Bacteria in the vagina during the estrous cycle in mares

Timjan Åkerholm

Independent Project • 30 credits
Swedish University of Agricultural Sciences, SLU
Faculty of Veterinary Medicine and Animal Science
Veterinary Medicine Programme

Uppsala 2023

Bacteria in the vagina during the estrous cycle in mares

Bakterier i vagina under brunstcykeln på ston

Timjan Åkerholm

Supervisor:	**Jane Morrell, Swedish University of Agricultural Sciences, Department of Clinical Sciences**
Assistant supervisor:	Ingrid Hansson, Swedish University of Agricultural Sciences, Department of Biomedical Sciences and Veterinary Public Health
Assistant supervisor:	Johanna Lindahl, Swedish University of Agricultural Sciences, Department of Clinical Sciences
Assistant supervisor:	Pongpreecha Malaluang, Swedish University of Agricultural Sciences, Department of Clinical Sciences
Examiner:	Görel Nyman, Swedish University of Agricultural Sciences, Department of Clinical Sciences

Credits:	30 credits
Level:	Second cycle, A2E
Course title:	Independent Project in Veterinary Medicine
Course code:	EX0869
Programme/education:	Veterinary Medicine Programme
Course coordinating dept:	Department of Clinical Sciences
Place of publication:	Uppsala
Year of publication:	2023
Cover picture:	Photograph by T. Åkerholm
Copyright:	All featured images are used with permission from the copyright owner.

Keywords:	Artificial insemination, bacteriology, breeding, embryo transfer, equine reproduction, estrous cycle, ovulation, stud farm, vagina

Swedish University of Agricultural Sciences
Faculty of Veterinary Medicine and Animal Science
Veterinary Medicine Programme

Abstract

An understanding of the normal bacterial flora of any organ is essential to provide the background to conditions and interventions that might cause the flora to change. In the vagina of the mare, a change of bacterial flora could be induced by introduction of semen, treatment with antibiotics, backflow from an unhealthy uterus etc. Previous studies that have aimed to map the vaginal bacterial flora of the mare are not all conducted in the same way, and results are not altogether consistent. Therefore, this study was designed to provide a deeper understanding of the bacterial flora of the mare vagina, and possible changes throughout the estrous cycle.

In total ten mares were included in the present study; the aim was to sample the cranial portion of the vagina of all ten mares on day 0 (ovulation), day 3, day 7 and day 14 of the estrous cycle. Seven mares were sampled according to plan, whereas three mares had some deviations in sampling days to fit their intended use as embryo recipients. The vaginal sampling was conducted with double-guarded occluded swabs from the cranial floor of the vagina, in a similar manner to taking uterine samples. Ovulation was determined by rectal palpation and ultrasonic examination, and the day 0 samples were taken within ± 24 h of ovulation. Swabs were brought to the laboratory in Aime's medium within 2-3 hours and were plated out immediately on agars. Results were registered as amount of growth (qualitatively), bacterial species and number of isolates.

The results showed that bacterial growth was highest on day 3 and 7, representing the beginning and middle of diestrous. The dominant bacteria in the mare vagina in the present study were *Escherichia coli* and *Streptococcus zooepidemicus*. Fluctuations throughout the cycle were observed but these two bacteria were the most isolated on each of the sampling days. *Escherichia coli* was especially dominant in maiden mares, compared to the mares that had had foals. An increase in bacterial diversity throughout the estrous cycle was observed, being highest on day 14. These results suggest that there are changes in the bacterial flora of the mare vagina throughout the normal estrous cycle. Further research is required to confirm the results in other populations.

Keywords: Artificial insemination, bacteriology, breeding, embryo transfer, equine reproduction, estrous cycle, ovulation, stud farm, vagina

Sammanfattning

Att känna till den normala bakteriefloran i ett organ är avgörande för att ge bakgrund till tillstånd och faktorer som kan göra att floran förändras. I stoets vagina kan en förändring induceras av introduktion av sperma, behandling med antibiotika, återflöde från en sjuklig livmoder etc. Tidigare studier som syftat till att kartlägga stoets vaginala bakterieflora är inte alla utförda på samma sätt, och resultaten överensstämmer inte alltid. Därför syftade denna studie till att ge en fördjupad kunskap om stoets bakterieflora i vaginan, och möjliga förändringar under brunstcykeln.

Totalt ingick tio ston i studien, där målet var att ta svabbprov kranialt i vaginan på alla tio ston på dag 0 (ovulation), dag 3, dag 7 och dag 14 i brunstcykeln. Sju ston provtogs enligt plan, medan tre ston hade vissa anpassningar i provtagningsdagarna för att inte sammanfalla med deras huvudsakliga uppgift som embryomottagare. Vaginalprovtagningen gjordes med dubbelskyddade provtagningspinnar, på liknande sätt som när livmoderprover tas, men istället togs provet kranialt i botten på vagina. Ovulation bestämdes genom rektal palpation och ultraljudsundersökning, och dag 0-prover togs inom ± 24 timmar i förhållande till ovulationen. Provtagningspinnarna transporterades till laboratorium i Aimes medium inom 2–3 timmar och spreds omedelbart på agarplattor. Resultaten av odlingarna bedömdes avseende mängd bakterier (kvalitativt) och bakteriearter.

Resultaten visade att bakterietillväxten var störst dag 3 och 7, vilket representerar början och mitten av diestrus. De dominerande bakterierna i stoets vagina i denna studie var *Escherichia coli* och *Streptococcus zooepidemicus*. Fluktuationer under cykeln observerades men dessa två bakterier var de mest isolerade på alla de olika provtagningsdagarna. *Escherichia coli* var särskilt dominerande hos ston som inte tidigare haft föl, jämfört med de ston som hade fått föl. En ökning av bakteriell mångfald genom brunstcykeln observerades, där dag 14 hade störst mångfald. Resultatet tyder på att det finns förändringar i bakteriefloran i stoets vagina under brunstcykeln, men att ytterligare forskning krävs för att bekräfta resultaten i andra populationer.

Nyckelord: Artificiell inseminering, bakteriologi, avel, embryotransfer, hästens reproduktion, brunstcykel, ovulation, stuteri, vagina

Table of contents

Abbreviations

AI	Artificial Insemination
AMR	Antimicrobial Resistance
BP	Baird Parker
CL	Corpus Luteum
ET	Embryo Transfer
FSH	Follicle-Stimulating Hormone
GnRH	Gonadotropin-Releasing Hormone
HNS	Hästnäringens Nationella Stiftelse
LH	Luteinizing Hormone
MALDI-TOF MS	Matrix-Assisted Laser Desorption Ionization Time of Flight Mass Spectrometry
MRS	De Man, Rogosa & Sharpe
$PGF_{2\alpha}$	Prostaglandin-F2α
SWB	Swedish Warmblood

1. Introduction

The bacterial flora of the equine vagina, and whether it changes during the different phases of the estrous cycle, has not been widely studied. When it comes to research about the equine reproductive tract, the uterus has been the organ in focus, mostly due to the contribution of endometritis to poor breeding results (Troedsson & Woodward 2016). In this study we will attempt to map the bacterial patterns in the vagina of healthy mares throughout the estrous cycle, to determine whether there is a change in the bacterial flora and if that change could be linked to the different phases of the estrous cycle.

The vaginal microbiota in other mammals, for example cows, during different stages in the estrous cycle has been more thoroughly studied. This is due to the role a healthy reproductive system (not only the uterus) plays in good breeding results and the economics of production animals, but also in the hope of finding probiotics for non-antibiotic treatment of infections in the reproductive tract (Otero *et al*. 1999; Quereda *et al.* 2020).

A deeper knowledge of the bacterial patterns in the vagina of healthy mares would contribute to better understanding of diseases and microbial abnormalities in the vagina, but also to the reactions in the vagina to local administration of medications, inseminations (artificial or natural) or embryo transfer. Although substances are deposited in the uterus during artificial insemination (AI), embryo transfer (ET) and most therapeutic treatments in the mare's reproductive organs, the vagina will most certainly be affected when discharge makes its way out of the uterus.

Artificial insemination is the most common form of breeding for racing and riding horses in Sweden (HästSverige, 2012), and is increasingly used globally (Aurich 2012). The presence of antibiotics in semen extenders and embryo transfer medias is very common, as their addition is controlled by law in Sweden (SJVFS 2015:1). Antibiotics are added to protect both the mare and the spermatozoa from bacteria. This means that antibiotics are transferred into the uterus, and later the vagina and environment, at almost every AI and ET performed. To be able to study the impact of antibiotics on the bacterial flora of the vagina, more information about the bacterial patterns during the normal estrous cycle would be helpful.

The objective of this study was, therefore, to map the bacterial flora of the cranial vagina of the mare during the estrous cycle, by sampling the vagina of ten healthy

mares at four time points relative to ovulation. The bacteria were cultured and identified to species level at the different time points to determine whether there was a change in the bacterial flora, either in number or in species present.

2. Literature Review

2.1 Reproductive organs of the mare

2.1.1 Anatomy and physiology

The mare's reproductive organs (excluding the higher hormonal organs involved such as hypothalamus, pineal- and pituitary gland) consist of ovaries and a tubular structure, comprising the oviducts, uterus, cervix, vagina, vestibulum and vulva (Brinsko *et al.* 2011). The udder may also be included as a reproductive organ.

Most cranially, in the abdominal cavity, are the left and right ovaries attached to the abdominal wall and the uterus by ligaments. In the centre of each ovary is the ovarian fossa, in which ovulations occur (Singh 2018). After ovulation, a corpus luteum (CL) is formed at the site of the old follicle (König & Liebich 2020).

Surrounding the ovarian fossa is the end of the oviduct, the infundibulum, which funnels the oocyte into the oviduct. The oviducts consist of three parts: infundibulum, ampulla and isthmus (McKinnon 2011). The ampulla-isthmus junction is where a possible fertilization will take place (Brinsko *et al.* 2011). Therefore, the timing of a conception (or insemination) must be calculated so that there are live spermatozoa present when the ovum reaches this point of the oviduct. The ovum will, if fertilized, migrate to the uterus (Kindahl 2004). An unfertilized ovum can either migrate to the uterus or remain and degenerate in the oviduct (Betteridge *et al.*, 1982).

The uterus of the horse consists of a body and two horns in a Y-shape (Figure 1). The uterus devolves into the cervix usually at the point where it enters the pelvic cavity (depending of course on the individual size and position of the uterus). The cervix is a physical barrier between the uterus and vagina (König & Liebich 2020). The cervix relaxes and opens normally only during estrous and parturition (Brinsko *et al.* 2011), making the uterus more vulnerable for infections at these time periods. Caudal to the cervix is the vagina, which is the last part of the internal reproductive organs, and the vestibule, which is a part of the external reproductive organs, as well as the vulva and clitoris (Sjaastad *et al.* 2016). The vulva of the mare has a vertical opening and serves as the first physical barrier towards the ingress of foreign material into the reproductive tract (Brinsko *et al.* 2011).

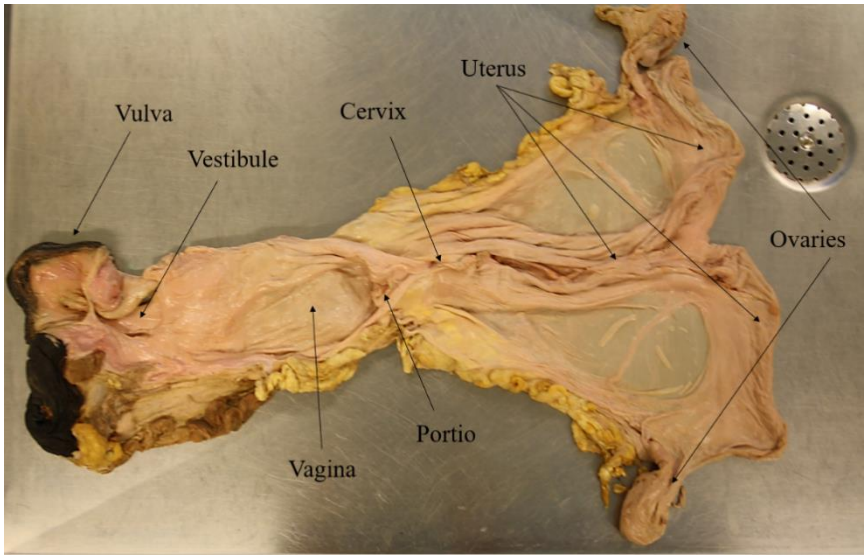

Figure 1. Anatomy of the mare reproductive organs. Photograph by T. Åkerholm.

2.1.2 The vagina

In this study we refer to the vagina as the tubular organ between the cervix and the vestibule. Cranially, the vagina meets the most caudal part of the cervix, the portio, that marks the entrance to the uterus. The transverse fold caudally that covers the external urethral orifice, separates the vagina and vestibule. The vestibulovaginal fold is a remnant of the hymen, and is, as well as the vulva and cervix, a physical barrier supposed to protect the mare's reproductive tract from foreign material (McKinnon 2011). A persistent hymen, extending from the vulvovaginal fold, can be present in maiden mares, but is usually easily ruptured (Paccamonti & Crabtree 2019).

The vagina is about 20 cm (15-25 cm) in length and is normally collapsed (Rodriguez-Martinez 2010; Brinsko *et al.* 2011; McKinnon 2011). During estrous (for conception) and parturition, the vagina can expand as wide as the pelvic bone (McKinnon 2011). The equine vagina contains no glandular structures, and therefore mucus is produced by the vestibule, cervix and uterus for lubrication (Brinsko *et al.* 2011).

The cornification of the superficial layer of epithelium is not as prominent during estrous in horses and therefore cytology cannot be used to determine stages of estrous (McKinnon 2011), as can be done, for example, in dogs (König & Liebich 2020).

2.2 The equine estrous cycle

Horses are seasonally polyestrous, long-day breeders, meaning that their sexual behaviour and estrous cycling begins as daylength increases (Crowell-Davis 2007). When the light hours become fewer again, the mares go into a period of anestrous, meaning they are not cycling. Here in Scandinavia, this results in a breeding season from April to September, with individual differences between mares (Kindahl 2010). The seasonality in sexual behaviour varies between mares, as according to Brinsko *et al.* (2011) about 15-20% of mares do not go into anestrous in wintertime, and this can vary from season to season within individuals.

The effect of light in initiating cyclical activity can be used to induce cycling earlier in the year, thus producing offspring born early the following year which might be preferable in certain breeds (mostly riding and racing horses). Other factors than light can also affect the onset of sexual behaviour in the spring, such as the weather, food supply and the mare's health (Aurich 2011).

The horse's seasonality in sexual activity can be divided into an ovulatory and anovulatory period, with transitional periods in between. The transitional period is often characterized by prolonged sexual behaviour, with or without a following ovulation (Darenius 2010; Aurich 2011). In Sweden this generally occurs in early spring and in autumn, although as stated earlier, this is not true for all mares (Kindahl 2010). In a summary of 14 studies published by McKinnon *et al.* (2011) the median age at which fillies had their first estrous was 15 months, ranging from 7.8 to 37 months.

The estrous cycle of the mare is generally 21-22 days (19-24 days), counted from one ovulation to the next (Kindahl 2010). The estrous, or follicular phase, is usually 4-7 days. However, the estrous phase has been shown to be highly variable, both between mares and in different parts of the season (longer in transitional periods). In a review of studies regarding the equine estrous cycle, Witherspoon (1971) concluded that the average length of the estrous phase is 6 days, but it can vary between 0 and 54 days. The diestrous, or lutheal phase, is often 14-15 days and generally considered not to differ as much, both throughout the season and between mares (Brinsko *et al.* 2011).

2.2.1 The hormonal regulation

The hormonal regulation of the mare's estrous cycle is complicated, as with most regulation of cyclical activity in females (Noakes & Robinson 2019). The following presentation of the equine reproductive hormonal play is a schematic overview, which can also be seen partly in Figure 2.

When the photo-period and the amount of light increases, the production of melatonin from the pineal gland will decrease. In mares, which are long-day breeders, this activates the hypothalamus to produce gonadotropin-releasing

hormone (GnRH) and start the ovulatory period of the year (Noakes & Robinson 2019). The pulsatile release of GnRH will, in its turn, stimulate the anterior pituitary gland to produce and release follicle-stimulating hormone (FSH) and luteinizing hormone (LH) (Kindahl 2010). As the name suggests, FSH stimulates growth of the follicles in the ovaries, whereas LH stimulates maturation of follicles, and in extension the production of estrogen, ovulation and luteinization (Brinsko *et al.* 2011). Growing, mature follicles will release estrogen and inhibin, which will suppress secretion of FSH and increase the release of LH, whereas the corpus luteum (CL) will release progesterone, which decreases the release of LH (Noakes & Robinson 2019). Another important hormone in the equine estrous cycle is prostaglandin-$F_{2\alpha}$ ($PGF_{2\alpha}$), released by the endometrium, which causes luteolysis of the CL and therefore a decrease in progesterone (Brinsko *et al.* 2011).

The estrous cycle begins (day -5 in the figure) when the levels of progesterone and prostaglandins are low, and mature follicles in the ovary are producing estrogen, which stimulates release of LH and thereby ovulation (day 0 in figure). At this point the levels of estrogen start to decline and the corpus luteum that is formed in the ovary after ovulation releases progesterone, which will be the dominating hormone during the diestrous phase (day 2-15 in figure).

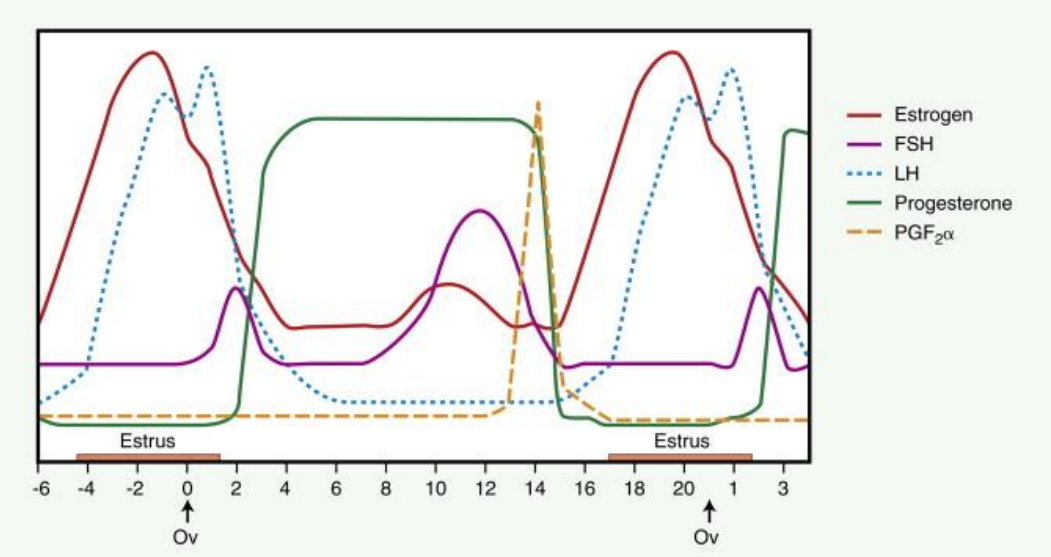

Figure 2. The hormonal waves during the mare estrous cycle. Retrieved from Manual of Equine Reproduction, Brinsko et al. *2011, with permission from Elsevier Books.*

Growth of follicles in the mare occurs in waves, with a group of follicles growing simultaneously. At the end of the wave, one (or occasionally two or three) follicle becomes dominant and ovulates (or regresses if a successful ovulation is not possible). The mare can have more than one follicular wave per cycle (Brinsko *et al.* 2011). Initiated in the middle of diestrous, the primary wave normally results in mature follicles at estrous. However, there can be a second wave of maturing follicles initiated during estrous, that might result in a secondary ovulation in the

middle of diestrous under the presence of progesterone (Donadeu & Pedersen 2008). Secondary waves, that can produce a diestrous ovulation, occur in most breeds but are believed to be most common in thoroughbreds (Donadeu & Pedersen 2008; Brinsko *et al.* 2011; McKinnon 2011).

By the end of diestrous, if no fertilization has taken place and no embryo has reached the uterus, the endometrium will release a surge of PGF2α (day 14 in figure) to break down the corpus luteum, which will decrease the progesterone levels and make way for a new estrous cycle (day 17 in Figure 2) (Brinsko *et al.* 2011).

2.2.2 Sexual behaviour of the mare

During estrous, the stallion generally becomes increasingly interested in the mare, and the mare's behaviour in response to the attracted stallion is a measurement of her sexual state (Crowell-Davis 2007). A mare in estrous will direct her hind-quarters towards the stallion, lower her pelvis and turn her tail. In this motionless position, the mare also usually everts the clitoris ("clitorial winking") and voids some urine. A mare not ready for mating will turn away, kick and/or vocalize when the stallion approaches and tries to interact (Aurich 2011).

When AI is used, the mare and stallion are not always in the same place, which makes observing classic estrous behaviour somewhat more difficult. In some stud farms a random stallion ("teasing stallion") can be used to interpret estrous (Dalin 2010a). Alternatively, the date of the estrous cycle can be determined by clinical and ultrasound examination of the reproductive tract.

2.2.3 Predicting ovulation

In this study, as well as in breeding, it was essential to be able to predict ovulation. The mare in question usually shows behavioural signs of estrous, which indicates when to start closer examinations. During clinical examination, an evaluation of the outer genitalia can also indicate if the mare is in heat. The vulva will become more relaxed and the colour of the vestibulum a darker pink, sometimes with shades of yellow/orange (Dalin 2010b). Finally, rectal palpation of the cervix, uterus and ovaries, often completed with a transrectal ultrasound examination, gives a more precis indication of the mare's sexual state.

During estrous the cervix is relaxed and is palpable as a soft mass at the pelvic brim. The uterine wall is thickened and edematous with decreased tonus, and a characteristic "wheel formation" is visible on ultrasound examination (Pinto & Frazer 2013). Big follicles are often palpable; before transrectal ultrasound became available, estimates of the follicle size, consistency and structures were the information available to the veterinarian (Dalin 2010b). With today's ultrasound, however, more detailed, and exact information about the uterus and ovaries can be

obtained (Brinsko *et al.* 2011). Ovulation usually occurs at the end of estrous, but the typical signs of estrous (relaxed, edematous membranes, wheel pattern etc.) have, in some cases, already started to decrease (Brinsko *et al.* 2011). In the ovary (Figure 3), the dominant follicle (or follicles) become irregular in shape and the wall thickens when ovulation is close (Brinsko *et al.* 2011; McKinnon 2011). The diameter of the ovulating follicle differs between breeds and mares, but is usually about 45 mm (Pinto & Frazer 2013).

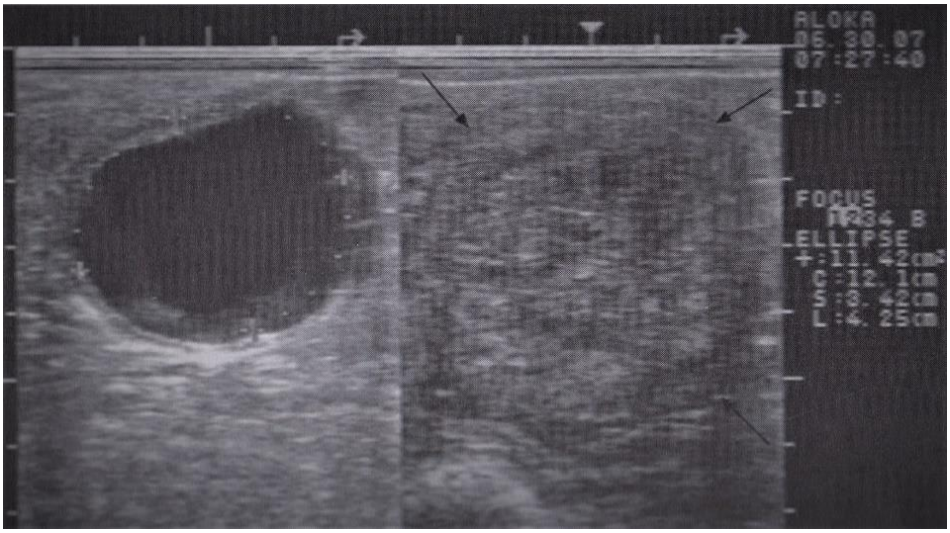

Figure 3. Ultrasonic picture of preovulatory follicle and a cross section of a uterus horn during late estrous. Retrieved from Equine Reproduction 2nd Edition, McKinnon et al. *2011, with permission from John Wiley and Sons.*

2.2.4 Irregularities of the equine estrous cycle

Various factors affect the regularity of the cycle.

Anestrous, i.e., when the mare shows no signs of estrous behaviour and has small, inactive, ovaries, occurs naturally during the winter but can in some cases continue into the otherwise ovulatory period. Anestrous occurs naturally while lactating and in prepubertal fillies, but can also be the result of poor physical condition, old age of the mare or some disease, condition or malformation of the reproductive or hormonal organs (Darenius 2010; Brinsko *et al.* 2011; McKinnon 2011).

Silent estrous means that the mare is cycling but shows no signs of estrous behaviour (Crowell-Davis 2007). This is not a big problem if the mare is examined routinely by rectal examination and ultrasound, since physically everything is functioning as it should. It is, of course, time-consuming, and is problematic if the mare is set up for natural breeding. According to Darenius (2010), silent estrous is most common in nervous mares, maiden mares, and mares with a foal at foot. It

must also be considered that preferences between mares and stallions exist, and one or both might not show any sexual behaviour because they simply do not like each other (McKinnon 2011).

Prolonged estrous or estrous with no following ovulation are irregularities associated mostly with the transitional period (Pinto & Frazer 2013). An an-ovulatory follicle can either become somewhat luteinized, regress slowly or become hemorrhagic (Darenius 2010).

Another complicating factor is that of multiple ovulations, either during the same estrous, or a secondary follicular wave that causes a diestrous ovulation. The rate of occurrence of multiple ovulations during the same estrous differs between breeds, but believed to be more common in horses than in smaller ponies (McKinnon 2011). The ovulation of two (rarely more) follicles during estrous is primarily a breeding problem, increasing the risk for twins which is undesirable in horses (Miller & Woods 1988). Diestrous ovulations can cause a prolonged luteal phase and therefore a disruption in cyclicity (Darenius 2010).

2.3 Equine breeding

2.3.1 Equine breeding in Sweden

Sweden has a long history of horse breeding, including a few native breeds. Horses have been used for transportation, in agriculture, war, competitions and for companionship (Fédération Équestre Internationale 2020). According to a report, made by Hästnäringens Nationella Stiftelse (HNS), the number of horses in Sweden registered with any of the 19 breed associations in 2021, born between 2001 and 2020 and not reported dead, was 255 000. The author of the report suggest that this number might be higher in reality, since an age restriction is set on this calculation and some horses in Sweden are still not registered with any breed association and do not have a passport. Of these 255 000 registered horses in 2021, the Swedish warmblood (31%) and the standardbred trotter (18%) are the most common breeds.

Artificial insemination was first used in Sweden during the 1970s, and at the time was mostly performed on standardbred trotters (Söderquist 2010). Since then, its use has increased rapidly and in 2020 AI was used in 95% of standardbred and 99% of Swedish warmblood horses in Swedish breeding (A-M. Dalin, personal correspondence). In the earlier mentioned breeding report made by HNS they conclude that AI is becoming more common in most breeds, but that natural mating is still used in Sweden; mostly in ponies, coldblood breeds, and the English thoroughbred.

2.3.2 Artificial insemination

A correctly-performed AI has proven to be preferable over natural mating in many ways (Söderquist 2010); there is faster breeding progress, since the semen from desirable stallions can be divided between more mares than with natural mating, prevention of transmission of venereal diseases and pathogenic bacteria between horses, less travelling and decreased risk for transmission of diseases between farms. On the other hand, if handled incorrectly or carelessly, pathogens may be spread more rapidly than with natural mating. An AI program also requires some equipment, and in Sweden one is obliged to apply for permission from the Swedish Board of Agriculture to carry out AI, and the veterinarians and technicians involved must attend courses before they are licensed to perform AI (SJVFS 2015:1).

The semen from the stallion is often collected using an artificial vagina after the stallion has mounted a phantom (Brinsko *et al.* 2011). In the laboratory, volume, color and possible contaminations are noted. Sperm motility and sperm concentration should also be measured (Söderquist 2010). Semen may be preserved either fresh, chilled or frozen, depending on how much time will elapse until it is deposited in a mare (Yates & Whitacre 1988). According to Söderquist (2010), in Sweden, one dose of semen should contain 500 million motile spermatozoa if it is to be used fresh, but 1000 million motile spermatozoa if the dose is to be chilled and sent to another stud farm.

The timing of insemination relative to ovulation, and the method of storing the semen between collection and insemination, is essential for a good result in AI. The semen should be deposited preferably approximately 48 hours before ovulation, but 72 hours before until 6 hours after ovulation has been proven to give acceptable or even normal pregnancy rates, depending also on the fertility of the stallion (Brinsko *et al.* 2011; Söderquist 2010). A mare is usually checked for ovulation every second day after insemination, and if no ovulation has occurred, she is inseminated again (Söderquist 2010). This continues until ovulation is confirmed, but as few inseminations as possible is always preferable, since the semen is deposited into the uterus and each entry can cause an infection or injury to the reproductive tract.

The viability of frozen and thawed semen differs between stallions but is generally lower than fresh semen and therefore needs to be inseminated closer to ovulation than fresh semen (Brinsko *et al.* 2011). This requires examination of the mare's ovaries at more frequent intervals then when using fresh semen, for acceptable pregnancy rates.

2.3.3 Embryo transfer

Since the introduction of this technique in horses in the 1970s, ET has become available at a few stud farms in Sweden (HästSverige 2018). During 2020, 24 ETs were performed on mares in Sweden; these were all fresh embryos rather than

frozen, according to statistics from the International Embryo Technology Society (IETS 2020).

The technique comprises the insemination of the donor mare, often a talented young mare still competing or a mare too old to bear the foal itself, followed by transfer of the embryo to a recipient mare, which will carry the pregnancy and give birth to the foal instead of the donor mare (Pinto & Frazer 2013). This procedure creates the opportunity for faster breeding results, since talented fillies with high breeding value can become mothers without having to carry the foal themselves, and can continue their carrier in competition. It is also possible for one mare to have more than one offspring per season, or to have a foal despite being too old or injured. One should consider, however, that different disciplines (racing, riding etc.) have different rules regarding registration of embryo transfer offspring.

As mentioned earlier, the donor mare is inseminated with semen from the chosen stallion. The cycle of the recipient mare (or mares) has to be synchronized with the donor, and ovulations should occur between one day before and three days after the donor (Gustafsson 2010; Scherzer 2011). The embryo reaches the uterus on day 6-6.5 (Scherzer 2011) and the flushing of the uterus to retrieve the embryo is usually done on day 7 after ovulation of the donor mare (Gustafsson 2010; Brinsko *et al.* 2011). All the backflow from the uterus passes through a filter, in which the embryo will be trapped. The embryo is then localized via microscope, washed and aspirated in a medium into a straw to be transferred into the recipient mare (HästSverige 2018).

2.3.4 Use of antibiotics in equine breeding

As with any use of medications, antibiotics should be used with restraint and the risk for antimicrobial resistance (AMR) must be considered by the prescribing veterinarian when choosing type and doses (SJVFS 2015:1). Antibiotics can reach the uterus via both systemic and local treatment (LeBlanc 2009).

Another very important way in which antibiotics can be introduced into the reproductive tract is through AI and ET. Both semen extenders and embryo transfer media contain small amounts of antibiotics, according to law within the European Union. The reason is to prevent spreading of bacteria between the stallion and mare, and to protect the semen/embryo during its time outside the equine body. According to a review by Malaluang *et al.* (2021), the use of antibiotics in semen extenders could be one of the main sources for development of AMR in the equine reproductive tract. The same review concludes that this low-level usage of antibiotics could possibly be replaced by other means of removing bacteria from semen, and keeping bacterial growth at a minimum, although further large-scale studies are needed.

2.4 Bacterial sampling of the equine vagina

Studies on bacterial sampling of the equine reproductive tract focus mostly on the uterus and the diagnosing of endometritis, especially contagious equine metritis (CEM), which is a serious venereal disease. To my knowledge, there are few studies in which bacteria from the vagina were sampled, and therefore few accounts of optimized techniques.

A study made by Christoffersen *et al.* (2015), concluded that when obtaining samples from the uterus the double-guarded, low-volume, lavage technique was the most accurate way of the methods available to diagnose endometritis. However, a lavage is not suitable for the vagina, since the vagina cannot contain the fluid in the same manner as the uterus.

In this project we chose to use a double-guarded occluded swab, as described for taking uterine swab samples. According to Blanchard *et al.* (1981), using a guarded swab will result in less chance of contamination, thus giving a more reliable result. When guarded swabs are used for uterine sampling (McKinnon 2011), the mare's tail should be tied up and preferably bandaged, and the outer genitalia should be thoroughly cleaned and dried. The person taking the sample can either guide the swab manually or use a vaginal speculum to enter the cervix. If the practitioner's hand is inserted to guide the swab, that person should wear a sterile rectal glove and insert one finger into the portio of the cervix, allow the swab to follow the finger and pass through the cervix into the uterus. In this study we did not pass through the cervix as the site of sampling was the cranial vagina. Once at the site of sampling, the swab is then pressed forward, to protrude from the two protective plastic straws that enclose it. The swab is then gently rotated to obtain as much material as possible without harming the organ wall. Before extracting the swab from the uterus or vagina it needs to be pulled back into the protective plastic straws, to avoid contamination on the way out (Dalin 2010b). The swab should then be put into a transport medium, preferably Aime's medium, until it reaches the lab (McKinnon 2011).

Figure 4. Swab used during sampling and the area of sampling in the vagina, portio marked with arrow. Photograph by T. Åkerholm.

3. Material and Methods

3.1 Study population

The study was conducted on a stud farm in Sweden in the early breeding season with horses (n=10) of different breeds and ages. All mares were part of the stud's embryo transfer recipient group, were stationed on the stud farm during breeding season, and were kept in a field without contact with visiting horses.

Mares were excluded if they had already been inseminated during this season or had received an embryo (either successfully or unsuccessfully), or had some treatment in the vagina or uterus (e.g. flushing of the uterus or local treatment with antibiotics, etc.) before or during our sampling period. Since the purpose of the study was to evaluate the bacterial changes during a normal estrous cycle, each mare served as its own control.

3.1.1 Mares

The mares consisted of three Swedish warmblood horses (SWB) and seven standardbred trotters. The ages ranged from three to twelve years, with a mean age of 8.6 years and a median age of 9.5 years. Six out of the ten mares had had at least one foal, whereas four mares had never had foals.

Table 1. The breeds, ages, and number of foals of each mare.

Horse:	Breed:	Age:	Number of foals:
A	Standardbred	6	1
B	Swedish warmblood	12	0
C	Swedish warmblood	10	1
D	Standardbred	9	1
E	Swedish warmblood	10	0
F	Standardbred	5	0
G	Standardbred	9	1
H	Standardbred	10	2
I	Standardbred	3	0
J	Standardbred	12	4

3.2 Examination and sampling

During heat, the ovaries were palpated rectally and examined with ultrasound every second day by the stud veterinarians, with closer intervals when ovulation was expected. Vaginal swab samples were to be taken on days 0, 3, 7, and 14 of the estrous cycle, where day 0 represents ovulation. Day 3 represents the period of transition from estrous to diestrous, day 7 represents diestrous, and day 14 the transition from diestrous to a new estrous. Seven mares were sampled on days 0, 3, 7, and 14 as planned. One horse was sampled on days 0, 3, 7, and 11. One horse was sampled on days 0, 3, 8, and 14. The tenth horse was sampled only three times, on days 0, 3, and 10. The deviation in timing for three of the mares was due to logistical reasons, and the fact that the mares' primary use was as embryo transfer recipients. Therefore, caution was needed with the timing of sampling to avoid disrupting the stud's breeding program.

On day 0, the samples were always taken within ± 24 h of the ovulation, which was confirmed with ultrasound. There was no ultrasound examination at the sampling on days 3, 7, and 14. Before each sample was taken, the mare's tail was wrapped and tied up, and the perineum and vulva were washed with lukewarm water and soap at least three times or until the area was visibly clean. The cleaned area was then dried with paper and controlled for cleanliness. Samples were taken with a sterile rectal glove, paraffine oil, and double-guarded occluded swabs. An assistant separated the vulval labia by touching only the outer parts of the vulva, thus minimizing the risk of bringing skin surface bacteria into the vagina and compromising the sample. The area of sampling was on the floor of the vagina, approximately one finger joint (2-3cm) caudally of the portio. The swab was turned clockwise and anti-clockwise several times to collect an adequate sample and then put into Amie's medium for transport to the lab. All sampling was performed by the same person.

3.3 Bacteriological analyses

All samples were taken directly to the lab and the analysis were initiated within 2-3 hours after sampling. The swab samples were spread on agar plates using standardised three streaks. To facilitate growth and identification of as many different bacteria as possible, a selection of agar plates were chosen in this project. Blood agar, which is an unselective, good growth medium for most bacteria, was used for all samples. Two plates were used, one was incubated in aerobic and the other one in anaerobic conditions. Lactose purple agar was also used on all samples, to identify bacteria that ferment lactose by a change of colour of the agar. MacConkey agar is a selective medium for gram negative bacteria and was used in all samples except one (97%), due to the fact that the supply of plates ran out. Baird

Parker (BP) is a selective medium for growth of *Staphylococcus spp*., and De Man, Rogosa & Sharpe (MRS) agar is selective for *Lactobacillus spp*. These two agars were used in 75% and 81% samples respectively, since they were not available at the start of the project. The plates were used at least once for swabs from each horse at some point during the sampling period, and for three horses, these plates were used for swabs on all sampling days.

The anaerobic blood agar plate was placed separately in an anaerobic jar or plastic bag with a commercial envelope to exclude oxygen. The other blood agar plate was incubated in aerobic condition together with lactose purple agar, MacConkey agar, and BP agar at 37°C. Bacterial growth was registered after 24h and 48h. The MRS agar was incubated in anaerobic conditions at 25°C, and bacterial growth was registered after five days.

After incubation, the growth on the plate was described qualitatively using a grading of no, poor, sparse, intermediate, or heavy growth. The plate was categorized as having no growth if there were no visible colonies after 48h. Poor growth means a few colonies could be seen only on the first streak with no growth on the second or third streak. A plate with sparse growth had a moderate number of colonies in the first streak and none or only a few colonies in the second streak, but still no growth in the third streak. Intermediate plates had a high number of colonies in the first streak and none or a few colonies in the second and/or third streak, whereas heavy growth means an increased number of colonies in the first streak and some growth in both the second and third streaks.

Visibly different colony types were noted and described by shape, color, and size. Three CFU (colony forming unit) of each colony type were re-cultured on blood agar plates and incubated, under aerobic or anaerobic conditions, depending on the incubation conditions for the first 48h. These plates were then incubated at 37°C for 24h to establish pure growth. Colonies from cultures with pure growth were then identified using Matrix- Assisted Laser Desorption Ionization Time of Flight Mass Spectrometry (MALDI-TOF MS); all identified colonies were then stored in cryotubes (containing glycerol and BHI) in the -80°C freezer.

3.4 Statistical analysis

The results of this study were transcribed into digital form and processed mainly in Microsoft Office Excel, creating lists, tables, and diagrams. The data analysis was performed using R v.4.1.2 software. Pearson correlation coefficients were calculated between the bacterial species using the cor.test function in the R environment, with $p < 0.05$ being considered significant. The result were plotted in R using corrplot v.0.92.

4. Results

Ten mares were included in the study. For nine of the mares, four swabs were taken, whereas for the remaining mare only three swabs were taken, resulting in 39 samples. From these 39 samples, the amount of growth was registered qualitatively, and the different bacterial species were identified.

4.1 Bacterial growth

The amount of growth differed between mares. For example, samples from mare J never produced more than sparse growth on any of the sampling days, whereas samples from mare H produced intermediate growth on both blood agar plates on all four days of sampling.

Of the 39 blood agar plates cultured under aerobic conditions, 17 showed intermediate growth (43.6%), 7 sparse growth (17.9%), 14 poor growth (35.9%), and one plate had no growth (2.6%). Similarly, for the blood agar plates cultured under anaerobic conditions, 39 showed growth; 22 showed intermediate growth (56.4%), 6 had sparse growth (15.4%), and 11 had poor growth (28.2%). All the blood agar plates cultured under anaerobic conditions produced some growth, corresponding to the growth on the aerobic plates for 32 of the 39 samples.

The lactose purple agar plates also consisted of 39 plates; 7 plates produced intermediate growth (17.9%), 5 showed sparse growth (12.8%), 21 plates had poor growth (53.8%), and 6 plates had no growth at all (15.4%). MacConkey agar plates were used on 38 samples, where 2 plates reached intermediate growth (5.3%), 1 with sparse growth (2.6%), 9 showed poor growth (23.7%), and a majority with 26 samples had no growth (68.4%).

Growth on the BP and MRS agar plates was poor or sparse, and most of the plates had no growth at all. Of the 29 BP plates, 2 plates showed sparse growth (6.9%), 9 showed poor growth (31.0%), and 18 had no growth (62.1%). For 31 MRS agar plates, 2 produced sparse growth (6.5%), 6 showed poor growth (19.4%), and 23 plates showed no growth at all (74.2%).

No sample produced heavy growth on any of the plates. The sampling days of each mare, and the amount of growth on all the different agar plates is shown in Appendix 1.

.

When studying the bacterial growth, only the blood agar plates were compared between the different days and horses, since the other agars were selective and not always used on all mares on all days. The individual patterns in amount of growth differed between almost all the mares; the same growth pattern was shared by two mares at best. The trend in the group, which can be seen in both the aerobic and anaerobic plates, is that there was least growth on day 14 (Figures 5 and 6), and a higher growth on day 7 (Figure 5) and 3 (Figure 6) for aerobic and anaerobic blood agar plates respectively. When comparing mares which had had foals and maiden mares (Figures 7 and 8), there is still a low growth on day 14 for both groups in both incubation conditions. The maiden mares follow the trend of hawing the highest growth on day 7 in aerobic conditions (Figure 7) and day 3 in anaerobic conditions (Figure 8), whereas the mares who had had foals have most growth on day 3 and 7 in aerobic conditions (Figure 7) and on day 0 and 3 in the anaerobic conditions (Figure 8).

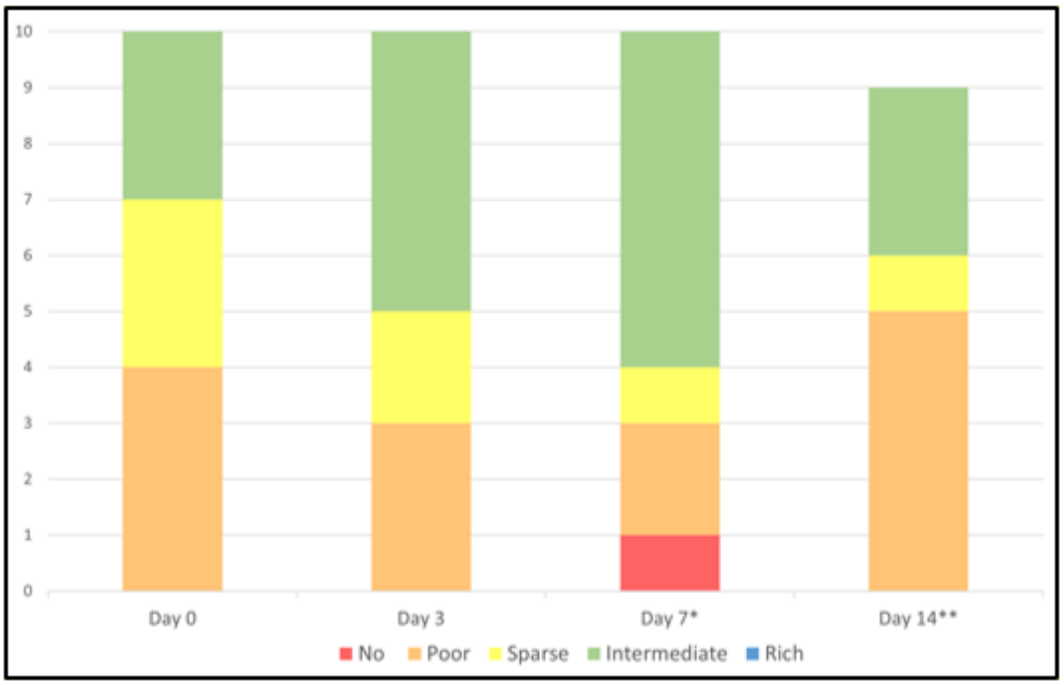

*Figure 5. The growth on aerobic blood agar plates. *Day 7 including one sample taken on day 8 and one on day 10. **Day 14 including one sample taken on day 11, only nine mares.*

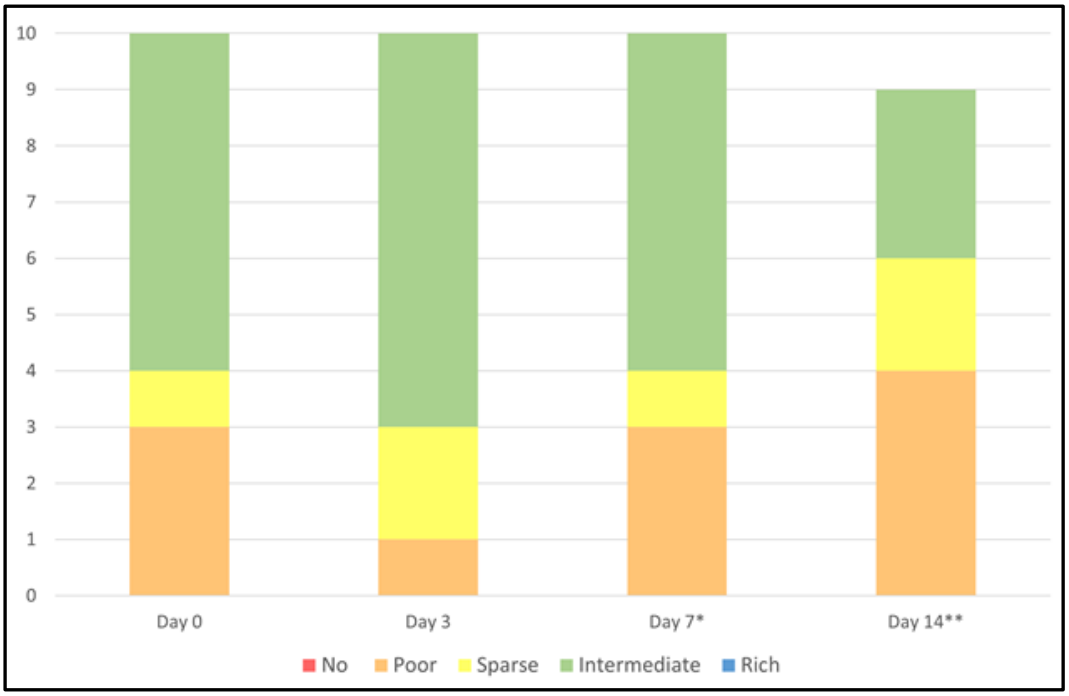

*Figure 6. The growth on anaerobic blood agar plates. *Day 7 including one sample taken on day 8 and one on day 10. **Day 14 including one sample taken on day 11, only nine mares*

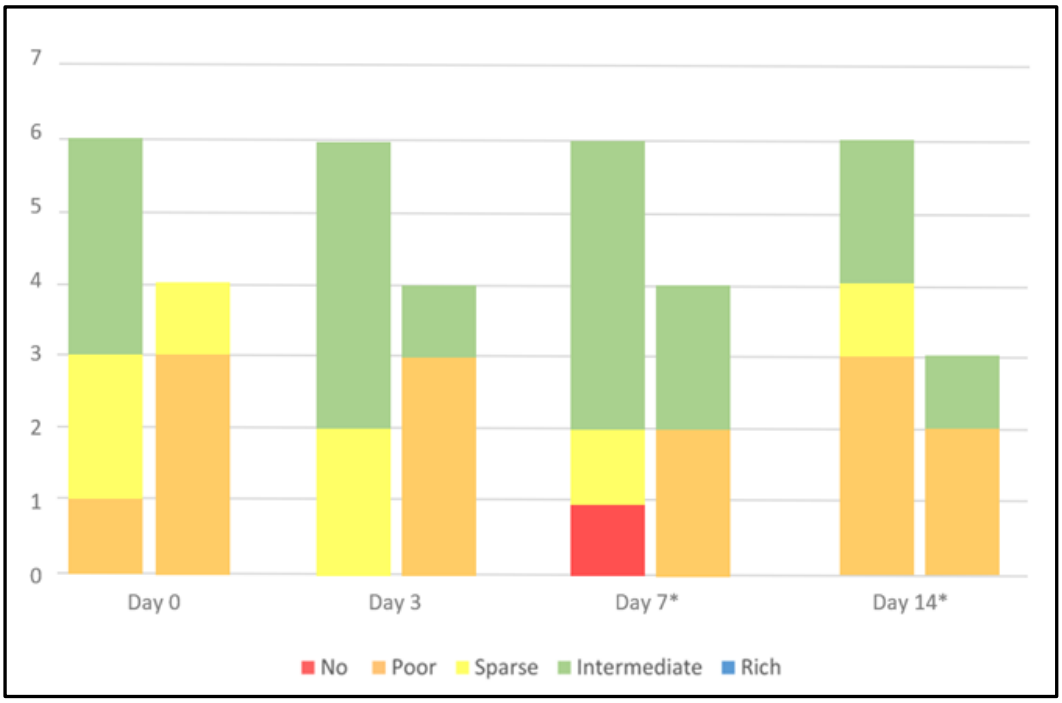

*Figure 7. The growth on aerobic blood agar plates on mares which had had foals (n=6) versus maiden mares (n=4). *Day 7 including one sample taken on day 8 and one on day 10. **Day 14 including one sample taken on day 11, only nine mares.*

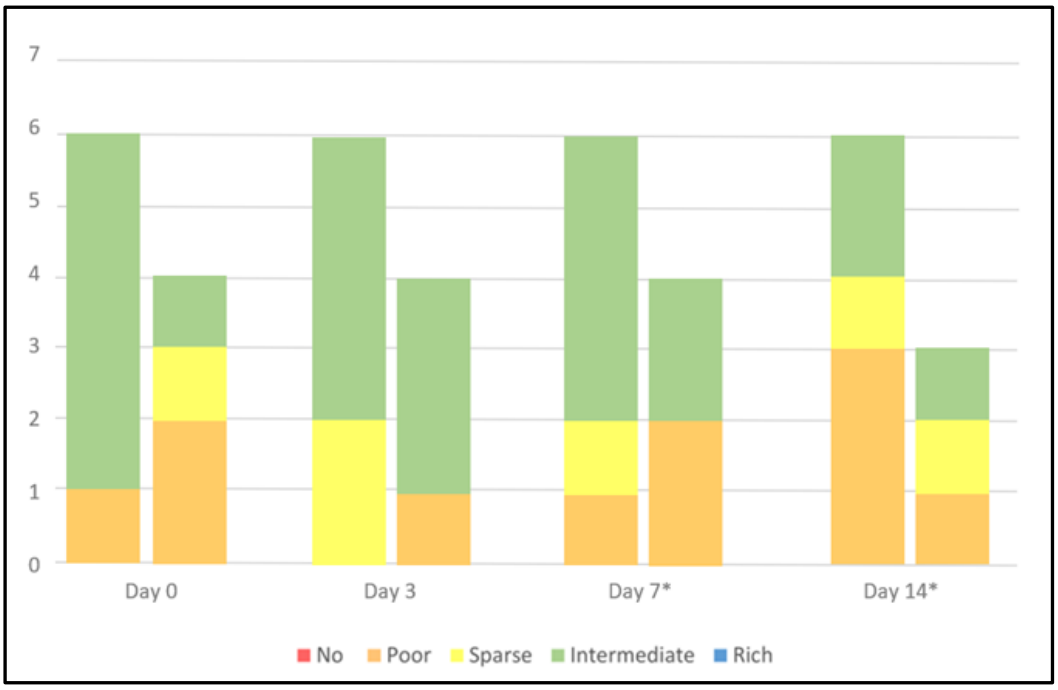

*Figure 8. The growth on anaerobic blood agar plates on mares which had had foals (n=6) versus maiden mares (n=4). *Day 7 including one sample taken on day 8 and one on day 10. **Day 14 including one sample taken on day 11, only nine mares*

4.2 Bacterial species

In total, 564 bacterial isolates were re-cultured, of which 323 (57.3%) could be identified by MALDI-TOF MS. Of the identified bacterial species (Table 2), 10 bacterial species were gram negative, consisting of 168 isolates, and 12 bacterial species were gram positive with 155 isolates. The most common phylum was *Firmicutes*, consisting of 11 species with 148 isolates. *Proteobacteria* was the second most common phylum, with only 5 different bacteria species but involving 139 isolates.

Concerning bacterial identification (Table 3), the dominant result in this study was “No identification” (42.7%), meaning that MALDI-TOF MS was not able to identify the isolate. These isolates are believed to be non-pathogenic environmental bacteria. Of the isolates that could be identified, 22 different bacteria were found. *Escherichia coli* (20.6%) and *Streptococcus zooepidemicus* (15.6%) were the two most frequently isolated.

Table 2. Bacteria identified from the equine vagina, gram staining, phylum and family.

Gram	Phylum	Family	Bacteria
-	*Bacteriodetes*	*Bacteroidaceae*	*Bacteroides fluxus*
-	*Bacteriodetes*	*Bacteroidaceae*	*Bacteroides fragilis*
-	*Bacteriodetes*	*Bacteroidaceae*	*Bacteroides thetaiotaomicron*
-	*Bacteriodetes*	*Prevotellaceae*	*Prevotella heparinolytica*
-	*Fusobacteria*	*Fusobacteriaceae*	*Fusobacterium varium*
-	*Proteobacteria*	*Pasteurellaceae*	*Actinobacillus equuli*
-	*Proteobacteria*	*Pasteurellaceae*	*Actinobacillus pleuropneumoniae*
-	*Proteobacteria*	*Pasteurellaceae*	*Actinobacillus rossii*
-	*Proteobacteria*	*Pasteurellaceae*	*Actinobacillus suis*
-	*Proteobacteria*	*Enterobacteriaceae*	*Escherichia coli*
+	*Actinobacteria*	*Actinomycetaceae*	*Arcanobacterium hippocoleae*
+	*Firmicutes*	*Clostridiaceae*	*Clostridium ramosum*
+	*Firmicutes*	*Enterococcaceae*	*Enterococcus casseliflavus*
+	*Firmicutes*	*Paenibacillaceae*	*Paenibacillus amylolyticus*
+	*Firmicutes*	*Staphylococcaceae*	*Staphylococcus borealis*
+	*Firmicutes*	*Staphylococcaceae*	*Staphylococcus capitis*
+	*Firmicutes*	*Staphylococcaceae*	*Staphylococcus epidermidis*
+	*Firmicutes*	*Staphylococcaceae*	*Staphylococcus haemolyticus*
+	*Firmicutes*	*Staphylococcaceae*	*Staphylococcus hominis*
+	*Firmicutes*	*Streptococcaceae*	*Streptococcus infantarius*
+	*Firmicutes*	*Streptococcaceae*	*Streptococcus thoraltensis*
+	*Firmicutes*	*Streptococcaceae*	*Streptococcus zooepidemicus*

Table 3. Number of isolates from the equine vagina on the different sampling days.

	A				B				C				D				E				F			G				H				I				J				Total	
	0	3	7	14	0	3	8	14	0	3	7	14	0	3	7	14	0	3	7	14	0	3	10	0	3	7	14	0	3	7	11	0	3	7	14	0	3	7	14		
Actinobacillus equuli																														3	1										**4**	**0,7 %**
Actinobacillus pleuropneumoniae																														2											**2**	**0,4 %**
Actinobacillus rossii													3										1															3	3	3	**13**	**2,3 %**
Actinobacillus suis																													3		1										**4**	**0,7 %**
Arcanobacterium hippocoleae	2	1											1		1																	1			1						**7**	**1,2 %**
Bacteroides fluxus								1																																	**1**	**0,2 %**
Bacteroides fragilis																				2		2							2		1					2		1	2		**12**	**2,1 %**
Bacteroides thetaiotaomicron																1					1																				**2**	**0,4 %**
Clostridium ramosum																					2			1						1											**4**	**0,7 %**
Enterococcus casseliflavus															1	1							3																		**5**	**0,9 %**
Escherichia coli	2					10	2	1	2							2	13	7	18	4		7	17		7				4	13							1	6	**116**	**20,6 %**
Fusobacterium varium													1			2		3						3																	**9**	**1,6 %**
No identification	4	5	4	3	3	7	2	6	1	9	5	3	6	11	5	3	5	11	7	8	7	3	5	11	6	6	10	8	15	11	5	5	10	8	4	2	10	5	2	**241**	**42,7 %**
Paenibacillus amylolyticus						3																													1					**4**	**0,7 %**
Prevotella heparinolytica								1										2												2											**5**	**0,9 %**
Staphylococcus borealis	1		7													4					1		2																		**15**	**2,7 %**
Staphylococcus capitis		1								1					3	8				1																					**14**	**2,5 %**
Staphylococcus epidermidis															1	1	1														1										**4**	**0,7 %**
Staphylococcus haemolyticus			1													2																									**3**	**0,5 %**
Staphylococcus hominis							1																	2												1					**4**	**0,7 %**
Streptococcus infantarius																							1																		**1**	**0,2 %**
Streptococcus thoraltensis	1								5																																**6**	**1,1 %**
Streptococcus zooepidemicus						10		6	8			4					2	8	11			5	2		3	8	6				1						8	5	1	**88**	**15,6 %**

4.2.1 Bacteria in mares

In the present study, *Escherichia coli* was found in 9 out of 10 mares on some occasion during the estrous cycle and was the most frequently isolated bacteria in the study. *Streptococcus zooepidemicus* was cultured from 7 out of 10 horses, making it the second most isolated bacteria. The third most frequently found bacteria, in half of the study population was *Bacteroides fragilis* (Table 4).

Table 4. Presence (x) or non-presence (o) of specific bacteria in the vagina of mares at some stage during the estrous cycle

	A	**B**	**C**	**D**	**E**	**F**	**G**	**H**	**I**	**J**	**Total**
Actinobacillus equuli	o	o	o	o	o	o	o	x	o	o	1
Actinobacillus pleuropneumoniae	o	o	o	o	o	o	o	x	o	o	1
Actinobacillus rossii	o	o	o	x	o	x	o	o	o	x	3
Actinobacillus suis	o	o	o	o	o	o	o	x	o	o	1
Arcanobacterium hippocoleae	x	o	o	x	o	o	o	o	x	o	3
Bacteroides fluxus	o	x	o	o	o	o	o	o	o	o	1
Bacteroides fragilis	o	o	o	o	x	x	o	x	x	x	5
Bacteroides thetaiotaomicron	o	o	o	x	o	x	o	o	o	o	2
Clostridium ramosum	o	o	o	o	o	x	x	x	o	o	3
Enterococcus casseliflavus	o	o	o	x	o	x	o	o	o	o	2
Escherichia coli	x	x	x	x	x	x	x	x	o	x	9
Fusobacterium varium	o	o	o	x	x	o	x	o	o	o	3
Paenibacillus amylolyticus	o	x	o	o	o	o	o	o	x	o	2
Prevotella heparinolytica	o	x	o	o	x	o	o	x	o	o	3
Staphylococcus borealis	x	o	o	x	o	x	o	o	o	o	3
Staphylococcus capitis	x	o	x	x	x	o	o	o	o	o	4
Staphylococcus epidermidis	o	o	o	x	x	o	o	x	o	o	3
Staphylococcus haemolyticus	x	o	o	x	o	o	o	o	o	o	2
Staphylococcus hominis	o	x	o	o	o	o	x	o	x	o	3
Streptococcus infantarius	o	o	o	o	o	x	o	o	o	o	1
Streptococcus thoraltensis	x	o	x	o	o	o	o	o	o	o	2
Streptococcus zooepidemicus	o	x	x	o	x	x	x	x	o	x	7

There were 166 bacterial isolates identified from the six mares that had foaled, and 157 identified isolates from the four maiden mares.

The two groups of mares had an equal number of isolates of *Streptococcus zooepidemicus* (44 isolates), making up about one fourth of the total bacterial isolates. However, maiden mares had more than double the number of *Escherichia coli* isolates (79 isolates) than the mares which had had foals (37 isolates), and in the maidens *Escherichia coli* constituted about half of the isolates identified. The number of isolates not identified in the mares ranged from 15 to 39 per mare, with no significant difference between mares which had had foals and maiden mares.

The mares which had foaled had a more diverse bacterial flora, consisting of 19 different bacteria, with *Escherichia coli* and *Streptococcus zooepidemicus* constituting about half of the isolates (Figure 9). In comparison, the flora of the maiden mares consisted of 17 different bacteria, with *Escherichia coli* and *Streptococcus zooepidemicus* constituting more than 75% of the isolates (Figure 10).

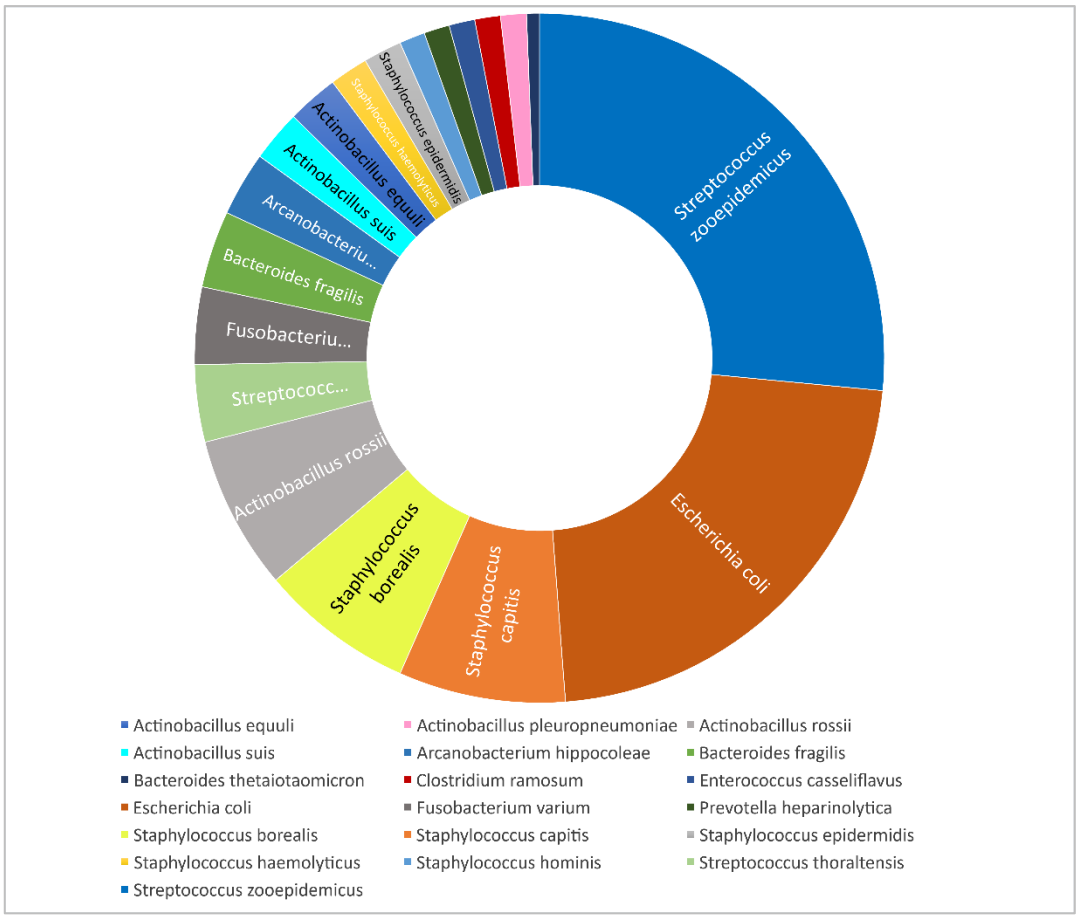

Figure 9. Number of bacteria isolates on all days from the vagina of mares that had had foals.

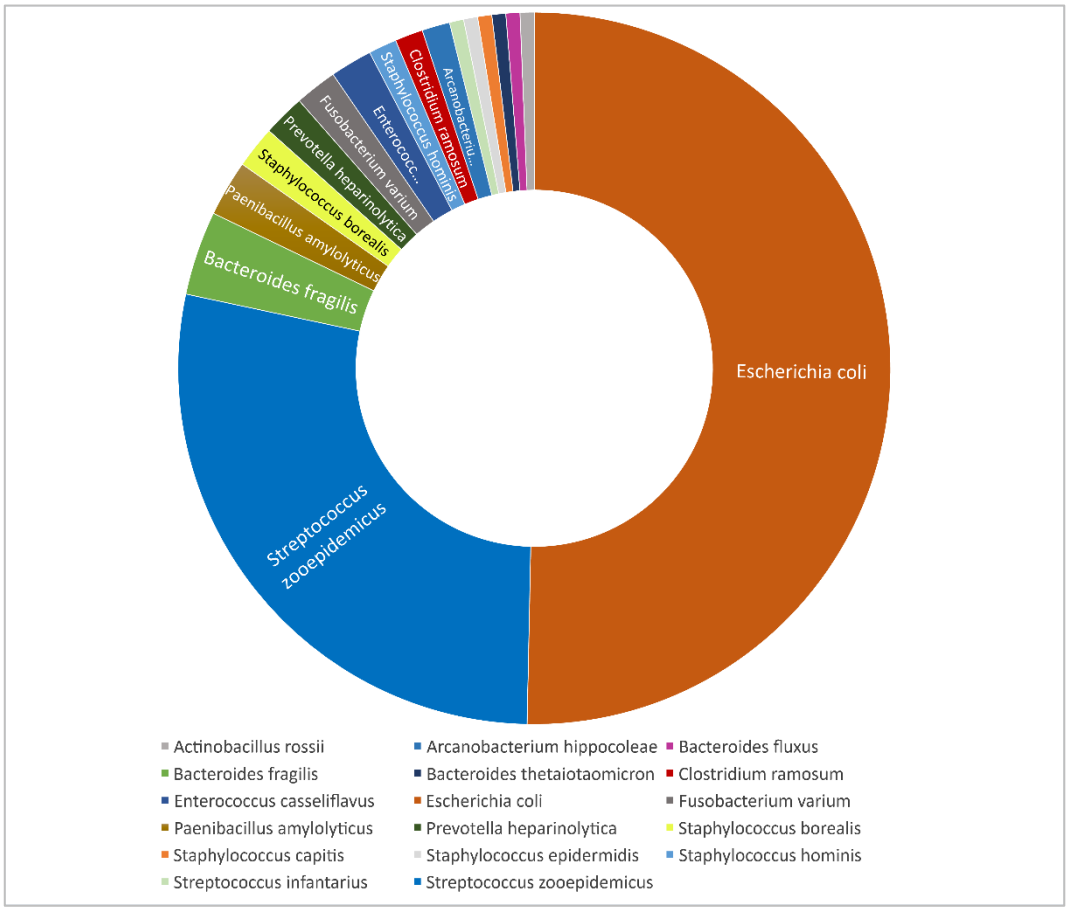

Figure 10. Number of bacteria isolates on all days from the vagina of maiden mares.

4.2.2 Bacterial species on different days

When comparing the bacteria between the different sampling days, the diversity in the study group rose from the beginning of the cycle (day 0 and 3) to the end (day 7 and 14) (Figure 11).

Four bacterial species were found on all four of the sampling days: *Actinobacillus rossii*, *Arcanobacterium hippocoleae*, *Escherichia coli* and *Streptococcus zooepidemicus*. Only *Escherichia coli* was found on all days in the same mare.

Four species were only found on one day: *Actinobacillus pleuropneumoniae*, *Bacteroides fluxus*, *Streptococcus infantarius* and *Streptococcus thoraltensis*. Of these four, only *Streptococcus tholartensis* was isolated on only one day in more than one horse.

The two most isolated bacteria species in this study, *Escherichia coli* and *Streptococcus zooepidemicus*, were isolated most often on day 7 and day 3 respectively (Figure 12).

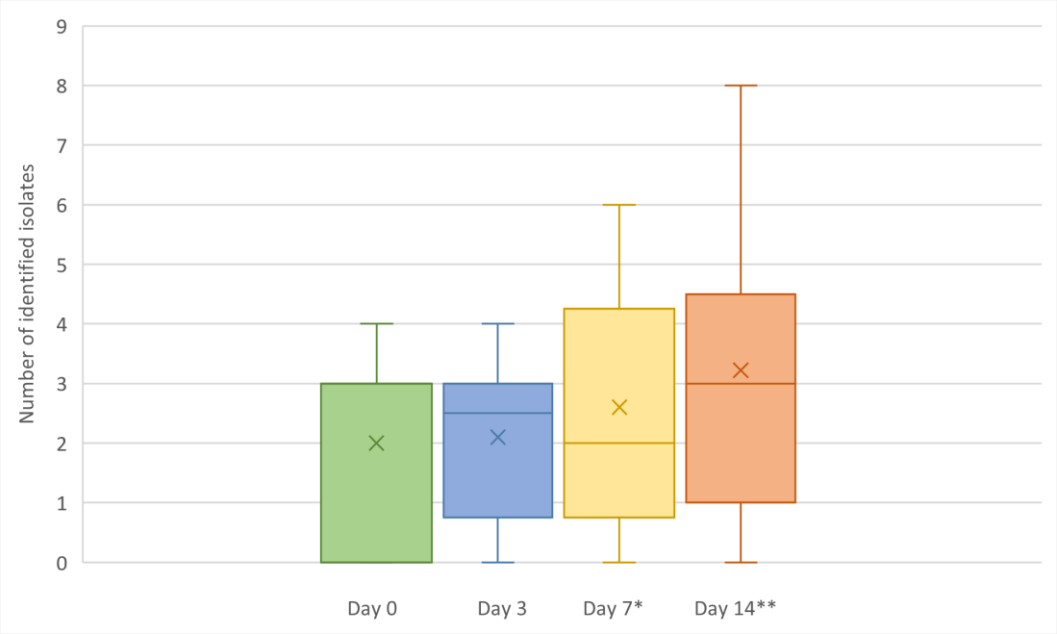

*Figure 11. Distribution of identified species from the vagina of the individual mares divided on sampling day. *Day 7 including one sample taken on day 8 and one on day 10. **Day 14 including one sample taken on day 11, only nine mares*

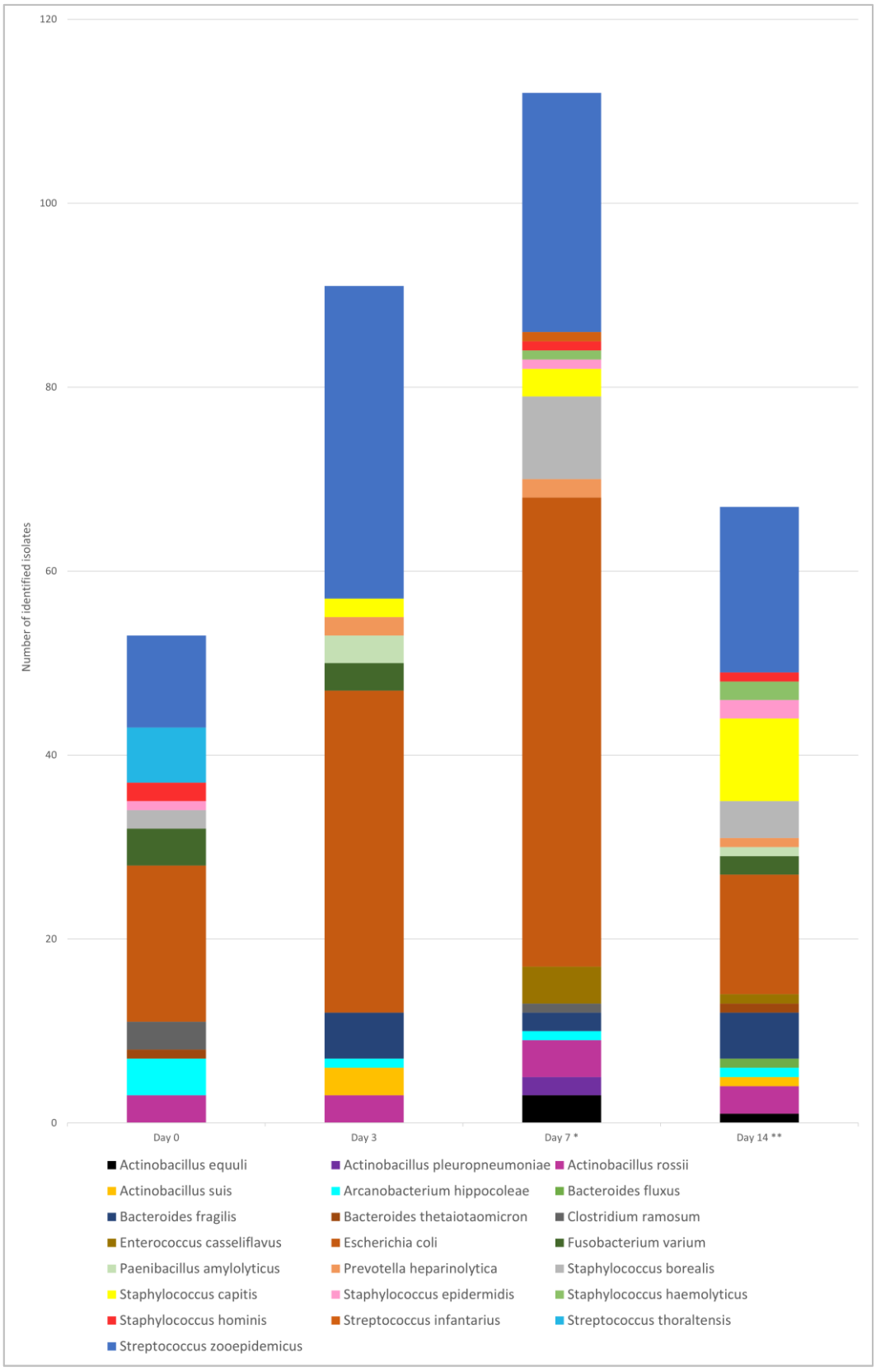

*Figure 12. Distribution of isolates of each identified bacterium from the equine vagina on the different sampling days. *Day 7 includes one sample taken on day 8 and one on day 10. **Day 14 includes one sample taken on day 11, only nine mares*

4.2.3 Bacterial patterns

The table below shows the different bacterial patterns in eight of the mares on all four days (F and H excluded due to sampling days that deviated from the others). In these eight mares a few patterns could be identified, such as *Bacteroides spp.* tended to appear towards the end of the cycle, whereas *Fusobacterium varium* and *Streptococcus tholartensis* appeared in the beginning. The two most isolated bacteria in this study, *Escherichia coli* and *Streptococcus zooepidemicus*, showed a variety of patterns. The difference between the two was that *Escherichia coli* was found in all compared mares, but might appear on only one day in the cycle, whereas *Streptococcus zooepidemicus* was not found in all mares, but when it was found it appeared on more than one day. However, most bacteria in this list had only a few isolates in total, making patterns hard to determine.

Table 5. Distribution of bacterial patterns in individual mares, x = present, o = non-present (including only identified bacteria and horse F and H excluded, day 7 includes one sample taken on day 8).

Bacteria	**Mare**	**Day 0**	**Day 3**	**Day 7**	**Day 14**
Actinobacillus rossii	D	x	o	o	o
Actinobacillus rossii	J	o	x	x	x
Arcanobacterium hippocoleae	A	x	x	o	o
Arcanobacterium hippocoleae	D	x	o	x	o
Arcanobacterium hippocoleae	I	x	o	o	x
Bacteroides fluxus	B	o	o	o	x
Bacteroides fragilis	E	o	o	o	x
Bacteroides fragilis	I	o	o	o	x
Bacteroides fragilis	J	o	x	x	o
Bacteroides thetaiotaomicron	D	o	o	o	x
Clostridium ramosum	G	x	o	o	o
Enterococcus casseliflavus	D	o	o	x	x
Escherichia coli	A	x	o	o	o
Escherichia coli	B	o	x	x	x
Escherichia coli	C	x	o	o	o
Escherichia coli	D	o	o	o	x
Escherichia coli	E	x	x	x	x
Escherichia coli	G	o	x	o	o
Escherichia coli	J	o	o	x	x
Fusobacterium varium	D	x	o	o	x
Fusobacterium varium	E	o	x	o	o
Fusobacterium varium	G	x	o	o	o
Paenibacillus amylolyticus	B	o	x	o	o
Paenibacillus amylolyticus	I	o	o	o	x
Prevotella heparinolytica	B	o	o	o	x
Prevotella heparinolytica	E	o	x	o	o
Staphylococcus borealis	A	x	o	x	o
Staphylococcus borealis	D	o	o	o	x
Staphylococcus capitis	A	o	x	o	o
Staphylococcus capitis	C	o	x	o	o
Staphylococcus capitis	D	o	o	x	x
Staphylococcus capitis	E	o	o	o	x
Staphylococcus epidermidis	D	o	o	x	x
Staphylococcus epidermidis	E	x	o	o	o
Staphylococcus haemolyticus	A	o	o	x	o
Staphylococcus haemolyticus	D	o	o	o	x
Staphylococcus hominis	B	o	o	x	o
Staphylococcus hominis	G	x	o	o	o
Staphylococcus hominis	I	o	o	o	x
Streptococcus thoraltensis	A	x	o	o	o
Streptococcus thoraltensis	C	x	o	o	o
Streptococcus zooepidemicus	B	o	x	o	x
Streptococcus zooepidemicus	C	x	o	o	x
Streptococcus zooepidemicus	E	x	x	x	o
Streptococcus zooepidemicus	G	o	x	x	x
Streptococcus zooepidemicus	J	o	x	x	x

4.3 Correlations between bacteria

The strongest correlation (r=0.81) appears between *Staphylococcus haemolyticus* and *Staphylococcus capitis*. *Staphylococcus haemolyticus* is also part of the second strongest correlation (r=0.79) with *Staphylococcus borealis*. The two most isolated bacteria in the study, *Escherichia coli* and *Streptococcus zooepidemicus*, have a correlation of r=0.38, suggesting that these two do not necessarily appear at the same time. No significant negative correlations could be found in this study.

We also looked for correlations on different sampling days, but after excluding horse F and H (due to deviated sampling days) and all bacteria with less than three isolates, not many bacteria remained. On day 0 only one significant correlation (r=0.97) can be found, between *Streptococcus zooepidemicus* and *Streptococcus thoraltensis*. On day 3 there were no significant correlations. Day 7 had one significant correlation (r=0.72) between *Escherichia coli* and *Streptococcus zooepidemicus*, making this the only day that these two bacteria share a significant correlation. On day 14 there were two significant correlations, between *Escherichia coli* and *Actinobacillus rossii* (r=0.78), and between *Staphylococcus capitis* and *Staphylococcus borealis* (r=0.99).

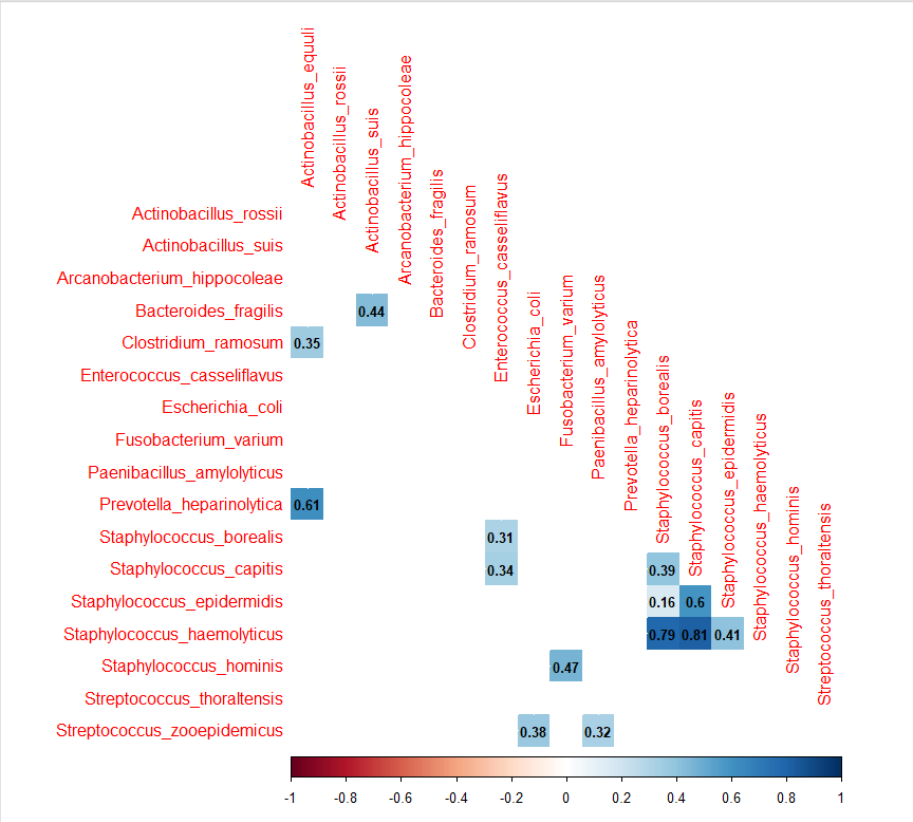

Figure 13. Correlations between identified bacteria from all mares on all days. Bacterial species with less than three isolates were excluded.

5. Discussion

The purpose of this study was to map the vaginal bacterial flora of healthy mares during the estrous cycle. Samples were taken at four time points throughout the cycle and results were registered as amount of growth, bacterial species, and number of isolates. To my knowledge, only a few studies have been conducted on the vaginal microbiota of mares.

The sampling period of this study (May) coincided with some mares still being in transitional period between inactivity and regular cyclic activity, making it more difficult to identify day 0, i.e. ovulation. Some mares showed a prolonged estrous or had an estrous without ovulation (or both), making the period of collecting samples somewhat longer than expected. It would be preferable to conduct a similar study when mares are showing regular cycles, later in the season, but that would require keeping a study population under the same supervision as brood mares, but without the intention of using them in breeding. However, the possibility of doing such a study on a busy stud farm is negligible.

The four sampling days chosen represented four different phases in the equine estrous cycle. In this project, day 0 represents ovulation, the grand finale of estrous, which was confirmed with ultrasound, and the samples were taken within ± 24 hours of the estimated time of ovulation. Whether this slight difference from the exact time of ovulation is relevant for the bacterial flora or not, we cannot know. However, finding the exact time of ovulation would require almost hourly checking of the mare, which was not an option in this project. Day 3 represents the beginning of diestrous with a decrease in estrogen and an increase in progesterone (Figure 2), released from the newly formed corpus luteum. According to Brinsko *et al.* (2011) the highest levels of progesterone are reached on day 6 after ovulation. By sampling mares on day 7 we should get a good representation of diestrous with progesterone being dominant. Day 14 marks the end of diestrous and preparation for a new estrous, with decreasing progesterone and a rise in estrogen. As with day 0, based on former research, we do not know whether sampling one mare on day 7 and one on day 10 makes any difference when comparing these mares. Since there are individualities and irregularities between mares, ultrasound examination and possibly also measurement of blood hormonal levels might be required at every sampling to be able to draw correct conclusions regarding the precise stage of the estrous cycle. In a study by Barba *et al.* (2020) eight Arabian mares were sampled

twice, once in estrous and once in diestrous, and inclusion criteria for the mares at each sampling were response on teasing, ultrasound examination and progesterone level in blood. The authors concluded that no significant difference in the vaginal microbiome between estrous and diestrous could be found in their study.

The results of the present study showed some growth, both aerobic and anaerobic, in the vagina of all mares on all sampling days. This is in contrast to a study by Hinrichs *et al.* (1988), where vaginal samples were taken once from each mare with a guarded swab through a sterile vaginal speculum from the cranial vaginal wall. Samples were plated within one hour on 5% sheep blood agar and McConkey agar, no enrichment media was used. Only aerobic incubation was performed. There was no growth found in 58% of the vaginal swabs, which is remarkable considering that the vagina is not a sterile environment, and a mixture of bacteria would be expected. In Hinrichs' study they suggest that the vulvovaginal fold might be "the major barrier to ascending bacterial contamination of the mare's reproductive tract". In the current study however, all mares were found to have bacterial growth in their vagina throughout the cycle. These contrasting results could be the result of different sampling and culturing techniques, for example, the method of transporting the sample to the laboratory, the agar plates used, and the area in which samples are taken in the vagina might affect the results.

When comparing growth on the four days of sampling, the mares showed a wide array of individual patterns. This type of study might benefit from registering the amount of growth quantitatively, to be able to make more precise comparisons between different days and different mares. Barba *et al.* (2020) measured the bacterial growth in CFU/mL; their study found higher counts on blood agar plates in estrous than in diestrous. With only two sampling days in their study, and no definition of the point in diestrous relative to ovulation, one cannot make a direct comparison to our results, but the trend in our study population was that growth on blood agar plates was highest during the beginning and middle of diestrous, on day 3 and 7.

For almost half of the isolates (42.7%) the MALDI-TOF MS was unable to produce an identification. This means that the bacteria in question were not in the MALDI-TOF MS database and therefore not identifiable at the present time. These bacteria are believed to be non-pathogenic bacteria, probably environmental, but in the current study they will remain unidentified (I. Hansson, docent in microbiology of veterinary medicine, personal communication).

The five phyla identified (*Firmicutes, Proteobacteria, Bacteriodetes, Fusobacteria, Actinobacteria*) are in accordance with similar studies of the bacterial flora of the mare's vagina (Barba *et al.* 2020; Husso *et al.* 2020), although the distribution might differ somewhat. None of the bacteria known to be venereal and pathogenic to the equine reproductive tract were found in this population, such as *Taylorella equigenitalis*, *Klebsiella pneumoniae,* or *Pseudomonas aeruginosa*

(McKinnon 2011). Only opportunistic and non-pathogenic bacteria were identified. The most isolated bacteria in the study, both in number of isolates and in the number of mares it was isolated from, was *Escherichia coli*, closely followed by *Streptococcus zooepidemicus*, making these two by far the most dominant bacteria in the study. Both these bacteria are known to be found in the reproductive tract of mares, and are considered possible causes for endometritis (McKinnon 2011). Only few studies have been conducted specifically sampling the vagina. Our results are not always consistent with these studies, but most importantly not all studies have presented bacterial identification to species and subspecies level, making comparisons between them difficult. Hinrichs *et al.* (1988) sampled 48 equine vaginas and did not isolate *Escherichia coli* or *Streptococcus zooepidemicus* from any of them, whereas Barba *et al.* (2020) reported some findings of *Streptococcus* spp, but no *Escherichia* spp in the eight mares sampled. In a study conducted by Scott *et al.* (1971), sampling the vagina of one hundred recently euthanized mares at a slaughterhouse, beta hemolytic streptococci, including *Streptococcus zooepidemicus*, were the most isolated bacteria, with coliforms being the second, including *Escherichia coli*. The presence of *Escherichia coli* was higher in the present study compared to some other similar studies, most likely due to the large proportion of bacteria identified in this study. Furthermore, some of those studies were performed a long time ago without access to current methods of identifying bacteria.

Furthermore, one has to consider the individual differences between mares and the fact that the surroundings can affect the bacterial flora. Husso *et al.* (2020) studied fourteen mare-foal pairs, investigating perinatal intestinal microbiota in foals, where bacterial samples were taken from the mare as well, including the vagina. They concluded that in the mares some similarities between the vaginal and fecal bacterial flora existed, suggesting fecal contamination of the vagina, although not as frequently as had been shown in similar studies on cows. Barba *et al.* (2020) refer to the work of Kamińska & Gajecka (2017), stating that in humans both geographic location and ethnicity influence the vaginal microbiota, and that further research is needed to investigate whether this is also true for the bacterial flora of the equine vagina.

As shown in the results, the bacterial diversity in this group of mares changes throughout the estrous cycle. According to Barba *et al.* (2020) the diversity in their study did not change between estrous and diestrous; however, their comparison was based on identification to phylum and genus level and only two sampling occasions, whereas in this present study the bacteria were identified to species level on four well-defined sampling occasions. Thus, the two studies are not completely equivalent.

When comparing bacterial patterns and correlations, two mares were excluded, since their sampling days deviated from the remaining mares. One mare sampled

on day 8 instead of 7 is included, since the difference is not believed to be highly relevant. To distinguish and compare bacterial patterns, a larger sample size would be needed. When calculating correlations, (apart from two of the mares mentioned earlier) we also chose to exclude all bacterial species with less than three isolates, to make the results easier to interpret. Of the remaining bacteria, it was interesting to note that no negative correlations were found when comparing the isolates of all the mares' sampling days. The staphylococci isolated in this study showed signify-cant correlations, and the strongest correlations of all, suggesting that they rarely appear alone in the vaginal bacterial flora. Although *Escherichia coli* and *Strepto-coccus zooepidemicus* were isolated in such high amounts in this study, their correlation was not stronger than r=0.38, suggesting that one is not particularly dependent of the other, and that they might appear in different phases of the estrous cycle.

Comparing mares which had had foals with maiden mares was not the original aim of this study, but since the study population consisted of mares with different breeding history, this became an option. When comparing bacterial growth patterns, the trend seen in the whole group for day 14 having the lowest growth is consistent in the two subgroups in both incubation conditions of the blood agar plates. As mentioned earlier, a quantitative measurement of the bacterial growth, as well as a larger sample size from the two groups, would be preferable to facilitate precise comparisons between the different groups and days. The results showed that *Escherichia coli* was isolated considerably more often from the four maiden mares (79 isolates) than from the six mares which had had foals (37 isolates). *Escherichia coli* were also a bigger part of the total bacterial flora in maidens (50.3%) than in mares that had had a foal (22.3%). The importance of this result is unclear and requires further investigation, but it could be an indication that the mating/-insemination and birth of a foal introduces new bacteria to the vagina that changes the bacterial flora in the vagina completely.

In conclusion, this study shows that bacteria colonise the vagina throughout the whole estrous cycle in mares of different ages and breeding history. *Escherichia coli* and *Streptococcus zooepidemicus* were the dominant vaginal bacteria in this population. *Escherichia coli* was even more dominant in maiden mares compared to mares that had had foals. The bacterial growth changed during the different phases of the estrous cycle, with highest growth in the beginning and middle of diestrous. This pattern of growth is observed both in maiden mares and in mares that had had foals. The results suggest that when comparing vaginal samples between different mares, or samples taken from the same mare at different occasions, the stage of the estrous cycle in which the sample is obtained could affect the results and should be noted. Further studies, with larger sample sizes and even more precis determination of ovulation, would be necessary to verify these results in other populations.

References

Aurich, C. (2011). Reproductive cycles of horses. *Animal Reproduction Science*, 124 (3), 220–228. https://doi.org/10.1016/j.anireprosci.2011.02.005

Aurich, J.E. (2012). Artificial insemination in horses—More than a century of practice and research. *Journal of Equine Veterinary Science*, 32 (8), 458–463. https://doi.org/10.1016/j.jevs.2012.06.011

Barba, M., Martínez-Boví, R., Quereda, J.J., Mocé, M.L., Plaza-Dávila, M., Jiménez-Trigos, E., Gómez-Martín, Á., González-Torres, P., Carbonetto, B. & García-Roselló, E. (2020). Vaginal microbiota is stable throughout the estrous cycle in Arabian mares. *Animals*, 10 (11), 2020. https://doi.org/10.3390/ani10112020

Betteridge, K.J., Eaglesome, M.D., Mitchell, D., Flood, P.F. & Beriault, R. (1982). Development of horse embryos up to twenty two days after ovulation: observations on fresh specimens. *Journal of Anatomy*, 135 (Pt 1), 191–209 PMID: 7130052

Blanchard, T.L., Garcia, M.C., Hurtgen, J.P. & Kenney, R.M. (1981). Comparison of two techniques for obtaining endometrial bacteriologic cultures in the mare. *Theriogenology*, 16 (1), 85–93. https://doi.org/10.1016/0093-691X(81)90116-3

Brinsko, S.P., Blanchard, T.L., Varner, D.D., Schumacher, J., Love, C.C., Hinrichs, K. & Hartman, D.L. (2011). *Manual of Equine Reproduction*. 3. ed. St. Louis: Mosby, an affiliate of Elsevier Ltd.

Christoffersen, M., Brandis, L., Samuelsson, J., Bojesen, A.M., Troedsson, M.H.T. & Petersen, M.R. (2015). Diagnostic double-guarded low-volume uterine lavage in mares. *Theriogenology*, 83 (2), 222–227. https://doi.org/10.1016/j.theriogenology.2014.09.008

Crowell-Davis, S.L. (2007). Sexual behavior of mares. *Hormones and Behavior*, 52 (1), 12–17. https://doi.org/10.1016/j.yhbeh.2007.03.020

Dalin, A.-M. (2010a). Oestrous behaviour and oestrous detection (Reproductive physiology in the mare). In: *Compendium on Equine Reproduction*. Department of Obstetrics and Gynaecology. 5th ed. Uppsala: Swedish University of Agricultural Sciences. 32-38.

Dalin, A.-M. (2010b). Gynaecological examination in the mare. In: *Compendium on Equine Reproduction*. Department of Obstetrics and Gynaecology. 5th ed. Uppsala: Swedish University of Agricultural Sciences

Darenius, K (2010). Ovarian abnormalities in the mare. In: *Compendium on Equine Reproduction*. Department of Obstetrics and Gynaecology. 5th ed. Uppsala: Swedish University of Agricultural Sciences

Donadeu, F. & Pedersen, H. (2008). Follicle development in mares. *Reproduction in Domestic Animals*, 43 (s2), 224–231. https://doi.org/10.1111/j.1439-0531.2008.01166.x

Fédération Équestre Internationale (2020). *Equestrian Nation: Sweden*. https://www.fei.org/stories/lifestyle/my-equestrian-life/equestrian-nation-sweden [2022-10-05]

Gustafsson, H. (2010). Embryo transfer in the horse. In: *Compendium on Equine Reproduction*. Department of Obstetrics and Gynaecology. 5th ed. Uppsala: Swedish University of Agricultural Sciences

Hinrichs, K., Cummings, M.R., Sertich, P.L. & Kenney, R.M. (1988). Clinical significance of aerobic bacterial flora of the uterus, vagina, vestibule, and clitoral fossa of clinically normal mares. *Journal of the American Veterinary Medical Association*, 193 (1), 72–75 PMID: 3417532

Husso, A., Jalanka, J., Alipour, M.J., Huhti, P., Kareskoski, M., Pessa-Morikawa, T., Iivanainen, A. & Niku, M. (2020). The composition of the perinatal intestinal microbiota in horse. *Scientific Reports*, 10 (1), 441. https://doi.org/10.1038/s41598-019-57003-8

Hästnäringens Nationella Stiftelse (2021). *Hästar och uppfödare i Sverige*. Stockholm: Hästnäringens Nationella Stiftelse & Hästnäringens Avelskommitté. https://hastnaringen.se/app/uploads/2021/12/avelsrapport-2021-hastar-och-uppfodare-i-sverige.pdf [2022-10-03]

HästSverige (2018). *Embryotransfer*. https://hastsverige.se/om-hastar/hastavel-uppfodning/avelsstoet/embryotransfer/ [2022-10-04]

HästSverige (2012). *Betäckning.* https://hastsverige.se/om-hastar/hastavel-uppfodning/avelsstoet/betackning/ [2022-11-10]

International Embryo Technology Society (2020). *2020 Statistics of embryo production and transfer in domestic farm animals.* https://www.iets.org/Portals/0/Documents/Public/News/December_2020.pdf [2022-11-01]

Kamińska, D. & Gajecka, M. (2017). Is the role of human female reproductive tract microbiota underestimated? *Beneficial Microbes*, 8 (3), 327–343. https://doi.org/10.3920/BM2015.0174

Kindahl, H. (2010). Reproducitve physiology in the mare. In: *Compendium on Equine Reproduction.* Department of Obstetrics and Gynaecology. 5th ed. Uppsala: Swedish University of Agricultural Sciences

König, H.E. & Liebich, H.-G. (2020). Female genital organs (organa genitalia feminina). In: König, H.E. & Liebich, H.-G. (red.) *Veterinary Anatomy of Domestic Animals: Textbook and Colour Atlas*. 7th, updated and extended edition. Stuttgart: Georg Thieme Verlag. 449-470.

LeBlanc, M.M. (2009). The current status of antibiotic use in equine reproduction. *Equine Veterinary Education*, 21 (3), 156–167. https://doi.org/10.2746/095777308X357621

Malaluang, P., Wilén, E., Lindahl, J., Hansson, I. & Morrell, J.M. (2021). Antimicrobial resistance in equine reproduction. *Animals : an Open Access Journal from MDPI*, 11 (11), 3035. https://doi.org/10.3390/ani11113035

McKinnon, A.O. (2011). *Equine Reproduction*. 2. ed. Ames, Iowa: Wiley-Blackwell.

Miller, A. & Woods, G.L. (1988). Diagnosis and correction of twin pregnancy in the mare. *Veterinary Clinics of North America: Equine Practice*, 4 (2), 215–220. https://doi.org/10.1016/S0749-0739(17)30637-5

Noakes D.E. & Robinson B. (2019). Reproductive physiology of the female. In: Noakes, D.E., Parkinson, T.J. & England, G.C.W. (red.) *Veterinary Reproduction and Obstetrics*. 10th ed. St. Louis (MO): W.B. Saunders. 2-34. https://doi.org/10.1016/B978-0-7020-7233-8.00001-X

Otero, C., Silva de Ruiz, C., Ibañez, R., Wilde, O.R., de Ruiz Holgado, A.A.P. & Nader-Macias, M.E. (1999). Lactobacilli and Enterococci isolated from the bovine vagina during the estrous cycle. *Anaerobe*, 5 (3), 305–307. https://doi.org/10.1006/anae.1999.0245

Paccamonti, D. & Crabtree, J.R. (2019). Equine infertility and stud medicine practice. In: Noakes, D.E., Parkinson, T.J., & England, G.C.W. (red.) *Veterinary Reproduction and Obstetrics*. 10th ed. St. Louis (MO): W.B. Saunders. 541–580. https://doi.org/10.1016/B978-0-7020-7233-8.00031-8

Pinto C. & Frazer G.S. (2013). Reproduction. In: Mair, T.S., Love S., Schumacher J., Smith R.K.W., Frazer G.S. (red.) *Equine Medicine, Surgery and Reproduction*. 2nd ed. United Kingdom: Elsevier Health Sciences. 283-308.

Quereda, J.J., Barba, M., Mocé, M.L., Gomis, J., Jiménez-Trigos, E., García-Muñoz, Á., Gómez-Martín, Á., González-Torres, P., Carbonetto, B. & García-Roselló, E. (2020). Vaginal microbiota changes during estrous cycle in dairy heifers. *Frontiers in Veterinary Science*, 7. https://www.frontiersin.org/articles/10.3389/fvets.2020.00371

Rodriguez-Martinez, H. (2010). Reproductive anatomy of the mare. In: *Compendium on Equine Reproduction*. Department of Obstetrics and Gynaecology. 5th ed. Uppsala: Swedish University of Agricultural Sciences

Scherzer, J. (2011) Artificial insemination and embryo transfer in mares. *Compendium on Continuing Education for the Practising Veterinarian*, 33(7):E1-5; quiz E5. PMID: 21870346.

Scott, P., Daley, P., Baird, G.G., Sturgess, S. & Frost, A.J. (1971). The aerobic bacterial flora of the reproductive tract of the mare. *The Veterinary Record*, 88 (3), 58–61. https://doi.org/10.1136/vr.88.3.58

Singh, B. (2018). The pelvis and reproductive organs of the horse. In: Singh, B. (red.) *Dyce, Sack, and Wensing's Textbook of Veterinary Anatomy*. 5th ed. Elsevier Ltd. 552-573.

Sjaastad, Ø.V., Sand O., Hove K. (2016). Reproduction. In: Sjaastad, Ø.V., Sand O., Hove K. (red.) *Physiology of Domestic Animals*. 3rd ed. Oslo: Scandinavian Veterinary Press. 795-850.

SJVFS 2015:1. *Statens jordbruksverks föreskrifter om seminverksamhet med hästdjur*. Jönköping: Statens jordbruksverk https://lagen.nu/sjvfs/2015:1

Söderquist, L. (2010). Artificial insemination. In: *Compendium on equine reproduction*. Department of Obstetrics and Gynaecology. 5th ed. Uppsala: Swedish University of Agricultural Sciences

Troedsson, M.H.T. & Woodward, E.M. (2016). Our current understanding of the pathophysiology of equine endometritis with an emphasis on breeding-induced endometritis. *Reproductive Biology*, 16 (1), 8–12. https://doi.org/10.1016/j.repbio.2016.01.003

Witherspoon, D.M. (1971). The oestrous cycle of the mare. *Equine Veterinary Journal*, 3 (3), 114–117. https://doi.org/10.1111/j.2042-3306.1971.tb04451.x

Yates, D.J. & Whitacre, M.D. (1988). Equine artificial insemination. *Veterinary Clinics of North America: Equine Practice*, 4 (2), 291–304. https://doi.org/10.1016/S0749-0739(17)30642-9

Popular science summary

Horse breeding has been conducted in Sweden for centuries, and strategies and techniques have developed or been introduced here from other countries over time. Today artificial insemination is very common in Swedish horse breeding and embryo transfer is on the increase. One of the critical points for good breeding results, however, is a healthy reproductive system. In this project the focus is on the mare's reproductive system, and more specifically the vagina and the bacterial flora that colonise it.

Many of the published studies of the reproductive tract of mares focused on the uterus, since endometritis is common and contributes to infertility among mares. In other mammals, for example cows, more effort has been put into researching the vagina as well as the uterus. In the research on cows, one of the aims is to find probiotics, that could possibly be used to improve the environment in the cow's vagina and reproductive tract in the same way that *Lactobacillus* has proven to be an important probiotic for humans. The use of probiotics could lead to less treatment with antibiotics in the reproductive tract, which of course would be desirable from a One Health point of view.

Treatment with antibiotics in the reproductive tract is mostly used to treat infection of the uterus and would be deposited into the uterus in liquid form. One must consider, however, that everything that is inside the uterus at some point makes its way out through the vagina. The introduction of antibiotics in the reproductive organs occur not only during treatment, but also during breeding with artificial insemination and embryo transfer. The semen extender and embryo transfer media both contain small amounts of antibiotics, to protect the semen, embryo and recipient mare. This use of antibiotics is controlled by law within Sweden and the European Union and is therefore common practice in Sweden. Recent studies have shown that this low-level usage of antibiotics most probably is one of the contributing factors to antimicrobial resistance in the mare's reproductive organs.

Thus, the purpose of this study was to map the vaginal bacterial flora during different phases of the estrous cycle, to gain more information about the bacteria normally inhabiting the vagina of the mare. This was done by sampling the vagina with a swab at four time points. The study population was 10 brood mares at a stud farm in Sweden, of different ages and breeds, and with different breeding history.

All mares were examined by rectal palpation of the uterus and ovaries, and after that by ultrasound, to determine the stage of the estrous cycle. Samples were to be taken on day 0, 3, 7, and 14, where day 0 represented the ovulation. Mares were examined until ovulation was confirmed, and our day 0 samples are taken within ± 24 h of the ovulation. Swabs were taken to the laboratory after sampling and spread on agar plates and incubated. Identification of bacterial colonies were made with Matrix-Assisted Laser Desorption Ionization Time of Flight Mass Spectrometry (MALDI-TOF MS), and results were registered in amount of growth (qualitatively) and bacterial species.

A total of 564 bacterial isolates were collected, of which 323 (57.3%) could be identified by MALDI-TOF MS. Of the 22 identified bacteria, 10 were gram negative and 12 were gram positive, and the two most isolated phyla were *Firmicutes* and *Proteobacteria*. Results showed that the bacterial growth was highest on day 3 and 7, representing the beginning and middle of diestrous, i.e., the period between two ovulations where progesterone is the dominating hormone. The most frequently isolated bacteria in the mare vagina in the present study were *Escherichia coli* and *Streptococcus zooepidemicus*. *Escherichia coli* was especially dominant in the maiden mares, compared to the mares which had had foals. We also observed an increase in bacterial diversity throughout the estrous cycle, being highest on day 14.

The results of this study suggests that there are changes in the bacterial flora of the mare vagina throughout the estrous cycle, but that further research is required to confirm the results in other populations. A larger number of samples and confirmation of stage of estrous cycle and hormone level at each sampling would be preferable, but would of course be both time consuming and expensive.

Acknowledgements

To the participating stud farm, the personal, horses and horse owners – I can’t thank you enough for your contribution to this project and your patience. A special thanks to the station veterinarians who let me observe their work for weeks and took time out of their busy schedule to explain everything they did.

I would also like to thank my supervisors for their support and encouragement during this work; Jane Morrell, Ingrid Hansson, Johanna Lindahl and Pongpreecha Malaluang. This project has been very educational and interesting, and I am glad that I got the opportunity to be a part of this research group.

A special thank you to Pongpreecha Malaluang for being an excellent co-driver on trips to the stud as well as putting in long hours working in the lab and later with the results. Thank you for all your help.

Appendix 1

Bacterial growth of respective plates after 48 hours of incubation.

		Aerobic blood agar	Anaerobic blood agar	Purple agar	MacConkey agar	BP agar	MRS agar
A	**0**	Intermediate	Intermediate	Poor	No	Not used	No
	3	Intermediate	Intermediate	Poor	No	Poor	No
	7	Intermediate	Intermediate	Poor	No	No	Poor
	14	Poor	Poor	No	No	No	No
B	**0**	Poor	Poor	Poor	No	No	Poor
	3	Intermediate	Intermediate	Sparse	Poor	Poor	Poor
	8	Poor	Poor	No	No	No	No
	14	Poor	Poor	Poor	Poor	Poor	No
C	**0**	Intermediate	Intermediate	Poor	No	Not used	Not used
	3	Sparse	Sparse	No	No	Not used	Not used
	7	No	Poor	Poor	No	No	No
	14	Poor	Poor	Poor	No	No	No
D	**0**	Sparse	Intermediate	Sparse	Not used	Not used	Not used
	3	Intermediate	Intermediate	Intermediate	No	Poor	No
	7	Intermediate	Intermediate	Poor	No	No	No
	14	Intermediate	Intermediate	Intermediate	Intermediate	Sparse	No
E	**0**	Sparse	Sparse	Poor	Poor	Poor	Poor
	3	Poor	Intermediate	Poor	Poor	No	Poor
	7	Intermediate	Intermediate	Intermediate	Intermediate	Poor	Sparse
	14	Poor	Sparse	Poor	Poor	No	No
F	**0**	Poor	Intermediate	Poor	No	Poor	No
	3	Poor	Poor	Poor	Poor	No	Poor
	10	Intermediate	Intermediate	Sparse	Sparse	Sparse	Sparse
G	**0**	Sparse	Intermediate	Sparse	No	Not used	Not used
	3	Intermediate	Intermediate	Intermediate	Poor	Not used	Not used
	7	Intermediate	Intermediate	Intermediate	No	No	No
	14	Sparse	Sparse	Sparse	No	No	No
H	**0**	Intermediate	Intermediate	Poor	No	Not used	Not used
	3	Intermediate	Intermediate	Intermediate	Poor	Not used	Not used
	7	Intermediate	Intermediate	Intermediate	Poor	Not used	Not used
	11	Intermediate	Intermediate	Poor	No	No	No
I	**0**	Poor	Poor	Poor	No	Not used	No
	3	Poor	Intermediate	No	No	No	No
	7	Poor	Poor	No	No	No	No
	14	Intermediate	Intermediate	Poor	No	No	No
J	**0**	Poor	Poor	No	No	No	No
	3	Sparse	Sparse	Poor	No	Poor	No
	7	Sparse	Sparse	Poor	No	Poor	No
	14	Poor	Poor	Poor	No	No	No

Publishing and archiving

Approved students' theses at SLU are published electronically. As a student, you have the copyright to your own work and need to approve the electronic publishing. If you check the box for **YES**, the full text (pdf file) and metadata will be visible and searchable online. If you check the box for **NO**, only the metadata and the abstract will be visible and searchable online. Nevertheless, when the document is uploaded it will still be archived as a digital file. If you are more than one author, the checked box will be applied to all authors. Read about SLU's publishing agreement here:

- https://www.slu.se/en/subweb/library/publish-and-analyse/register-and-publish/agreement-for-publishing/.

☑ YES, I hereby give permission to publish the present thesis in accordance with the SLU agreement regarding the transfer of the right to publish a work.

☐ NO, I do not give permission to publish the present work. The work will still be archived and its metadata and abstract will be visible and searchable.