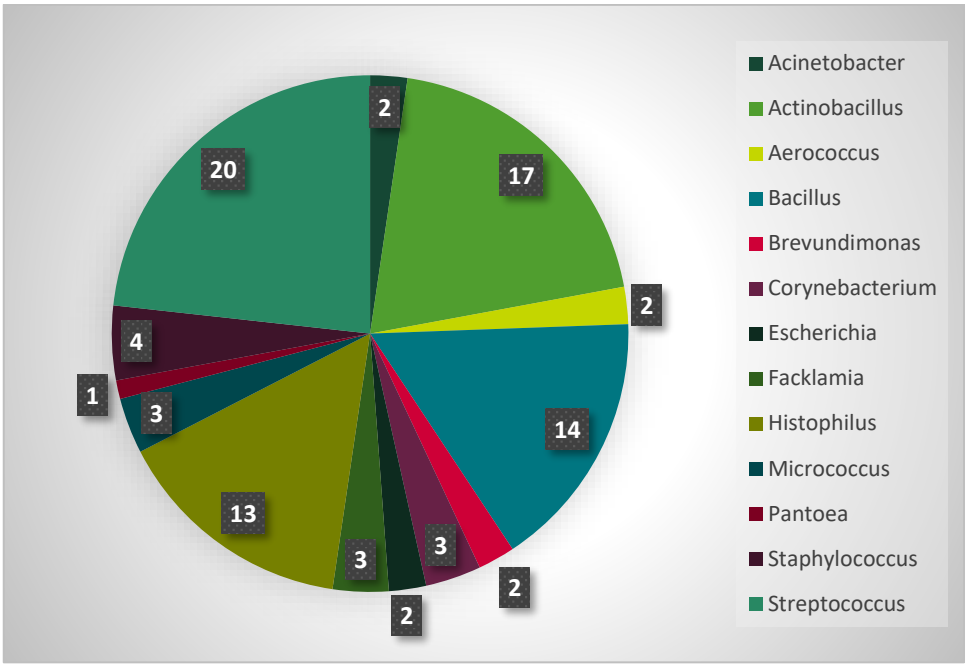

Figure 4. Results from sample 2 (6 days post AI) on a genus level (n=26).

The most diverse genera found were *Staphylococcus*, *Bacillus* and *Micrococcus*. The genera *Staphylococcus* was represented by *Staphylococcus borealis*(n=1), *Staphylococcus epidermidis*(n=2) as well as *Staphylococcus* that were unable to be identified on a species level in two heifers. Both *Bacillus* and *Micrococcus* were also represented by two identifiable species each, and some instances where only the genera was identified. Regarding only the genera being identified, this was the case in five heifers regarding *Bacillus* sp. and in one heifer regarding *Micrococcus* sp. The identified species in these two genera were represented by *Bacillus licheniformis*(n=4), *Bacillus pumilus*(n=5), *Micrococcus flavis*(n=1) and *Micrococcus luteus*(n=1). Two species of *Acinetobacter* were detected within the samples, *Acinetobacter lwoffi* in one heifer and *Acinetobacter speudolnoffi* in another heifer. Also, *Aerococcus* had more than one species represented, namely *Aerococcus spyro* and *viridans*, as seen in Figure 5. In total 20 species were identified in the samples, as well as 3 instances when only the genus was identified.

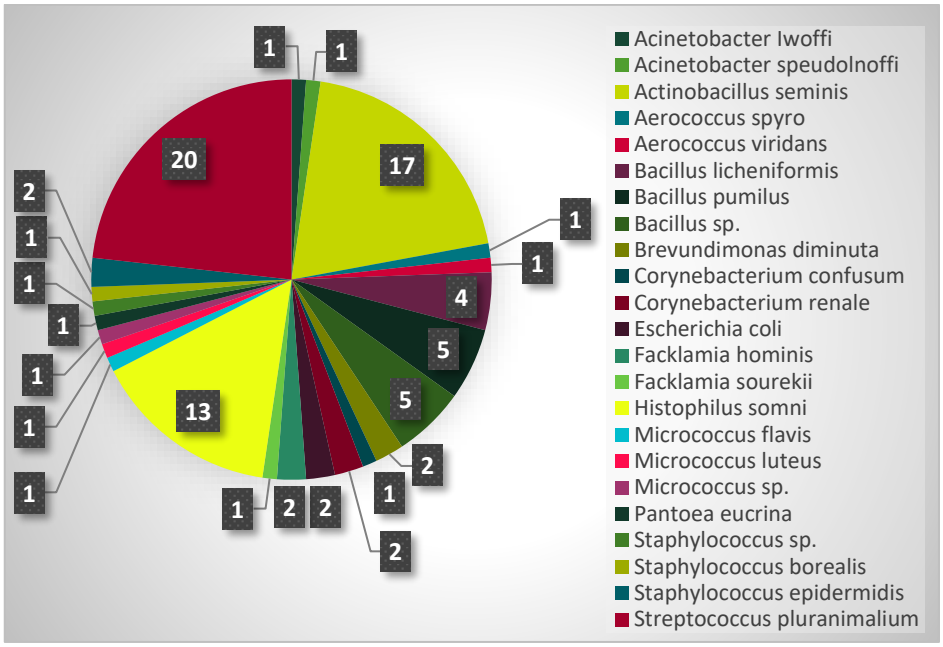

Figure 5. Results from sample 2 (6 days post AI) on a species level (n=26).

4.3 Vaginal flora in pregnant and non-pregnant heifers

After insemination, all heifers were examined using ultrasound to confirm pregnancy at Day 40 post insemination as per farm protocol, and the result was noted in the farm journal.

The five most representative bacteria isolated in heifers that became pregnant (n = 20) at first insemination vs. those that did not (n= 6) are shown in Figure 6 and 7 below. *Staphylococcus* spp. were more often isolated from the group of heifers that did not get pregnant at first insemination.

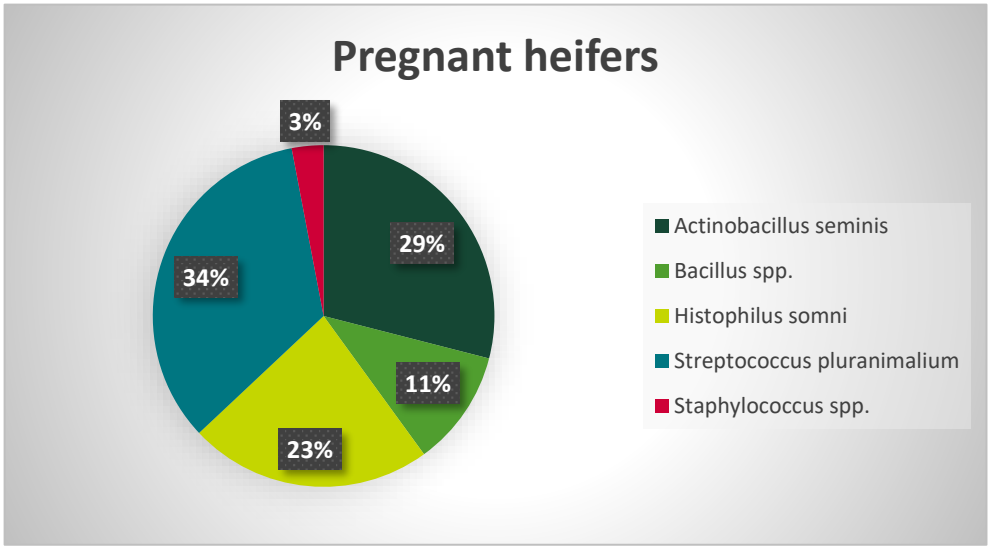

Figure 6. The five most representative bacteria on a species level isolated on day 0 and 6 from heifers that became pregnant.

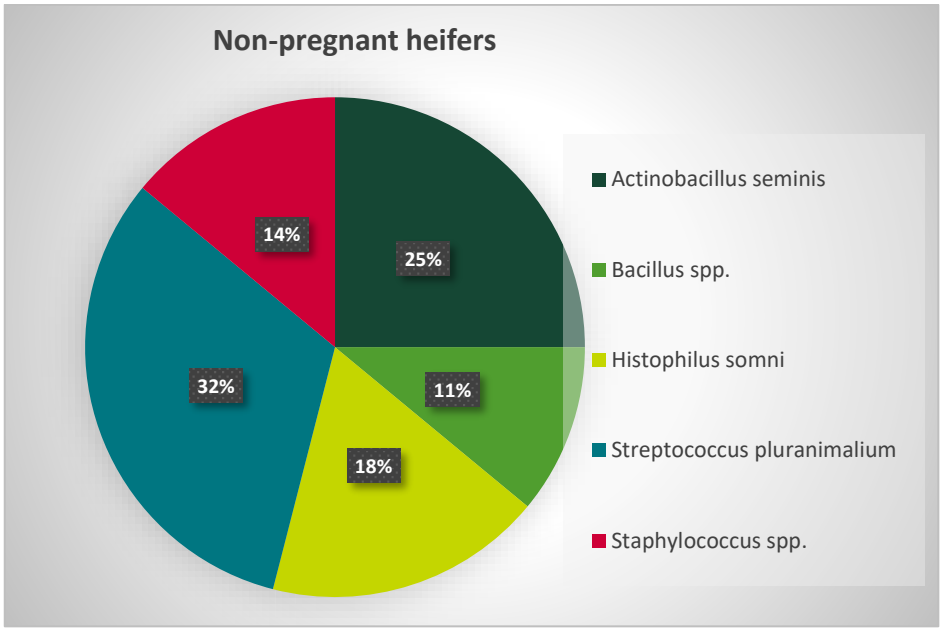

Figure 7. The five most representable bacteria on a species level isolated on day 0 and 6 from heifers that did not become pregnant.

5. Discussion

This study was conducted to investigate what constitutes the normal vaginal flora in healthy heifers, how it changes after insemination and whether differences exist between heifers that become pregnant at first insemination and heifers that do not. This was warranted because of the relative lack of knowledge on the subject, and to learn more about bacterial effects on fertility in healthy animals.

In comparison to the studies by Petit *et al.* (2009), Zambrano-Nova *et al.* (2011) and Rodrigues *et al.* (2015), we also found that the vaginal flora of these heifers contained *Bacillus* spp. and *Staphylococci* spp. However, the bacteria most commonly found differed slightly, with our results showing *Actinobacillus* spp., *Streptococcus* spp. and *Histophilus somni* being more common than previously shown. Contrary to the findings of Chao *et al.* (2015), we did not find *Lactobacillus* to be dominant.

No previous studies investigated the differences in vaginal bacteria before and after artificial insemination, and more studies on this subject would be beneficial. Our findings suggest that the bacterial flora at the second sampling is more diverse than during the first sampling, but due to our study not including a control group we cannot know whether this is due to some effect from the cycle itself or from the insemination. Furthermore, our study does not investigate where these other bacteria come from. They might stem from the insemination itself in the form of contamination from semen samples, from some other type of contamination, or they could come from the environment. We have not investigated whether these bacteria would be present without the insemination, and in that case be a part of normal development of the heifer.

Regarding the differences found in the normal vaginal microbiota in heifers that got pregnant by the first insemination versus the heifers that did not, our results suggest that the presence of *Staphylococcus* is in some way linked to difficulties getting pregnant. This correlation should be furthered studied to better understand it.

The insemination dose in cattle is either 0.25 or 0.5 ml and the amount of antibiotics is small; therefore the likelihood of these antibiotics influencing the vaginal flora is low. During artificial insemination the semen sample is deposited inside the uterus, but the liquid portion comes out through the vagina via backflow. Therefore, some antibiotics will be present in the vagina of some animals, and could affect the microbiota there.

Finding data on the normal vaginal bacterial flora in healthy cattle, and specifically healthy heifers, was difficult due to the relatively small number of publications on the subject.

Further studies are needed to assess why the microbiota changes from before insemination to after, for example if it is in fact due to the insemination

procedure, if it comes from some other type of contamination or if it is simply an effect of the heifer maturing and growing older. To do this, several control groups would be needed, such as non-inseminated heifers, heifers inseminated without antibiotics in the semen sample, or other studies that include investigating changes in microbiome in relation to hormonal changes in the animals.

One limitation with the chosen method for identifying bacteria is that not all bacteria are culturable, meaning that we cannot grow them on agar plates, and therefore they would not be identified using our method. Another limitation with the chosen method is the manual identification of bacterial colonies, the higher diversity in samples collected 6 days after insemination could be attributed to increased experience in colony phenotype differentiation.

These problems could have been avoided using a different method, for example the 16S rRNA gene sequencing method, where bacterial rRNA-coding DNA is sequenced to identify all bacteria present in a sample (Schmidt & Relman 1994). However, this method does not distinguish between viable bacteria and DNA fragments. The plating method was chosen to be able to further analyse the isolated bacteria to investigate development of antibiotic resistance as a result of artificial insemination.

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Popular science summary

This research was performed to gain a better understanding of what types of bacteria live in the vagina of healthy heifers. Before this study, most of the research centred around the bacteria found in the vagina of sick cows, but without any knowledge of what is normal, it is difficult to know what is abnormal. To better understand what bacteria are making cows sick in different stages of life, it is good to know what bacteria they already carry when they are still healthy.

Different factors affect which bacteria are normally present in a cow vagina, for example different anatomical variations that makes contamination more likely to occur, such as having a vulva that is angled in such a way that feces are likely to enter the vagina. Another factor that affects what bacteria are present in the vagina is which stage of the oestrous cycle the cow is in.

We conducted this study by taking bacterial samples from the inner vaginas of healthy heifers before and after insemination. We analysed what bacteria were present by growing the bacteria on agar plates, after which the colonies were identified using a method called Matrix-Assisted Laser Desorption/Ionization Time of Flight Mass Spectrometry, or MALDI-TOF MS for short.

What was discovered during this study was that the majority of heifers have mostly the same types of bacteria in their vagina, and that after they have been inseminated the types of bacteria are more diverse. However, we cannot know if this change is due to the insemination, from the stage of the cycle, or for any other reason, due to this study not containing any control animals that were not inseminated. These results differed from some previous research but agreed with most articles on the subject.

We also found that *Staphylococcus* spp. were more often found in the vagina of heifers that do not get pregnant at their first insemination compared to those that do get pregnant.

There are some limitations to the method we used for identifying bacteria, such as the fact that we are not able to grow all bacteria on agar plates. One of the reasons the method was chosen was the possibility to save samples for further investigation on the development of antibiotic resistance, which would not have been possible using other methods of identification.

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