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Bacteria in the healthy equine vagina during the estrous cycle

P. Malaluang [a], T. Åkerholm [a], G. Nyman [a,c], J. Lindahl [a,d], I. Hansson [b], J.M. Morrell [a,*]

[a] Clinical Sciences, Swedish University of Agricultural Sciences, Uppsala, Sweden
[b] Biomedical Sciences and Veterinary Public Health, Swedish University of Agricultural Sciences, Uppsala, Sweden
[c] Lövsta Stud, Upplands Väsby, Sweden
[d] Current Affiliation Department of Animal Health and Antibiotic Strategies, National Veterinary Institute, Uppsala, Sweden

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ABSTRACT

An understanding of the normal bacterial microbiota of any organ is essential to provide the background to conditions and interventions that might cause the microbiota to change. In the vagina of the mare, a change of bacterial microbiota could be induced by introduction of semen, treatment with antibiotics, discharge from an unhealthy uterus etc. Previous studies on equine vaginal bacteria 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 microbiota of the mare vagina, and possible changes throughout the estrous cycle. The cranial portion of the vagina was sampled on day 0 (ovulation), day 3, day 7, and day 14 of the estrous cycle. The vaginal sampling was conducted with double-guarded occluded swabs from the cranial floor of the vagina. 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 Amies medium within 2–3 h and were plated out immediately on both selective and non-selective agars. Results were registered as amount of growth (qualitatively), bacterial species and number of isolates. Bacterial growth was highest on day 3 and 7, representing the beginning and middle of diestrus. The dominant bacteria were *Escherichia coli* and *Streptococcus zooepidemicus*. *Escherichia coli* was especially dominant in maiden mares, compared to the mares that had foaled. 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 microbiota of the mare vagina throughout the normal estrous cycle.

1. Introduction

The focus for research on the equine reproductive tract is usually the uterus, partly due to the contribution of endometritis to poor breeding results [1]. As a result, little is known about the bacterial microbiota of the equine vagina during the different phases of the estrous cycle. In contrast, the vaginal microbiota in production animals was thoroughly studied, because of the role a healthy reproductive system plays in achieving good breeding results and hence in the economics of livestock production. Furthermore, the uterine microbiome in livestock was studied to find probiotics for non-antibiotic treatment of infections in the reproductive tract [2,3]. Increased knowledge about the bacteria in the vagina of healthy mares would contribute to a better understanding of diseases and microbial abnormalities in the vagina, as well as 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 therapeutic treatments in the mare's reproductive tract, the vagina is also affected when fluid deposited in the uterus passes out by backflow.

Artificial insemination is commonly used for breeding sport and riding horses worldwide, apart from thoroughbreds [4]. The presence of antibiotics in semen extenders and embryo transfer media is very common, and is required by various directives e.g. in the European Union Council Directive 2019/6. Antibiotics are added to semen extenders to prevent disease in inseminated mares and deterioration of sperm quality in stored semen samples. As a result, the uterus is exposed to antibiotics at almost every AI and ET performed; these antibiotics later leave the uterus by backflow, thus exposing bacteria in the vagina and the environment to antibiotics [5]. Such exposure could induce development of antimicrobial resistance [6,7]. Knowledge about the bacteria present during the normal estrous cycle is needed to understand the impact of antibiotics on the bacterial microbiota of the vagina.

In the mare, the estrous cycle of approximately 21 days is

* Corresponding author. Clinical Sciences, SLU, Box 7054, SE.75007, Uppsala, Sweden.
E-mail address: jane.morrell@slu.se (J.M. Morrell).

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characterised by alternating periods of estrogen and progesterone dominance [8]. During estrus, for the 5 days preceding ovulation, estrogen concentrations are high and progesterone concentrations low. Estrogen concentrations start to decline in the 24h preceding ovulation, reaching a nadir approximately 4 days after ovulation. Progesterone concentrations rise precipitously 48h after ovulation, reaching a peak at the same time as estrogen is at its lowest level. They remain high until 14 days after ovulation when they fall abruptly. Estrogen concentrations start to rise again after the onset of declining progesterone concentrations.

Our hypothesis was that the changing hormones during the estrous cycle would influence the bacterial communities colonising the vagina. The objective of this study was, therefore, to map the bacterial microbiota of the cranial vagina of the mare by sampling at several times during the estrous cycle.

2. Material and methods

2.1. Mares

The study population consisted of 10 mares, 3–12 years old, six of which had previously had foals. Three were Swedish warmblood horses (SWB) and seven were standard bred trotters (Table 1). The mares were destined to be recipients in an embryo transfer program later in the season but had not received any interventions at the time of sampling for this study. An examination of the external genitalia and palpation of the ovaries was carried out when the mares arrived at the stud and also at the start of the breeding season. The mares were housed and cared for according to national regulations for breeding equids. The swabbing was done under ethical approval (number 5.8.18–15533/2018) and the study was approved by the head of the ethical committee at our university.

2.2. Swabbing

During estrus, as determined by behavioural signs and the appearance of the external genitalia, the ovaries were palpated rectally and examined with ultrasound every second day by the stud veterinarians, and at closer intervals when ovulation was expected. Vaginal swab samples were 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 early 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, sampling could only take place if it did not disrupt the stud's breeding program.

Table 1
Bacterial growth from each mare after 48 h of incubation on different agar plates from vaginal swabs of 10 mares sampled at ovulation (Day 0) and on days 3, 7 and 14 thereafter.

Horse	Day	Breed	Age	No. of foals	Aerobic blood agar	Anaerobic blood agar	Purple agar	McConkey agar	BP agar	MRS agar
A	**0**	Standardbred	6	1	Interm.	Interm.	Poor	No	Not used	No
	3				Interm.	Interm.	Poor	No	Poor	No
	7				Interm.	Interm.	Poor	No	No	Poor
	14				Poor	Poor	No	No	No	No
B	**0**	Swedish Warmblood	12	0	Poor	Poor	Poor	No	No	Poor
	3				Interm.	Interm.	Sparse	Poor	Poor	Poor
	8				Poor	Poor	No	No	No	No
	14				Poor	Poor	Poor	Poor	Poor	No
C	**0**	Swedish Warmblood	10	1	Interm.	Interm.	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**	Standardbred	9	1	Sparse	Interm.	Sparse	Not used	Not used	Not used
	3				Interm.	Interm.	Interm.	No	Poor	No
	7				Interm.	Interm.	Poor	No	No	No
	14				Interm.	Interm.	Interm.	Interm.	Sparse	No
E	**0**	Swedish Warmblood	10	0	Sparse	Sparse	Poor	Poor	Poor	Poor
	3				Poor	Interm.	Poor	Poor	No	Poor
	7				Interm.	Interm.	Interm.	Interm.	Poor	Sparse
	14				Poor	Sparse	Poor	Poor	No	No
F	**0**	Standardbred	5	0	Poor	Interm.	Poor	No	Poor	No
	3				Poor	Poor	Poor	Poor	No	Poor
	10				Interm.	Interm.	Sparse	Sparse	Sparse	Sparse
G	**0**	Standardbred	9	1	Sparse	Interm.	Sparse	No	Not used	Not used
	3				Interm.	Interm.	Interm.	Poor	Not used	Not used
	7				Interm.	Interm.	Interm.	No	No	No
	14				Sparse	Sparse	Sparse	No	No	No
H	**0**	Standardbred	10	2	Interm.	Interm.	Poor	No	Not used	Not used
	3				Interm.	Interm.	Interm.	Poor	Not used	Not used
	7				Interm.	Interm.	Interm.	Poor	Not used	Not used
	11				Interm.	Interm.	Poor	No	No	No
I	**0**	Standardbred	3	0	Poor	Poor	Poor	No	Not used	No
	3				Poor	Interm.	No	No	No	No
	7				Poor	Poor	No	No	No	No
	14				Interm.	Interm.	Poor	No	No	No
J	**0**	Standardbred	12	4	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

Notes: no growth = no visible colonies after 48h; poor growth = a few colonies could be seen only on the first streak; sparse growth = a moderate number of colonies in the first streak and none in or third streak, intermediate growth = a high number of colonies in the first and second streak but not in the third streak; and heavy growth = an increased number of colonies in the first streak and growth in both the second and third streaks.

On day 0, the samples were taken within ±24 h of ovulation, as confirmed by ultrasound. Before each sample was taken, the mare's tail was wrapped, and the perineum and vulva were washed with lukewarm water and chlorhexidine soap at least three times or until the area was visibly clean. The cleaned area was then dried with paper and checked for cleanliness. Samples were taken with a sterile rectal glove, paraffin oil, and double-guarded occluded swabs. An assistant separated the vulval labia, touching only the outer parts of the vulva, thus minimizing the risk of transferring bacteria from the surface of the skin into the vagina. The area sampled was on the floor of the vagina, approximately one finger joint (2–3 cm) caudal to the cervix (Fig. 1). The swab was turned clockwise and anti-clockwise several times to collect an adequate sample and then put into Amies transport medium with charcoal (Copan Diagnostics, Inc. Murrieta, CA, USA) to the laboratory. All sampling was performed by the same person.

2.3. Bacteriological analysis

The swabs were taken directly to the laboratory and streaked on to agar plates within 2–3 h of sampling. A selection of agar plates was used to facilitate growth and identification of as many different bacteria as possible. Blood agar (National Veterinary Institute [SVA], Uppsala, Sweden), a non-selective, good growth medium for most bacteria, was chosen for all samples. Two plates were prepared, one incubated in aerobic conditions and the other in anaerobic conditions. In addition, lactose purple agar (SVA, Uppsala, Sweden), was used for all samples, to identify bacteria that ferment lactose. MacConkey agar (SVA, Uppsala, Sweden), is a selective medium for gram negative bacteria and was used in all samples except one. Baird Parker (BP) (Oxoid, Basingstoke, UK), is a selective medium for growth of *Staphylococcus* spp., and De Man, Rogosa & Sharpe (MRS) agar (Oxoid, Basingstoke, UK), is selective for *Lactobacillus* spp. These two agars were not available at the start of the project, but subsequently became available for 75 and 81% of the samples, respectively. For three horses, these plates were used for swabs taken on all sampling days. For the remaining mares, the plates were used at least once for swabs from each horse at some point during the sampling period.

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 conditions together with lactose purple agar, MacConkey agar, and BP agar at 37 ± 1 °C; the plates were examined for bacterial growth after 24h and 48h. The MRS agar was incubated in anaerobic conditions at 25 ± 1 °C; bacterial growth was reported after five days.

After incubation, the growth on the plate was described qualitatively. The following criteria were used: no growth = no visible colonies after 48h; poor growth = a few colonies could be seen only on the first streak; sparse growth = a moderate number of colonies in the first streak and none in the second or third streak, intermediate growth = a high number of colonies in the first and second streak but not in the third streak; and heavy growth = an increased number of colonies in the first streak and growth in both the second and third streaks.

Visibly different colony types differing in shape, color, and size were re-cultured on blood agar plates at 37 ± 1 °C for 24h to obtain pure growth. Colonies were then identified using Matrix-Assisted Laser Desorption Ionization Time of Flight Mass Spectrometry (MALDI-TOF MS) (Bruker Daltonics, Billerica, MA, USA). The mass spectrum of bacterial isolates was compared with those of known bacterial strains in the database (Bruker Daltonics, Billerica, MA, USA). All identified colonies were then stored in cryotubes (containing glycerol and BHI) at −80 °C.

Fig. 1. Swab used during sampling (left) and the area of sampling in the vagina (right). The entrance to the cervix is marked with an arrow.

2.4. Statistical analysis

The data analysis was performed using R v.4.1.2 software. Pearson correlations between bacterial species were calculated using the cor.test function in the R environment, with $p \leq 0.05$ being considered significant. Associations between different groups, i.e. foaled mares vs. maiden mares, were assessed using Fisher's exact test and a p-value of ≤0.05 was considered significant.

3. Results

Swabs were taken at four time points for nine of the 10 mares, and at three time points for the remaining mare, resulting in 39 samples. The amount of growth differed between mares (Table 1), and between sampling days. For example, samples from mare J only produced sparse growth on all of the sampling days, whereas samples from mare H produced intermediate growth on both blood agar plates on all four days of sampling.

The bacterial growth on different days varied among mares; similar growth patterns were seen in only one or, at most, two mares. The trend seen on both the aerobic and anaerobic plates was that there was poorest growth on day 14 (Fig. 2), and a heavier growth on day 3 and 7 for anaerobic and aerobic blood agar plates respectively. When comparing foaled mares and maiden mares (Figs. 3 and 4), there was less growth on day 14 in both groups for both aerobic and anaerobic incubation conditions. Growth was heaviest in maiden mares on day 7 in aerobic conditions and day 3 in anaerobic conditions (Fig. 4), whereas the most growth for the foaled mares occurred on days 3 and 7 in aerobic conditions and on day 0 and 3 in anaerobic conditions. Foaled mares showed intermediate growth more often than maiden mares (aerobic growth 13/24 for foaled mares compared to 4/15 for maiden mares; anaerobic growth 15/24 for foaled mares compared with 7/15 for maiden mares), although there was only a trend towards significance ($p = 0.1$).

In total, 564 bacterial isolates were detected, of which 323 could be identified by MALDI-TOF MS (Table 2). Ten bacterial species (168 isolates) were gram negative, and 12 bacterial species (155 isolates) were gram positive. The most common phylum was *Bacillota* (previously called *Firmicutes*), consisting of 11 species with 148 isolates. *Pseudomonadota* (previously called *Proteobacteria*) was the second most common phylum, with 5 species involving 139 isolates. There was no match in the database for almost half (43%) of the isolates, which were believed to be non-pathogenic environmental bacteria. Of the isolates that could be identified, 22 species were found, of which *Escherichia coli* (9 out of 10 mares) and *Streptococcus zooepidemicus* (7 out of 10 mares) were the most frequently isolated. The third most frequently found bacteria, in half of the mares, was *Bacteroides fragilis* (Table 2). In eight mares, *Fusobacterium varium* and *Streptococcus tholartensis* appeared in the beginning of the cycle whereas *Bacteroides* spp. tended to appear towards the end of the cycle. The two most frequently isolated bacteria in this study, *Escherichia coli* and *Streptococcus zooepidemicus*, showed a variety of patterns. *Escherichia coli* was found in almost all mares (9 out of 10) but might appear on only one day in the cycle, whereas *Streptococcus zooepidemicus* was not found in all mares, but it appeared on more than one day. However, patterns were hard to determine since most bacteria did not appear frequently.

Some associations between different bacteria were observed (Fig. 5). *Staphylococcus haemolyticus* appeared in association with *Staphylococcus capitis* ($r = 0.81$) and with *Staphylococcus borealis* ($r = 0.79$). However,

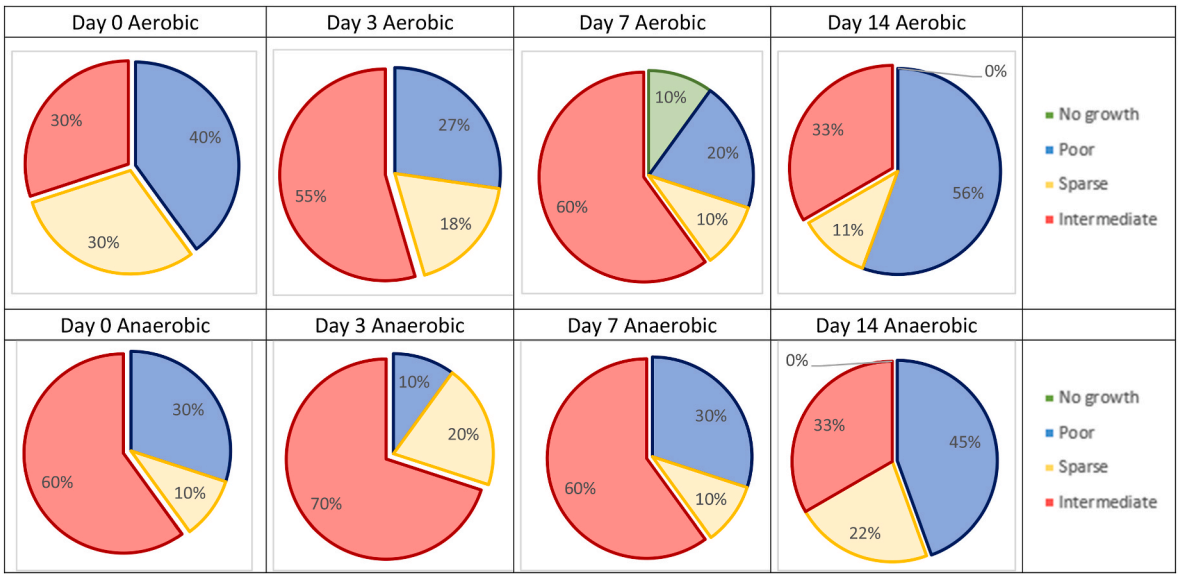

Fig. 2. Growth on aerobic (above) and anaerobic (below) blood agar plates from vaginal swabs taken at four time points during the estrous cycle: at ovulation (Day 0) and on days 3, 7 and 14 thereafter (n = 39). Note: *Day 7 included one sample taken on day 8 and one on day 10. **Day 14 included one sample taken on day 11, and contains only nine mares.
Notes: no growth = no visible colonies after 48h; poor growth = a few colonies could be seen only on the first streak; sparse growth = a moderate number of colonies in the first streak and none in or third streak, intermediate growth = a high number of colonies in the first and second streak but not in the third streak; and heavy growth = an increased number of colonies in the first streak and growth in both the second and third streaks.

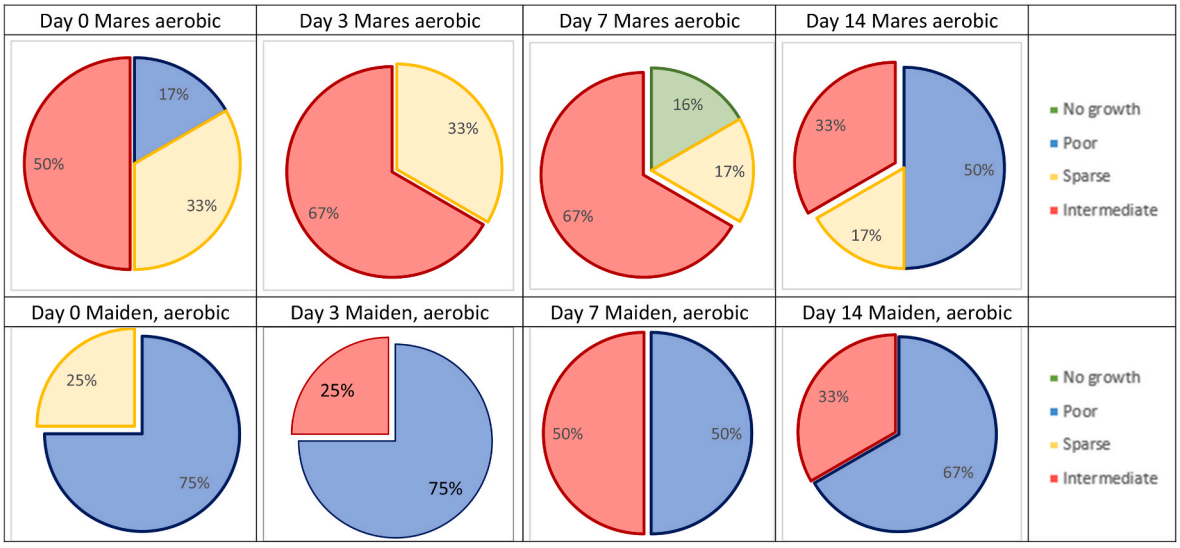

Fig. 3. Growth on aerobic blood agar plates for vaginal swabs taken from foaled mares (n = 6), above, versus maiden mares (n = 4), below. Samples were taken at ovulation (Day 0) and on days 3, 7 and 14 thereafter.
Note: *Day 7 included one sample taken on day 8 and one on day 10. **Day 14 included one sample taken on day 11, only nine mares. No growth = no visible colonies after 48h; poor growth = a few colonies could be seen only on the first streak; sparse growth = a moderate number of colonies in the first streak and none in or third streak, intermediate growth = a high number of colonies in the first and second streak but not in the third streak; and heavy growth = an increased number of colonies in the first streak and growth in both the second and third streaks.

the two most frequently isolated species in the study, *Escherichia coli* and *Streptococcus zooepidemicus*, were not strongly correlated (r = 0.38), indicating that these two do not necessarily appear at the same time in the same mare. No significant negative correlations were observed.

Associations between bacteria on different sampling days were more difficult to determine since only a few bacteria remained after excluding mares F and H (due to sampling days that deviated from the rest of the group) and all bacteria with less than three isolates (Fig. 4). Only one significant correlation (r = 0.97) was found on day 0, between *Streptococcus zooepidemicus* and *Streptococcus thoraltensis*. There were no significant correlations on day 3. One significant correlation (r = 0.72) occurred on Day 7, between *Escherichia coli* and *Streptococcus zooepidemicus*. 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).

4. Discussion

The purpose of this study was to map the vaginal bacterial microbiota of healthy mares during the estrous cycle. Samples were taken at

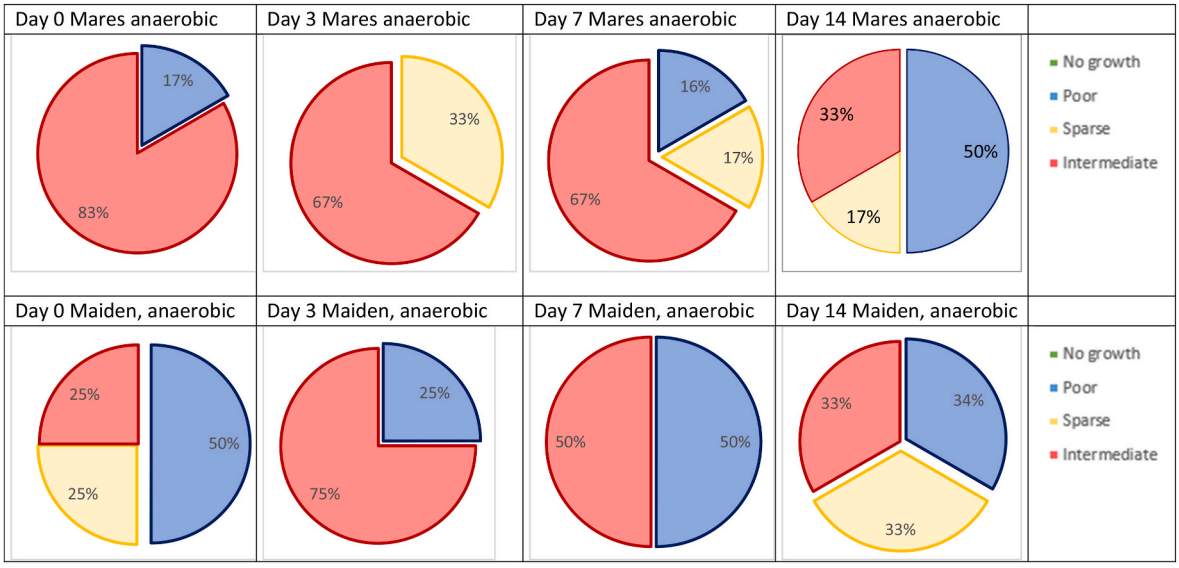

Fig. 4. Growth on anaerobic blood agar plates for vaginal swabs taken from mares that had foaled (n = 6), upper row, versus maiden mares (n = 4), lower row. Notes: *Day 7 included one sample taken on day 8 and one on day 10. **Day 14 included one sample taken on day 11, only nine mares. No growth = no visible colonies after 48h; poor growth = a few colonies could be seen only on the first streak; sparse growth = a moderate number of colonies in the first streak and none in or third streak, intermediate growth = a high number of colonies in the first and second streak but not in the third streak; and heavy growth = an increased number of colonies in the first streak and growth in both the second and third streaks.

Table 2
Bacteria identified from vaginal swabs from ten mares sampled at ovulation (Day 0) and on days 3, 7 and 14 14 days thereafter. The number of isolates is shown in the first column for each day and the number of mares is shown in parentheses.

	Day 0	(No. of mares)	Day 3	(No. of mares)	Day 7 + 8	(No. of mares)	Day 10 + 11	(No. of mares)	Day 14	(No. of mares)	Total	(No. of mares)	% of Total	% of mares
Actinobacillus equuli	0	(0)	0	(0)	3	(1)	1	(1)	0	(0)	4	(1)	0.7%	10%
Actinobacillus pleuropneumoniae	0	(0)	0	(0)	2	(1)	0	(0)	0	(0)	2	(1)	0.4%	10%
Actinobacillus rossii	3	(1)	3	(1)	3	(1)	1	(1)	3	(1)	13	(3)	2.3%	30%
Actinobacillus suis	0	(0)	3	(1)	0	(0)	1	(1)	0	(0)	4	(1)	0.7%	10%
Arcanobacterium hippocoleae	4	(3)	1	(1)	1	(1)	0	(0)	1	(1)	7	(3)	1.2%	30%
Bacteroides fluxus	0	(0)	0	(0)	0	(0)	0	(0)	1	(1)	1	(1)	0.2%	10%
Bacteroides fragilis	0	(0)	5	(3)	2	(1)	1	(1)	4	(2)	12	(5)	2.1%	50%
Bacteroides thetaiotaomicron	1	(1)	0	(0)	0	(0)	0	(0)	1	(1)	2	(2)	0.4%	20%
Clostridium ramosum	3	(2)	0	(0)	1	(1)	0	(0)	0	(0)	4	(3)	0.7%	30%
Enterococcus casseliflavus	0	(0)	0	(0)	1	(1)	3	(1)	1	(1)	5	(2)	0.9%	20%
Escherichia coli	17	(3)	35	(5)	34	(4)	17	(1)	13	(4)	116	(9)	20.6%	90%
Fusobacterium varium	4	(2)	3	(1)	0	(0)	0	(0)	2	(1)	9	(3)	1.6%	30%
Paenibacillus amylolyticus	0	(0)	3	(1)	0	(0)	0	(0)	1	(1)	4	(2)	0.7%	20%
Prevotella heparinolytica	0	(0)	2	(1)	2	(1)	0	(0)	1	(1)	5	(3)	0.9%	30%
Staphylococcus borealis	2	(2)	0	(0)	7	(1)	2	(1)	4	(1)	15	(3)	2.7%	30%
Staphylococcus capitis	0	(0)	2	(2)	3	(1)	0	(0)	9	(2)	14	(4)	2.5%	40%
Staphylococcus epidermidis	1	(1)	0	(0)	1	(1)	1	(1)	1	(1)	4	(3)	0.7%	30%
Staphylococcus haemolyticus	0	(0)	0	(0)	1	(1)	0	(0)	2	(1)	3	(2)	0.5%	20%
Staphylococcus hominis	2	(1)	0	(0)	1	(1)	0	(0)	1	(1)	4	(3)	0.7%	30%
Streptococcus infantarius	0	(0)	0	(0)	0	(0)	1	(1)	0	(0)	1	(1)	0.2%	10%
Streptococcus thoraltensis	6	(2)	0	(0)	0	(0)	0	(0)	0	(0)	6	(2)	1.1%	20%
Streptococcus zooepidemicus	10	(2)	34	(5)	24	(3)	3	(2)	17	(4)	88	(7)	15.6%	70%
Identified	53		91		86		31		62		323		57.3%	
No identification	52		87		53		10		39		241			

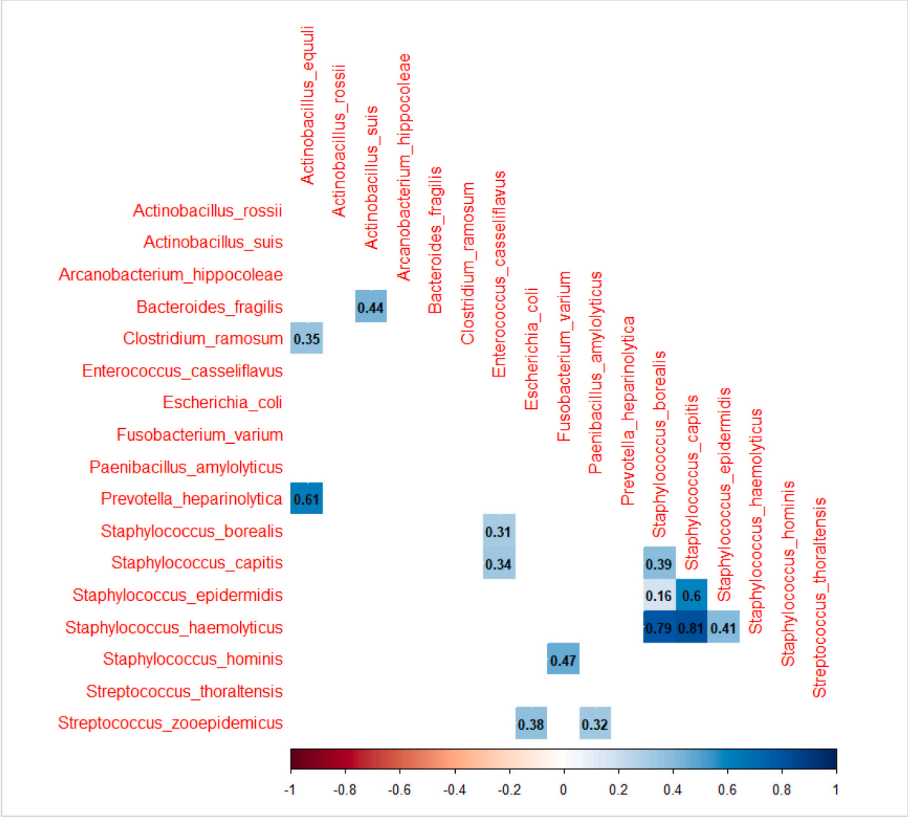

Fig. 5. Correlations between different bacteria (compiled from all mares on all days, excluding species with fewer than three isolates).
Notes: colored squares indicate correlations. The strength of the correlation is indicated by the color, blue being positive and red negative. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

four time points throughout the cycle and results were registered as amount of growth, bacterial species, and number of isolates. The four sampling days chosen represented four different phases in the equine estrous cycle and were chosen with reference to a previous study from our group [6]. In this study, day 0 represents ovulation, which was confirmed with ultrasound, and the samples were taken within ±24 h of the estimated time of ovulation. At this stage, estrogen should be high and progesterone low. Day 3 represents the phase of the cycle where there is decreased estrogen and increased progesterone, released from the newly formed corpus luteum. The highest levels of progesterone are reached on day 4–12 after ovulation [8]. Day 14 marks the end of diestrous and preparation for a new estrous, with decreasing progesterone and a rise in estrogen.

The results of the present study showed growth of aerobic as well as anaerobic bacteria, in the vagina of all mares on all sampling days. This is in contrast to a study by Hinrichs et al. [9], where no growth was 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 the authors suggested that ascending bacterial contamination of the reproductive tract might be hindered by the vulvovaginal fold. In contrast, in the current study, 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 of the vagina from which samples were taken. In a study by Barba et al. [10], eight Arabian mares were sampled twice, once in estrous and once in diestrous; the inclusion criteria for the mares at each sampling were response on teasing, ultrasound examination and progesterone level in blood. The authors concluded that there were no significant differences in the vaginal microbiome between estrous and diestrous. However, they had fewer sampling points than in the present study and did not state the timing of the diestrous sample relative to ovulation.

The mares in the present study showed considerable individual variation in the amount of growth on the four days of sampling. However, since growth was not recorded quantitatively, precise comparisons between different days and different mares were not possible. Barba et al. [10] 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, compared to estrous.

The five phyla identified (*Bacillota, Pseudomonadota, Bacteroidota, Fusobacteriota, Actinmycetota*, previously named *Firmicutes, Proteobacteria, Bacteriodetes, Fusobacteria, Actinobacteria*) are in accordance with other studies of the bacterial microbiota of the mare's vagina [10,11]. None of the bacteria known to be venereal or pathogenic to the equine reproductive tract were found in this population, such as *Taylorella equigenitalis, Klebsiella pneumoniae,* or *Pseudomonas aeruginosa* [12]. Only non-pathogenic and opportunistic bacteria, i.e. microorganisms that are found in healthy host animals but which may cause diseases in certain circumstances, were detected. The most isolated bacteria in the study, both in the number of isolates and in the number of mares, was *Escherichia coli*, closely followed by *Streptococcus zooepidemicus*, making these two by far the most dominant bacteria in the study. Both these bacteria are found in the reproductive tract of mares, and are considered to be possible causes of endometritis [12]. However, they are also known as opportunistic bacteria included in the normal respiratory and intestinal tract, respectively, in healthy horses [13,14]. Few studies have specifically sampling the vagina, and not all studies have presented

bacterial identification to species and subspecies level, making comparisons between them difficult. In the study by Hinrichs et al. [9], *Escherichia coli* or *Streptococcus zooepidemicus* were not isolated from the vagina of 48 mares, whereas Barba et al. [10] reported *Streptococcus* spp., but no *Escherichia* spp. in the eight mares that they sampled. In a study conducted by Scott et al. [15], 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. However, some of those studies were performed previously without access to current methods of identifying bacteria. In this study, MALDI-TOF MS was used for identification, with the result that 57.3% of the isolates could be identified. This was not unexpected since a large proportion of the bacteria in the environment, especially uncommon anaerobic bacteria, are not included in the MALDI-TOF database, which limits identification [16].

As shown in the results, the bacterial diversity in this group of mares changed throughout the estrous cycle. According to Barba et al. [10] 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.

Comparing mares that had already had foals with maiden mares was not one of the original aims of this study, but this became an option since the study population consisted of mares with different breeding history. The poorest growth was seen on day 14 in both groups. *Escherichia coli* was represented more frequently in the bacterial microbiota in maiden mares (50.3%) than in mares that had foaled (22.3%). The importance of this result is unclear, but it could be an indication that mating or insemination and birth of a foal introduces new bacteria to the vagina, resulting in a permanent change in the bacterial microbiota. Individual differences between mares, and their environment, can affect the bacterial microbiota. Husso et al. [11] studied fourteen mare-foal pairs, concluding that fecal contamination of the vagina occurs, although not as frequently as in similar studies on cows. In humans, both geographic location and ethnicity influence the vaginal microbiota ([17], cited by Ref. [10]). Further research is needed to investigate whether this is also true for the bacterial microbiota of the equine vagina.

Recent studies using 16S rRNA sequencing highlighted the influence of progesterone on vaginal microbial diversity in dairy cows [3,18], whereas estradiol did not appear to affect microbial communities during estrus [19]. In contrast, the composition of bacterial communities in the uterus of beef cows was seen to vary according to the circulating concentrations of both estradiol and progesterone [20]. The latter authors speculate that shifts in the relative abundance of different organisms and concomitant changes in pH in the uterus may contribute to fertility.

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

CRediT authorship contribution statement

P. Malaluang: ConceptualizationConceptualisation, Methodology, Investigation, Data curation, writing-editing. **T. Åkerholm:** Investigation, Methodology, Data curation, writing-editing. **G. Nyman:** Methodology, writing-editing. **J. Lindahl:** Methodology, Data curation, writing-editing. **I. Hansson:** ConceptualizationConceptualisation, Validation, Data curation, VisualizationVisualisation, writing-editing. **J.M. Morrell:** ConceptualizationConceptualisation, Data curation, VisualizationVisualisation, writing-editing, Project administration, Funding acquisition.

Declaration of competing interest

None.

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References

[1] Troedsson MHT, Woodward EM. Our current understanding of the pathophysiology of equine endometritis with an emphasis on breeding-induced endometritis. Reprod Biol, 2106;16:8–12. https://doi.org/10.1016/j.repbio.2016.01.003.
[2] Otero C, Silva de Ruiz C, Ibañez R, Wilde OR, de Ruiz Holgado AAP, Nader-Macias ME. Lactobacilli and Enterococci isolated from the bovine vagina during the estrous cycle. Anaerobe 1999;5(3):305–7. https://doi.org/10.1006/anae.1999.0245.
[3] Quereda JJ, Barba M, Mocé ML, Gomis J, Jiménez-Trigos E, García-Muñoz Á, et al. Vaginal microbiota changes during estrous cycle in dairy heifers. Front Vet Sci 2020;7. https://www.frontiersin.org/articles/10.3389/fvets.2020.00371.
[4] Aurich JE. Artificial insemination in horses—more than a century of practice and research. J Equine Vet Sci 2012;32:458–63. https://doi.org/10.1016/j.jevs.2012.06.011.
[5] Malaluang P, Wilén E, Lindahl J, Hansson I, Morrell JM. Antimicrobial resistance in equine reproduction. Animals : an Open Access Journal from MDPI 2021;11:3035. https://doi.org/10.3390/ani11113035.
[6] Malaluang P, Wilén E, Frosth S, Lindahl J, Hansson I, Morrell JM. Vaginal bacteria in mares and the occurrence of antimicrobial resistance. Microorganisms 2022;10:2204–21.
[7] Malaluang P, Wilén E, Frosth S, Lindahl JF, Hansson I, Morrell JM. Antimicrobial resistance in vaginal bacteria in inseminated mares. Pathogens 2023;12:375–95.
[8] Brinsko SP, Blanchard TL, Varner DD, Schumacher J, Love CC, Hinrichs K, et al. Manual of equine reproduction, vol. 3. St. Louis: Mosby, an affiliate of Elsevier Ltd; 2011.
[9] Hinrichs K, Cummings MR, Sertich PL, Kenney RM. Clinical significance of aerobic bacterial microbiota of the uterus, vagina, vestibule, and clitoral fossa of clinically normal mares. J Am Vet Med Assoc 1988;193(72–5). PMID: 3417532.
[10] Barba M, Martínez-Boví R, Quereda JJ, Mocé ML, Plaza-Dávila M, Jiménez-Trigos E, et al. Vaginal microbiota is stable throughout the estrous cycle in Arabian mares. Animals 2020;10. https://doi.org/10.3390/ani10112020.
[11] Husso A, Jalanka J, Alipour MJ, Huhti P, Kareskoski M, Pessa-Morikawa T, et al. The composition of the perinatal intestinal microbiota in horse. Sci Rep 2020;2020(10):441. https://doi.org/10.1038/s41598-019-57003-8.
[12] McKinnon AO. *Equine reproduction*. 2. Ames, Iowa: Wiley-Blackwell; 2011.
[13] Hansson I, Johansson KE, Persson M, Riihimäki M. The clinical significance of Nicoletella semolina in horses with respiratory disorders and a screening of the bacterial flora in the airways of horses. Vet Microbiol 2013;2013(162):695–9. https://doi.org/10.1016/j.vetmic.2012.09.016.
[14] Johns I, Verheyen K, Good L, Rycroft A. Antimicrobial resistance in faecal Escherichia coli isolates from horses treated with antimicrobials: a longitudinal study in hospitalised and non-hospitalised horses. Vet Microbiol 2012;2012(159):381–9. https://doi.org/10.1016/j.vetmic.2012.04.010.
[15] Scott P, Daley P, Baird GG, Sturgess S, Frost AJ. The aerobic bacterial microbiota of the reproductive tract of the mare. Vet Rec 1971;88:58–61. https://doi.org/10.1136/vr.88.3.58.
[16] Li Y, Shan M, Zhu Z, Mao X, Yan M, Chen Y, Zhu Q, Li H, Gu B. Application of MALDI-TOF MS to rapid identification of anaerobic bacteria. BMC Infect Dis, 2109;19:941. https://doi.org/10.1186/s12879-019-4584-0.
[17] Kamińska D, Gajecka M. Is the role of human female reproductive tract microbiota underestimated? Benef Microbes 2017;8:327–43. https://doi.org/10.3920/BM2015.0174.

[18] Gohil P, Patel K, Purohit K, Chavda D, Puvar A, Suthar VS, et al. Metagenomic analysis of bacterial communities during estrous cycle in Bos indicus. Trop Anim Health Prod 2022;54:118.
[19] Messman RD, Contreras-Correa ZE, Paz HA, Perry G, Lemley CO. Vaginal bacterial community composition and concentrations of estradiol at the time of artificial insemination in Brangus heifers. J Anim Sci 2020;98:1–9.
[20] Poole RK, Pickett AT, Oliveira Filho RV, de Melo GD, Palanisamy Chitlapilly, Dass VS, et al. Shifts in uterine bacterial communities associated with endogenous progesterone and 17β-estradiol concentrations in beef cattle. Domest Anim Endocrinol 2023:106766.