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– a study on mares in a stud environment

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Abstract

In Sweden and in many other countries, artificial insemination (AI) is frequently used in horse breeding. The technology makes it possible to use genetic material from horses in different geographical locations in an effective way, minimizing the risk of infection or even injury to both horses and stud personnel. Semen collection is performed with a high hygienic standard but despite this, it is seldom possible to obtain an ejaculate that does not contain bacteria. Bacteria in the ejaculate can cause disease in the mare, or result in a deterioration in sperm quality, thereby reducing the chance of the mare becoming pregnant after insemination. To ensure disease control and maintain sperm quality, addition of antibiotics to the semen is therefore required to reduce the number of bacteria in the ejaculate. To protect the spermatozoa during storage, a semen extender containing both buffering and nutrient-rich components, but also antimicrobial substances (AMS) is added. During insemination, the semen dose is deposited in the mare's uterus, which initiates a response from the local immune system. This results in an immunological and mechanical cleansing of the deposited material through the cervix uteri, which is an important function for maintaining a healthy uterine environment and enabling a pregnancy. The mare's vaginal bacterial flora is therefore exposed to the antibiotics contained in the extender, which could theoretically increase the risk of developing antibiotic resistance in the bacterial flora.

In this study, vaginal swabs were obtained from 39 mares of varying breeds and ages in northern Sweden, with the aim of investigating the normal vaginal bacterial flora and how its resistance pattern is affected by the exposure to the antibiotics (penicillin and gentamicin) contained in the semen extender INRA-96, and if resistant bacteria are present in the normal vaginal flora of mares. The mares were categorized into either an exposed group consisting of breeding mares at a stud farm, or a control group consisting of mares that had never been inseminated. Swabs were taken from a predetermined area in the cranial vagina just before the first insemination of the season (D0), with a follow-up sample after three days (D3). The control group was sampled only once, as far as possible in connection with estrus. The samples were sent by post to the Department of Biomedical Science and Veterinary Public Health, SLU, Uppsala for analyses. Isolated bacteria were identified by Matrix-Assisted Laser Desorption / Ionization Time of Flight Mass Spectrometry (MALDI-TOF MS). Bacterial species that could be isolated from both D0 and D3 in the same mare were analysed regarding antimicrobial resistance. A comparison was also made between the bacteria and resistance pattern of bacteria from mares that had never been inseminated. A total of 971 bacterial isolates were isolated from the 39 mares, with *E. coli* being by far the most common isolated bacterium (48.6%). The bacteria included in the antimicrobial susceptibility testing were *Staphylococcus simulans, Streptococcus equi* ssp. *zooepidemicus, Streptococcus dysgalactiae* and *Enterococcus faecalis*. Resistant isolates were identified in all species except *Enterococcus faecalis.* These isolates showed resistance to penicillin, oxacillin, fusidic acid, trimethoprim, erythromycin, clindamycin, nitrofurantoin and tetracycline, depending on species.

Exposure to antibiotics did not affect the resistance pattern in the vaginal bacterial flora in this study, but there was a difference in the bacterial species obtained before and after insemination and between the exposed group and the control group. Resistant bacteria were found in the vaginal flora of both groups.

Keywords: Antibiotics, antimicrobial resistance, bacteriology, artificial insemination, horse breeding, reproductive techniques, semen extender, stud farm

Sammanfattning

I Sverige och i många andra länder är det vanligt att använda sig av artificiell insemination (AI) inom hästaveln. Tekniken gör det möjligt att på ett smittskyddssäkert och effektivt sätt använda sig av genetiskt material från hästar som befinner sig på olika geografiska platser, med minskad risk för skador på såväl hästar som stuteripersonal. Spermasamling utförs med hög hygienisk standard, men trots de höga kraven på strikt hygien går det sällan att framställa ett ejakulat som inte innehåller bakterier. Bakterier riskerar att orsaka infektioner och försämra spermakvalitén, vilket minskar chansen för stoet att bli dräktigt. För att säkerställa smittskyddet och bibehålla spermakvalitén krävs därför en efterföljande beredning av sperman för att reducera mängden bakterier i ejakulatet. För att skydda spermierna under tiden spermadoser lagras och transporteras tillsätts därför en spädningsvätska innehållande dels buffrande och näringsrika komponenter, men även antimikrobiella substanser (AMS). Vid inseminationen av stoet initieras en respons från det lokala immunförsvaret, vilket resulterar i en immunologisk och mekanisk rensning av det deponerade materialet ut genom cervix uteri. Detta är en viktig funktion för att upprätthålla en hälsosam livmodermiljö och därav möjliggöra en dräktighet. Stoets vaginala bakterieflora exponeras i och med livmoderns mekaniska rensning för de i spädningsvätskan ingående antibiotikumen, vilket teoretiskt kan öka risken för utveckling av antibiotikaresistens hos bakterierna.

I denna studie provtogs 39 ston av varierande raser och åldrar i norra Sverige, med syftet att undersöka den normala vaginala bakteriefloran hos ston och hur dess resistensmönster påverkas av exponeringen för de i spädningsvätskan INRA-96 ingående antibiotikumen (penicillin och gentamicin) samt undersöka om resistenta bakterier förekommer i den normala vaginala bakteriefloran. Stona kategoriserades i antingen en exponerad grupp bestående av avelsston på stuteri, samt en kontrollgrupp bestående av ston som aldrig inseminerats. Den exponerade gruppen provtogs från ett förutbestämt område i kraniala vagina just innan den för säsongen första inseminationen (D0) samt med ett uppföljande prov efter tre dagar (D3). Kontrollgruppen provtogs enbart en gång, i högsta möjliga mån i samband med att stoet brunstade. Proverna skickades via post och analyserades på Institutionen för biomedicin och veterinär folkhälsovetenskaps laboratorium vid Sveriges lantbruksuniversitet (SLU) i Uppsala. Påvisade bakterier identifierades via Matrix-Assisted Laser Desorption/Ionization Time of Flight Mass Spectrometry (MALDI-TOF MS). Bakteriearter som kunde isoleras från både D0 och D3 hos samma sto resistenstestades. Resistensmönstret för dessa bakteriearter jämfördes sedan mellan D0 och D3 samt mot kontrollgruppens resistensresultat av samma bakterieart. Totalt påvisades 971 bakterieisolat från de 39 stona, av vilken *E. coli* var den överlägset vanligast isolerade bakterien (48.6%). De bakterier som valdes ut för resistensundersökning var *Staphylococcus simulans, Streptococcus equi* ssp. *zooepidemicus, Streptococcus dysgalactiae* och *Enterococcus faecalis*. Resistenta isolat konstaterades hos alla inkluderade bakteriearter utom *Enterococcus faecalis* mot (beroende på bakterieart) penicillin, oxacillin, fusidinsyra, trimetoprim, erythromycin, clindamycin, nitrofurantoin och tetracyklin.

I denna studie påverkade inte exponering av antibiotika i spädningsvätskan resistensmönstret hos den vaginala bakteriefloran. Däremot påverkades vilka bakteriearter som isolerades innan och efter insemination samt mellan den exponerade gruppen och kontrollgruppen. Resistenta bakterier förekom i den vaginala bakteriefloran hos de båda grupperna.

Nyckelord: antibiotika, antibiotikaresistens, bakteriologi, artificiell insemination, hästavel, reproduktionsteknik, spädningsvätska, stuteri

Content

Abbreviations

AI	Artificial insemination
AMR	Antimicrobial resistance
AMS	Antimicrobial substances
CFU	Colony forming unit
D0	The first sample taken just before the first insemination
D3	The sample taken three days after the last insemination
DNA	Deoxyribo-nucleic-acid
ECOFF	Epidemical cut-off
EUCAST	The European Committee of Antimicrobial Susceptibility Testing
FA	Fusidic acid
HGF	Horizontal gene transfer
MALDI-TOF MS	Matrix-Assisted Laser Desorption/Ionization Time of Flight Mass Spectrometry
MDR	Multidrug-resistant
MIC	Minimum Inhibitory Concentration
MHB	Mueller Hinton broth
MRS	De Man, Rogosa, Sharpe
PDR	Pan drug-resistant
PMN	Polymorphonuclear neutrophils
$PGF_2\alpha$	Prostaglandin $F_2\alpha$
SLC	Single Layer Centrifugation
SLU	Swedish University of Agricultural Sciences
XDR	Extensively drug-resistant
WHO	World Health Organization

Introduction

Artificial insemination (AI) is frequently used for horse breeding in Sweden and other countries. Transporting semen doses instead of living animals, reduces the risk of injuries to horses and working stud personnel, and also minimizes the risk of spreading pathogens between different horse populations.

The process of collecting semen from the stallion is done with strict attention to hygiene: However, contamination of the ejaculate occurs either directly from the stallion or personnel, or indirectly from the environment, before it is inseminated into the mare. Furthermore, the semen extender acts as a nutrient medium for bacteria, leading to potentially considerable growth of contaminating microbes. Apart from the risk of spreading pathogenic bacteria, which can have a detrimental effect on fertility (Samper & Tibary, 2006), it is also known that bacteria in semen can cause a deterioration in sperm quality (Aurich & Spergser, 2007), which itself can affect the fertility. Therefore, it is important to try to reduce the bacterial contamination of semen for AI and thus, the semen extender contains antimicrobial substances (AMS). The uterus normally develops an acute endometritis after insemination due to foreign substances, such as spermatozoa or debris contaminating from the reproductive tract of the stallion, which activate the immune system of the mare. This response is required to cleanse the uterus of debris and bacteria deposited during mating and is essential for maintained uterine health and the chance of pregnancy. The immune response initiates a myometrial contraction which presses unwanted material (residues of the semen dose, pathogenic microorganisms, cellular debris and inflammatory by-products) through the cervix and out through the vagina (Troedsson, 1999). This means that it is not only bacteria in the semen dose that are exposed to AMS present in the extender, but also the normal vaginal bacterial flora.

The increasing development of resistance in bacteria in our immediate environment is a worldwide problem for both animal and human health (World Health Organization, 2020), which implies that further studies are needed to investigate and potentially revise our use of antibiotics. Studies are therefore needed to evaluate whether exposure to AMS in semen extenders affects the microbiome of the reproducetive tract and therefore the potential need for alternative antimicrobial methods to control bacteria in animal inseminates.

The objectives of this study were thus to investigate the presence of resistant bacteria in the vaginal flora of mares exposed to a semen extender, INRA-96, which includes the antibiotics penicillin (27 mg/l) and gentamicin (76 mg/l) (apart from the antimycotic substance Amphotericin-beta). Specific aims were as follows: i) to

investigate whether insemination of semen extender containing antibiotics affects the pattern of antibiotic resistance in the vaginal bacterial flora; and ii) to determine if there are resistant bacteria in the normal vaginal bacterial flora of mares that have never been inseminated.

This study is part of a larger PhD-project that is ongoing at the Department of Clinical Sciences at the Swedish University of Agricultural Sciences (SLU). I helped the PhD student with a pilot study at SLU before I went to the stud to start collecting the material for the present study. During the pilot study, I learnt how to swab the vagina of the mare and how to do the microbiology, i.e. plating out and culturing under different conditions, selecting bacterial colonies for subculture, and finally identifying them with MALDI-TOF MS. While at the stud, I did all the swabbing myself, sending the material to the PhD student back in the laboratory for culture and identification. After the breeding season, I returned to SLU, where I sub-cultured the isolates that the PhD student had frozen for me, I checked the identity and did the antibiotic susceptibility testing as shown in this report.

Literature review

Artificial insemination

Horse breeding in Sweden is today conducted largely via AI with fresh, chilled or frozen semen (HästSverige, 2012) and has been used regularly in Sweden since the 1970s (Department of Obstetrics and Gynecology, 2010). The use of AI makes it possible to breed animals that would otherwise require long transportation for natural mating, which entails the risk of injury for both horses and stud personnel.

With AI, semen collected from the stallion with strict attention to hygiene is deposited via an insemination catheter in the uterus of the mare. In Sweden, semen collection and insemination may only be performed by trained personnel authorized by the Swedish Board of Agriculture (SJVFS 2015:1). Before insemination, the ejaculate is prepared by separating the gel fraction and checking the volume, sperm concentration and sperm motility to allow the correct insemination dose to be calculated, before the ejaculate is mixed with an extender. The extender typically contains water, glucose, milk or egg yolk derivatives, and antimicrobial substances. The inclusion of AMS in the semen extender is required by national and international regulations to prevent growth of microbes (Morrell & Wallgren, 2014). The semen dose can then be used fresh if the mare is in the same place as the stallion, or alternatively it can be cooled or even frozen in liquid nitrogen to be transported to the mare. Various AMS are incorporated in the extender, depending on the type of extender used. The addition of AMS contributes to increased sperm survival and also reduces the risk of transmission of pathogenic bacteria from stallion to mare (Pickett *et al*., 1999). Bacteria reduce sperm motility and cause membrane damage (Aurich & Spergser, 2007), thus decreasing the likelihood of the mare becoming pregnant.

However, some antibiotic substances have a detrimental effect on the spermatozoa (Varner *et al.*, 1997; Aurich & Spergser, 2007) which favors using a mix of different agents to reduce the detrimental effect of each component (Morrell & Wallgren, 2014). Avoiding bacterial contamination of the ejaculate and bacterial growth when collecting and preparing the semen is difficult, as the bacteria can come directly from the stallion, the environment or the staff. Contamination can also appear from the laboratory environment (Morrell *et al.,* 2014) and the ejaculate can be contaminated from the artificial vagina despite a high hygienic standard (Dean *et al.*, 2012).

Bacteria of the equine reproductive organs

Bacteria can be assumed to be present on all body surfaces, hence also on the reproductive organs of the horse. In stallions, bacteria are found on both the penis and in the distal urethra. These bacteria are primarily of a non-pathogenic nature and constitute the normal bacterial flora (Pickett *et al.*, 1999) together with a variety of fungal species (Rota *et al.*, 2011). However, there are opportunistic bacteria that do not cause disease in the stallion, but which can cause diseases such as endometritis when they enter the mare's reproductive system. Thus, they may contribute to animal suffering and large financial losses for the horse owner by making it difficult for the mare to become pregnant. Stallions may also carry primary pathogens, thus contributing to the spread of venereal diseases (Pickett *et al*., 1999). In a recent study by Al-Kass *et al.* (2020), the bacterial content of stallion semen in Sweden was studied. A large number of bacterial genera (83) were reported, with some variation among stallions. The results differed to other scientific reports, probably because the Swedish study involved identifying bacteria from 16S sequencing rather than culturing and isolating bacteria, although extraneous factors such as environment, geographic location and husbandry may also contribute to these differences (Al-Kass *et al.*, 2020).

Physiologic defense mechanisms

The immune system in the mare's uterus is designed to maintain an optimum environment for the mare to become pregnant. Bacteria normally enter the uterus in connection with mating or insemination and at foaling (Pickett *et al*., 1999), but can also naturally enter the uterus owing to any anatomical dysfunction in the reproductive tract (Troedsson, 1999). An anatomical abnormality known to affect the fertility of the mare is changed conformation of the vulva, which often leads to pneumovaginae (Hemberg *et al.*, 2005). Bacteria are found in the uterus during the first few days postpartum, but these are usually eliminated by the immune system before the foal-heat (Pickett *et al*., 1999), combined with the uterine involution. A healthy uterus being colonized by bacteria or exposed to spermatozoa quickly develops an inflammatory reaction in the endometrium (acute endometritis) to enable clearance of foreign materials from the uterine lumen. This local immune reaction is usually initiated by the migration of polymorphonuclear neutrophils (PMNs) after bacterial or sperm antigens create a local release of several different PMN-chemotactic mediators, including interleukin (IL) 1, interferon gamma (IFNγ) and prostaglandin $F_2\alpha$ ($PGF_2\alpha$). These mediators are produced by a variety of cell types in the endometrium and transported into the uterine lumen (Troedsson, 1999; Hurtgren, 2006; Troedsson & Woodward, 2016). The cascade of mediators leads to increased vascular permeability, chemotaxis, opsonization prior to phagocytosis, activation of membrane lipases and lysis of target organisms (Troedsson, 1999).

This clearance initiates a mechanical drainage of the unwanted materials (bacteria, excess spermatozoa, seminal plasma and inflammatory products) through cervix uteri within 24 hours in a healthy mare (Evans *et al.*, 1986). Myometrial contrac-

tions are initiated by $PGF_2\alpha$, which regulates the myometrial activity together with oxytocin which also cause a relaxation of cervix uteri (Troedsson, 1999). This drainage can sometimes be affected in older maiden mares, since the cervix becomes extended and tightened, preventing the uterus from clearing itself of excess fluid (Hurtgren, 2006). The vaginal bacterial flora could thus be affected by the emptying of the uterus after insemination, due to the presence of the AMS in the semen extender.

Normal vaginal bacterial flora

A normal, healthy, vaginal flora upholds a defense barrier against pathogenic or opportunistic bacteria in the reproductive tract by competing for nutrients and physiological niche. There are a few reports in the scientific literature on the bacterial species that have been isolated from the normal vaginal bacterial flora in horses.

In mares, the bacterial flora in the caudal part of the reproductive tract (vagina to clitoral fossa) usually consists of a mixed flora, containing both commensal and environmental bacteria (Scot *et al.*, 1971; Hinrichs *et al*., 1988; Ricketts *et al*., 1993).

Singh (2009) compared the vaginal bacterial flora of 54 infertile and 12 healthy breeding mares in northern India. The bacteria found in the healthy breeding mares after aerobic culture were *Klebsiella pneumoniae* ssp. *aerogenes, Escherichia coli, Enterococcus faecalis, Enterococcus cecorum, Enterobacter agglomerans, Proteus mirabilis, Proteus vulgaris, Streptococcus mitis* and *Streptococcus vestibularis* (Singh, 2009). No bacteria could be isolated in one mare, although the previously mentioned bacteria could be isolated from the other mares in various combinations of mixed flora. Singh (2009) stated that there may be significant differences in the vaginal bacterial flora and their resistance pattern between infertile and healthy breeding mares. In another study, growth of *Lactobacillus* spp. was reported from 18 of 26 vaginal bacterial samples from healthy mares. *Enterococcus faecalis* and *Enterococcus faecium* were also found (Fraga *et al.*, 2007). Aiming to investigate different subpopulations of *Streptococcus equi* ssp. *zooepidemicus* and its association with endometritis in mares, Rasmussen *et al.* (2013) isolated these bacteria in the cranial vagina of 12 of 18 mares. Other bacteria reported from the vagina of mares are *Corynebacterium* sp., gamma-staphylococci, filamentous rods (*Actinomyces, Lactobacillus*), alpha- and beta-hemolytic streptococci (Hinrichs *et al.*, 1988). Scott *et* al. (1971) studied the vaginal flora of 100 slaughtered mares whereas samples were taken within four hours post mortem by separating the tissues with a sterile scalpel and swab the lumen. The three most common bacteria were *Streptococcus equi* ssp. *zooepidemicus,* coiliform bacteria and *Pseudomonas* sp. (Scott *et al.*, 1971).

Antimicrobial treatment (local or intravaginal) may affect the commensal microflora in horses (Singh *et al*., 2009). It was also shown that systemic treatment with ceftiofur, injected intramuscular in healthy mares, resulted in a concentration in

both serum and endometrial tissue above the minimum inhibitory concentration (MIC) for *Streptococcus equi* ssp. *zooepidemicus* and *E. coli* for over 24 h after one single treatment (Witte *et al.*, 2010).

Resistance development

Bacteria can have a natural or acquired resistance to different types of antibiotics. This resistance is either a result of genetic mutations or a selection pressure in nature, and resistance occurs both in pathogenic and non-pathogenic bacteria. A variety of resistance genes can be found in the environment (Wright, 2010). Since natural selection is a risk factor for acquired antimicrobial resistance (AMR), antibiotic exposure can enable natural selection of resistant genes to occur, as those bacteria can grow with less competition from other bacterial strains in their environment. This selection also explains why topical use of antibiotics contributes to antibiotic resistance. One study suggested that this process needs a higher concentration of antibiotics than we usually see in a non-clinical environment but can happen in case of pollution of antibiotics (Martinez, 2012). Another study concluded that even a small amount of antibiotic-usage in poultry could result in a considerable development of AMR-strains of *Clostridium perfringens* (Johansson *et al.*, 2004).

When a bacterium has acquired resistance genes, these can be passed on from one bacterium to another in three known horizontal ways in nature: conjugation, transduction and transformation (Davies & Webb, 1998).

Conjugation

During conjugation, a transfer of a resistance-bearing genetic sequence (plasmid) takes place between the bacteria when they are in contact with each other. In addition, mainly gram-positive bacteria can also conjugate via transposons (Davies & Webb, 1998).

Transduction

During transduction, genetic material is transferred between the bacteria via bacteriophages or phages which then infect the host cell and take control of the host's deoxyribo-nucleic-acid (DNA) and protein synthesis. The infected cell can thus form new phages that carry resistance-bearing genetic material and spread it further. Transduction is considered to be one of the more ineffective ways to spread antibiotic resistance as the phages can usually only infect closely related bacterial species (Davies & Webb, 1998).

Transformation

With transformation, the bacterium takes up exogenous DNA from another bacterium and incorporates it into its own DNA if it fits into the homology of its own genome (Davies & Webb, 1998). Compared to plasmids, transformed DNA (transposomes) cannot replicate by itself, but when it is incorporated into a plasmid

it can transfer parts of its DNA to other plasmids and be transmitted (Rang & Dale, 2011).

The resistance genes can then be spread vertically from mother to daughter cell. This horizontal gene transfer (HGT) is central for the spread of novel and known resistance genes beyond specific clones (Bengtsson-Palme *et al.*, 2018) and makes it possible to spread genes in the environment and to other bacterial species (Martinez, 2012). It is generally assumed that the acquisition of an antibiotic resistance determinant confers a fitness cost (Andersson & Levin, 1999) since the bacteria which have received resistance genes by HGT start to produce novel genetic elements, but at a higher metabolic cost. This means that in the absence of selection, resistant bacteria will, in time, be outcompeted by susceptible ones (Martinez, 2012).

Definition of resistance

A bacterial strain is considered resistant if it has a higher MIC than the corresponding wild-type strain of the same bacterium (Martinez *et al*., 2015). Depending on the susceptibility testing, a bacterium can be classified into different resistance categories. A bacterium showing acquired non-susceptibility to at least one agent in three or more antimicrobial categories is defined as multidrug-resistant (MDR). Bacteria only susceptible to one or two antimicrobial categories are defined as extensively drug-resistant (XDR) and the ones that are non-susceptible to all agents in all antimicrobial categories are defined as pandrug-resistant (PDR) (Magiorakos *et al.*, 2012). Examples of bacteria that are known for their ability to acquire resistance genes to all currently available antimicrobial agents are for example *Klebsiella pneumoniae* and *Acinetobacter* spp. (Magiorakos *et al.*, 2012), which are reported as common bacteria in the vaginal flora of the mare (Singh, 2008).

Resistant bacteria in horses

When antimicrobial susceptibility testing of bacteria from horses was performed, different bacterial species and resistance pattern were found, varying from time to time and from one population of horses to another (Sternberg, 1999). In a recent study by Balamurugan *et al.* (2020), AMR was reported from cervical bacterial samples in breeding mares in India. The susceptibility test focusing on *E. coli*, *Streptococcus* spp. beta hemolytic group and *Staphylococcus* spp. showed that the resistance pattern for the same bacterial species varied between different mares. Their results for *E. coli* showed a higher sensitivity to ofloxacin, azithromycin, gentamicin and amikacin, and higher resistance to tetracycline, cefotaxime, amoxicillin+clavulanate and amikacin. The same study of Balamurugan *et al.* (2020) showed that *Streptococcus* spp. beta hemolytic group had a high sensitivity to cefotaxime, amoxicillin+clavulanate and azithromycin and higher resistance against tetracycline, amikacin and gentamicin All three pathogens in the study showed resistance to tetracycline. Resistant isolates of *E. coli* and beta-hemolytic streptococci were found from uterine swabs in a Swedish study. Among 104 *E. coli* isolates, resistance was most common to cephalothin, streptomycin, trimethoprim-

sulphamethoxazole and ampicillin, while among the 31 beta-hemolytic streptococci isolates, resistance was most common to gentamicin, neomycin, oxytetracycline and trimethoprim-sulphamethoxazole (Albihn *et al.*, 2003).

Repeated use of antibiotics against endometritis and in semen extenders has occurred for decades (Kenney *et al.*, 1975 cited by Albihn *et al.*, 2003) and both are suggested as risk factors for developing AMR in the bacterial flora. Since variable resistance to common antibiotics in bacteria from uterine samples of mares with fertility problems has been found, they concluded that a proper microbial diagnosis and antimicrobial susceptibility testing are required for successful antimicrobial treatment of endometritis (Albihn *et al.*, 2003). Al-Kass *et al.* (2020) also suggest a microbial diagnosis is needed to make an informed decision about the use of antibiotics. Even when a specific bacterium is manually inoculated in the uterus during estrus, this species may not necessarily be recovered days later when the acute infection is established (Evans *et al.*, 1986), which also enhances the need for microbial identification and susceptibility testing.

Alternative methods to AMS in semen extenders

Creating a favorable environment for sperm survival during storage is essential for conception. Sperm survival can be enhanced by adding nutritious and pH-buffering substances and reducing the storage temperature. Adding antibiotics may be beneficial in reducing bacterial growth but can also be detrimental to sperm quality (Varner *et al.*, 1997; Aurich & Spergser, 2007).

Single Layer Centrifugation

Colloid centrifugation can be used to physically separate spermatozoa from bacteria in an ejaculate. This technique is a relatively simple procedure and has shown to prolong the survival of boar spermatozoa at room temperature with no need of antibiotics in the semen extender (Morrell & Wallgren, 2011).

In Single Layer Centrifugation (SLC), a colloid formulation with high density is poured into a centrifugation tube and extended semen is carefully layered on top of it. The preparation is then centrifugated at 300∗g for 20 min, before aspirating the supernatant and most of the colloid. The sperm pellet can then be harvested using a sterile pipette (Morrell & Wallgren, 2014). It was shown that semen quality is improved in various species after SLC, including stallions, and there is a considerable decrease of the number of bacteria in stallion semen. In a study by Morrell *et al.* (2014), SLC with Androcoll-E was studied for its effectiveness in separating stallion spermatozoa from ejaculates, when different bacterial species were added in different loads. They concluded that SLC with Androcoll-E was effective in removing bacteria from stallion semen (i.e., 81% to >90%), although removal was correlated to the bacterial load and bacterial species, since some species could accompany the spermatozoa through the colloid (Morrell *et al.*, 2014).

Cationic antimicrobial peptides

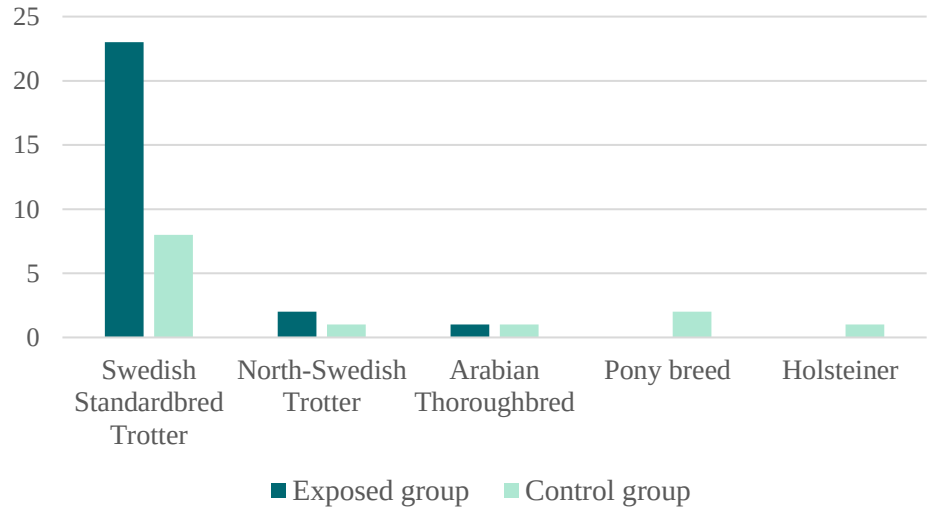
Cationic antimicrobial peptides were investigated as an additive to semen extenders for boars by Schulze *et al.* (2014). The peptides interact and destabilized bacterial cell membranes directly, resulting in an antimicrobial effect that is impossible for the bacteria to counteract. Therefore, they cannot develop resistance to these substances. These peptides should only have a minor impact on eucaryotic cells, because of differences in the charges of eucaryotic (negative) and procaryotic (positive) cell membranes. Specifically, one cyclic hexapeptide (in combination with a reduced amount of gentamicin) did not affect sperm quality or pregnancy rates following AI and was considered to be a suitable candidate for further studies as an alternative or supplement to conventional antibiotics in boar semen preservation. However, other types of peptides were shown to have unwanted effects on spermatozoa, such as decreased sperm motility and a negative effect on the sperm membrane (Schulz *et al.*, 2014).

Material and methods

Mares

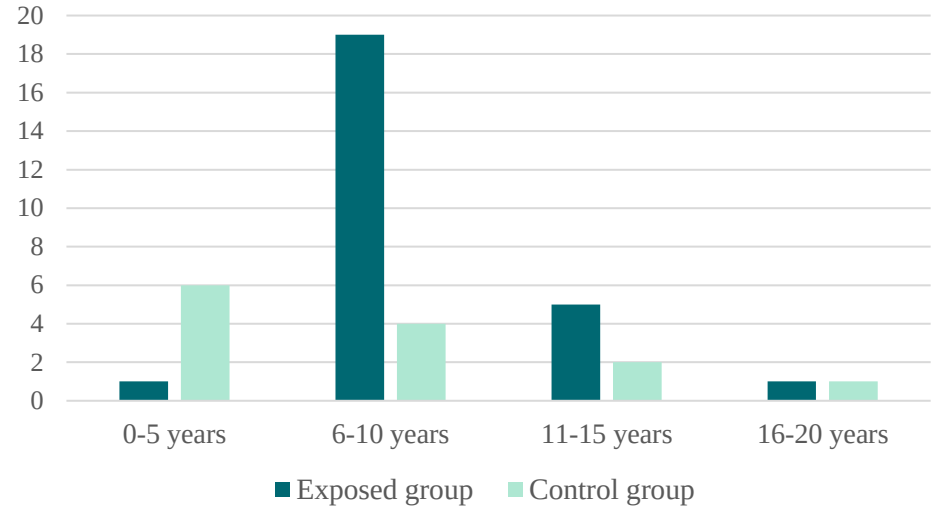
The mares (n = 39) included in the study were of different breeds, and aged 2 to 20 years, see Figure 1 and 2. They were all housed under similar conditions in the north part of Sweden; the sampling technique was the same for all mares. Breeds included in the study were Swedish Standardbred Trotters (n = 31), North-Swedish Trotter (n = 3), Arabian thoroughbred (n = 2), Holstein (n = 1) and pony breeds (n = 2). The mares were divided into two groups; mares in the *exposed group* were exposed to INRA-96 (penicillin and gentamicin) through insemination (n = 26), whereas those in the *control group* consisted of non-exposed mares (n = 13).

Figure 1. Distribution of breeds between the exposed group and the control group.



The exposed group and part of the control group were housed at the same stud farm in northern Sweden, while the remaining mares in the control group were housed within 50 kilometers of the stud farm under similar husbandry conditions.

Figure 2. Age distribution between the mares in the exposed group and the control group.



Exposed group

The 26 mares in the exposed group were aged 5 to 20 years (average age 9 years) and mainly consisted of Swedish Standardbred Trotters. The mares were largely Swedish-born. Of the 26 mares in the exposed group, 22 mares had had foals before (between 1 to 12 foals) and the remaining four mares were maiden mares. Fifteen of the mares had a nursing foal at foot. Two mares had aborted their foals earlier during the season; one mare aborted twins in the middle of her pregnancy while the other mare aborted her foal one month before the due date after an injury. The inclusion criterion for the mares in the exposed group was no previous exposure to semen extenders through insemination during the breeding season in 2020. The exclusion criteria were previous insemination during the breeding season in 2020 and mares that for any reason were systemically treated with antibiotics during the sampling period.

Control group

The 13 mares in the control group were aged 2 to 19 years (average age 7.7 years) and consisted mainly of young mares, competition mares and other riding horses of a variety of breeds. Two mares were imported from other European countries (The Netherlands and Ireland). The inclusion criterion was that they had never been inseminated or had not been inseminated within the previous 10 years. None of the mares had been systemically treated with antibiotics at the time of sampling.

Sampling technique

The sampling was carried out between March and August. For the exposed group, a day 0 (D0) sample was taken in association with the first insemination of the mare this season, where the mare was sampled before the semen dose was deposited in the uterus. Since 15 of the 26 mares in the exposed group had foals at foot, the D0 sample was taken in association with insemination at the foal heat, approximately 7-10 days postpartum. For all mares, an additional sample was taken three days later (D3). In case of insemination in a later cycle, due to failure to conceive or early pregnancy loss, further sampling was carried out at the same interval, noting that the mare had been inseminated already this season. In the control group, the mares were sampled only once, with the sample being taken as close as possible in conjuncttion with the mare's estrous. Estrous was indicated by the owners report of the mare's behavior and was confirmed by examining the color of the genital mucosa and wrinkles on the vulva lips, in connection with taking the sample. When possible, the mare's behavior in the presence of a teaser stallion was also determined (n = 7).

The sampling was performed with the mare confined in an examination stock. The tail was covered before the mare's perineal area and vulva were cleaned until they were visibly free of dirt, with at least three washes with soap and clean lukewarm water. The vulva and surrounding skin were then dried with bleached paper which was then inspected for cleanliness. If dirt was detected at this stage, the washing procedure was repeated until no visible dirt could be detected. The mare was sampled using a high hygienic standard with the hand in a clean rectal glove, sterile paraffin oil and a double-guarded occluded swab, avoiding touching the vulva lips. The sample was taken within a predetermined area of the cranial vagina, approximately three centimeters distal to the fornix vagina. The anatomical area had been pre-selected after previous inspection of organs from the slaughterhouse. The sampling area was identified by palpating portio and then moving caudally a distance of approximately one finger joint length. The sterile swab was in contact with the ventral vaginal wall for about 15 seconds while being rotated, to ensure that an adequate sample was collected.

The swab was then transferred directly into Aime's medium and marked with the mare's name, date and time of sampling. While waiting to be sent to the laboratory at SLU, the samples were stored at refrigerator temperature to prevent bacterial overgrowth. Samples were sent to the laboratory at ambient temperature; they were sent out only on Monday-Wednesday to reduce the risk of being stored in uncontrolled conditions over the weekend.

Bacteriology

Since a mixed flora was expected, several culture media were used to increase the chances of growth. When the samples arrived at SLU, cultures were made directly from the swab on (in the following order) cattle or horse blood agar plates (SVA, Uppsala, Sweden) (one plate each for aerobic and anaerobic culture environment, respectively), Baird Parker agar, McConkey agar, lactose purple agar (SVA, Uppsala, Sweden) and de Man Rogosa Sharpe (MRS) agar which is a selective culture medium for lactic bacteria. The blood agar plate labeled for anaerobic culture was placed in separate anaerobic jars with Anaerogen™ (Oxoid, Basingstoke, UK) with an oxygen indicator, to facilitate checking that the correct culture environment had been achieved.

The plates were incubated in 37°C, with bacterial growth being registered after 24 and 48 h. The MRS-agar plates were incubated anaerobically at 25°C for 5 days. Visibly different colony types from the initial culture plates were noted; a representtative colony of each type was plated out again to obtain a pure culture. These colonies were re-cultured on a blood agar plate and was incubated for 24 h in a 37°C incubator (anaerobic or anaerobic environment depending on the first screening agar) before checking growth in pure culture i.e. the presence of only one bacterial type.

Bacterial identification

Identification of bacteria in pure-cultures was performed via Matrix-Assisted Laser Desorption Ionization Time of Flight Mass Spectrometry (MALDI-TOF MS). Identified bacterial isolates were then immersed in cryotubes with a nutrient solution (BHI) and glycerol and were stored at –70°C for further analyzes of antimicrobial resistance.

Antimicrobial susceptibility testing

Susceptibility testing was performed on the bacteria that could be identified from both the D0 sample and the D3 sample from the same mare, in order to compare the resistance patterns of the bacteria before and after exposure to the antibiotics in the semen extender. For mares inseminated in more than one cycle, susceptibility testing was always performed on the last D3 sample. Cultures from the isolates found on both D0 and D3 were plated directly from the cryotubes on to horse blood agar and incubated for 24 h at 37°C. Streptococci were incubated in a CO_2 incubator (5%), in contrast to other bacteria. Only pure cultures were used for resistance testing and were checked against bacterial strains suggested by EUCAST (2020).

For resistance determination of *Staphylococcus simulans, Streptococcus equi* subsp. *zooepidemicus* and *Streptococcus dysgalactiae* the resistance panel *Thermo Scientific ™ Sensititre ™ 18-24 hour MIC and Breakpoint Susceptibility Plates* was used. For resistance determination of *Enterococcus faecalis* the resistance panel *Thermo Scientific™ Sensititre™ Gram Positive MIC Plates* was used.

Bacteria of pure cultures were homogenized in 1.0 ml NaCl and mechanically checked for turbidity of 0.50 (± 0.05) McFarland before 50 µl of the solution were added to 11 ml Mueller Hinton broth (MHB). For resistance testing of *Streptococcus dysgalactiae* 3% (330 µl) horse blood was added to the MHB. Of this solution, 50 µl was inoculated into the wells of the panel. The wells were sealed, and the panels were incubated at 37°C for 18-24 h. In connection with the susceptibility testing, a control of purification of the bacterial suspension was performed on horse blood agar. A viable count was performed to determine the concentration on a randomly selected broth suspension, diluted 1/50 with sterile water where 1µl diluted broth suspension was distributed on horse blood agar and incubated at 37°C for 24 h.

Epidemical cut-off (ECOFF) values for determining susceptibility were obtained from the European Committee on Antimicrobial Susceptibility Testing (EUCAST, 2020). The ECOFF values classify isolates with acquired reduced susceptibility as 'non wild-type'. In this paper, non-wild type isolates are called 'resistant', in agree with the Swedish Veterinary Antibiotic Resistance Monitoring report (Swedres-Svarm, 2019).

Statistical analysis

The statistical analyses were made in RStudio (2020) to compare (i) the species of bacteria isolated from *D0 and D3* and *the control group and D0*, and (ii) the antimicrobial susceptibility results between D0 and D3 in those samples where one (or more) isolate had a MIC-value above the ECOFF from EUCAST.

In the first case, the null hypothesis, H_0, was for (i) that the species of bacteria that could be isolated from D0 and D3 and control group and D0, did not depend on exposure to antibiotics in semen extenders. The Pearson's Chi-squared test was used, with significance set at $p = 0.05$.

In the second case, the null hypothesis, H_0, was that the pattern of AMR in the vaginal bacterial flora in mares was independent of exposure to antibiotics in semen extenders.

Difference in resistance between D0 and D3 for *S. simulans* (penicillin, oxacillin and fusidic acid) and *S. dysgalactiae* (erythromycin, nitrofurantoin and tetracycline) from Table 5 and 7, were analyzed by Fischer's Exact Test, with significance set at $p = 0.05$.

Tendencies for both cases was defined as differences between $0.10 \geq p \geq 0.05$.

Results

Sampled mares

In total we obtained 81 swabs from the 39 mares included in this study, swabs from three mares in the control group and one mare in the exposed group, did not present any growth from the first sample. Since we did not have any D0 sample with which to compare a D3 sample, and the mare had already been inseminated, the mare in the exposed group was excluded from further sampling. Two breeding mares were systemically treated once with Trimethoprim-sulfadiazine (Hippotrim® vet., 45 g, 288,3 mg/g + 58,0 mg/g) due to intrauterine fluid, which excluded them from further sampling, and colonies from their swabs were not chosen for susceptibility testing.

The mares were inseminated between 1-5 times (median 2 inseminations/mare; mean 2.2 inseminations/mare during the breeding season) (time between D0 to D3, where D3 refers to the sample taken three days after the last insemination and therefore is the sample used for resistance testing). Five mares were inseminated in more than one cycle. Almost all of the mares with a foal at foot were inseminated (and hence sampled for the D0 sample) during the foal heat.

Most of the samples reached the laboratory within two-four days, although a few samples arrived after 7 days due to national holidays prolonging the transport.

Bacteriological findings

In this study 971 bacterial isolates were obtained from swabs taken in the cranial vagina of 35 mares (Table 1) i.e. excluding the mares whose swabs did not produce any growth on D0. The most common bacteria isolated from the vagina was *E. coli* (48.6%), followed by *Streptococcus dysgalactiae* (11.3%), *Acinetobacter schindleri* (9.3%), *Acinetobacter lwoffi* (6.3%), *Streptococcus equi* subsp. *zooepidemicus* (4.2%) and *Enterococcus faecalis* (2.5%). Unfortunately, 6.3% of the isolates showed “No identification” on MALDI-TOF MS.

Table 1. Distribution of 971 bacterial isolates identified in each sampling category.

Bacteria identified by MALDI-TOF MS	**Control group**	**Exposed group D0**	**Exposed group D3***	**Total**
Escherichia coli	73	179	220	472
Streptococcus dysgalactiae	5	48	57	110
Acinetobacter schindleri	20	46	24	90
Acinetobacter lwoffi	22	21	18	61
No identification	26	17	18	61
Streptococcus equi ssp. *zooepidemicus*	9	8	24	41
Enterococcus faecalis	4	6	14	24
Klebsiella oxtoca	0	0	14	14
Enterococcus casseliflavus	3	0	8	11
Serratia marcescens	0	11	0	11
Staphylococcus schleiferi	0	0	10	10
Staphylococcus capitis	3	4	0	7
Aerococcus viridans	0	6	0	6
Staphylococcus vitulinus	0	4	2	6
Corynebacterium casei	3	3	0	6
Staphylococcus simulans	0	3	1	4
Corynebacterium callunae	0	0	3	3
Pantoea agglomerans	0	0	3	3
Streptococcus equinus	0	1	1	2
Streptococcus gallolyticus	0	0	2	2
Other bacteria**	4	13	10	27
Total number of isolates:	172	370	429	971

**D3 represents the summarized number of isolates from each bacterial specie, obtained from the last D3 sample of each mare (after approximately 2 inseminations).*
***Other bacteria includes: Acinetobacter sp., Acinetobacter bohemicus, Acinetobacter johnsonii, Acinetobacter kookii, Arthrobacter gandavensis, Enterobacter kobei, Enterococcus mundtii, Kluyvera intermedia, Lactococcus raffnolactis, Lelliottia amnigena, Paenibacillus amylolyticus, Pseudomonas fulva, Rahnella aquatilis, Staphylococcus aureus, Staphylococcus haemolyticus, Streptococcus canis, Streptococcus hyovaginals and Streptococcus thoraltensis.*

The top five most isolated bacterial species were present in samples both from the control group and from the breeding mares with various frequencies, see Table 2.

Table 2. Occurrence of bacterial species among the mares in each sampling category.

	Control group	**Exposed group D0**	**Exposed group D3**
Escherichia coli	70%	64%	87%
Streptococcus dysgalactiae	10%	52%	65%
Acinetobacter schindleri	20%	24%	17%
Acinetobacter lwoffi	40%	24%	30%
Enterococcus faecalis	10%	8%	9%
Streptococcus equi ssp. *zooepidemicus*	50%	12%	35%

In the exposed group, 21 out of 26 mares showed growth of the same bacterial species both at D0 and D3. In 15 out of these 21 mares the bacterial species were: *Staphylococcus simulans, Streptococcus equi* subsp. *zooepidemicus, Streptococcus dysgalactiae* and *Enterococcus faecalis*. These bacteria were chosen for the susceptibility testing. In addition to these bacteria, many mares also had *Eschericia coli*, but there were too many isolates to be included in this study. These isolates will be analyzed in another study (PhD project) at SLU.

Species of bacteria detected

The top seven most isolated species of bacteria found on D0 were different to those found on D3 (Table 3; $p < 0,01$). The six most isolated species found in control mares were different to those from D0 in exposed mares (Table 4; $p < 0.0000002$).

Table 3. Comparison of the number of bacterial species isolated from D0 and D3.

	E. coli	***S. dysgalactiae***	***A. schindleri***	***A. lwoffi***	***No identification***	***S. equi* ssp. *zooepidemicus***	***E. faecalis***	**Others**
D0	179	48	46	21	17	8	6	45
D3	220	57	24	18	18	24	14	54

Pearson's Chi-squared test, $\chi^2 = 19.928$, *df = 7*

$p < 0.01$

Table 4. Comparison of bacterial species isolated from control mares and D0.

	E. coli	***S. dysgalactiae***	***A. schindleri***	***A. lwoffi***	***No identification***	***S. equi* ssp. *zooepidemicus***	**Others**
Control	73	5	20	22	26	9	17
D0	179	48	46	21	17	8	51

Pearson's Chi-squared test, $\chi^2 = 41.948$, *df = 6,*

$p < 0.0000002$

There were no differences in bacterial species isolated from the last D3 sample, regardless of whether the mare had been inseminated in one or more than one cycle.

Antimicrobial susceptibility testing

The results of the susceptibility testing are presented in Table 5-11. Breakpoint values from EUCAST (2020) are marked with a vertical line, representing the MIC-value where a bacterial isolate is considered resistant, since it has a MIC above the corresponding wild-type strain of the same bacterium.

Exposed group

Table 5. MIC for Staphylococcus simulans, exposed group (1 horse, 4 isolates). Breakpoint values from EUCAST (2020) are marked with a vertical line.

µg/ml	*<0.03*	*0.06*	*0.125*	*0.25*	*<0.5*	*0.5*	*<1*	*1*	*>1*	*2*	*>2*
Penicillin, D0	*1*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*2*	*0*	*0*
Penicillin, D3	*1*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*
Oxacillin, D0	*0*	*0*	*0*	*2*	*0*	*1*	*0*	*0*	*0*	*0*	*0*
Oxacillin, D3	*0*	*0*	*0*	*1*	*0*	*0*		*0*	*0*	*0*	*0*
Fusidic acid, D0	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*1*	*0*	*2*	*0*
Fusidic acid, D3	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*1*	*0*
Erythromycin, D0	*0*	*0*	*0*	*0*	*3*	*0*	*0*	*0*	*0*	*0*	*0*
Erythromycin, D3	*0*	*0*	*0*	*0*	*1*	*0*	*0*	*0*	*0*	*0*	*0*
Gentamicin, D0	*0*	*0*	*0*	*0*	*3*	*0*	*0*	*0*	*0*	*0*	*0*
Gentamicin, D3	*0*	*0*	*0*	*0*	*1*	*0*	*0*	*0*	*0*	*0*	*0*
Tetracycline, D0	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*3*	*0*	*0*	*0*
Tetracycline, D3	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*1*	*0*	*0*	*0*

Table 6. MIC for Streptococcus equi ssp. zooepidemicus, exposed group (2 horses, 8 isolates). Breakpoint values from EUCAST (2020) are marked with a vertical line.

µg/ml	<0.03	0.06	0.12	<0.25	0.25	>0.25	<0.5	0.5	>0.5	<1	1	>1	2	>2	4	8	<16	32	>64
Penicillin, D0	5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Penicillin, D3	3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Oxacillin, D0	0	0	0	5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Oxacillin, D3	0	0	0	3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Erythromycin, D0	0	0	0	0	0	0	5	0	0	0	0	0	0	0	0	0	0	0	0
Erythromycin, D3	0	0	0	0	0	0	3	0	0	0	0	0	0	0	0	0	0	0	0
Clindamycin, D0	0	0	0	0	0	0	5	0	0	0	0	0	0	0	0	0	0	0	0
Clindamycin, D3	0	0	0	0	0	0	3	0	0	0	0	0	0	0	0	0	0	0	0
Gentamicin, D0*	0	0	0	0	0	0	0	0	0	5	0	0	0	0	0	0	0	0	0
Gentamicin, D3*	0	0	0	0	0	0	0	0	0	3	0	0	0	0	0	0	0	0	0
Nitrofurantoin, D0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	5	0	0
Nitrofurantoin, D3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	3	0	0
Tetracycline, D0	0	0	0	0	0	0	0	0	0	0	0	0	5	0	0	0	0	0	0
Tetracycline, D3	0	0	0	0	0	0	0	0	0	0	0	0	3	0	0	0	0	0	0
*Trimethoprim**, D0*	0	0	0	5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
*Trimethoprim**, D3*	0	0	0	3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

* No breakpoint for gentamicin is available for *Streptococcus* sp. from EUCAST (2020)
** Trimethoprim:sulfamethoxazole in the ratio 1:19. Breakpoints are expressed as the trimethoprim concentration.

Table 7. MIC for *Streptococcus dysgalactiae*, exposed group (10 horses, 90 isolates). *Breakpoint values from EUCAST (2020) are marked with a vertical line.*

µg/ml	*<0.03*	*0.06*	*0.12*	*<0.25*	*0.25*	*>0.25*	*<0.5*	*0.5*	*>0.5*	*<1*	*1*	*>1*	*2*	*>2*	*4*	*>4*	*8*	*<16*	*32*	*>64*
Penicillin, D0	*48*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*
Penicillin, D3	*42*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*
Oxacillin, D0	*0*	*0*	*0*	*48*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*
Oxacillin, D3	*0*	*0*	*0*	*42*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*
Erythromycin, D0	*0*	*0*	*0*	*0*	*0*	*0*	*47*	*0*	*0*	*0*	*1*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*
Erythromycin, D3	*0*	*0*	*0*	*0*	*0*	*0*	*42*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*
Clindamycin, D0	*0*	*0*	*0*	*0*	*0*	*0*	*48*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*
Clindamycin, D3	*0*	*0*	*0*	*0*	*0*	*0*	*42*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*
Gentamicin, D0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*4*	*0*	*39*	*5*	*0*	*0*	*0*	*0*
Gentamicin, D3*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*1*	*0*	*37*	*4*	*0*	*0*	*0*	*0*
Nitrofurantoin, D0	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*39*	*9*	*0*
Nitrofurantoin, D3	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*35*	*6*	*1*
Tetracycline, D0	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*6*	*0*	*39*	*3*	*0*	*0*	*0*	*0*
Tetracycline, D3	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*2*	*0*	*37*	*3*	*0*	*0*	*0*	*0*
*Trimethoprim**, D0*	*0*	*0*	*0*	*48*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*
*Trimethoprim**, D3*	*0*	*0*	*0*	*41*	*0*	*0*	*0*	*1*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*

* No breakpoint for gentamicin is available for *Streptococcus* sp. from EUCAST (2020)
** Trimethoprim:sulfamethoxazole in the ratio 1:19. Breakpoints are expressed as the trimethoprim concentration.

Table 8. MIC for *Enterococcus faecalis*, exposed group (2 horses, 20 isolates). *Breakpoint values from EUCAST (2020) are marked with a vertical line.*

µg/ml	*0.06*	*0.12*	*0.25*	*>0.25*	*<0.5*	*0.5*	*>0.5*	*<1*	*1*	*>1*	*2*	*>2*	*4*	*>4*	*<8*	*8*	*>8*	*16*	*32*	*>64*	*>128*
Vancomycin, D0	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*5*	*0*	*0*	*1*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*
Vancomycin, D3	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*9*	*0*	*0*	*4*	*0*	*1*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*
Teicoplanin, D0	*0*	*0*	*0*	*0*	*6*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*
Teicoplanin, D3	*0*	*0*	*0*	*0*	*14*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*
Ciprofloxacin, D0	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*6*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*
Ciprofloxacin, D3	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*3*	*0*	*11*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*
Tigecycline, D0	*6*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*
Tigecycline, D3	*12*	*2*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*
Linezolid, D0	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*6*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*
Linezolid, D3	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*14*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*
Gentamicin, D0	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*5*	*0*	*0*	*1*	*0*	*0*	*0*
Gentamicin, D3	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*13*	*0*	*0*	*1*	*0*	*0*	*0*
Ampicillin, D0	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*5*	*0*	*1*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*
Ampicillin, D3	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*14*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*

Control group

Table 9. MIC for Streptococcus equi ssp. zooepidemicus, control group (5 horses, 9 isolates). Breakpoint values from EUCAST (2020) are marked with a vertical line.

µg/ml	<0.03	0.06	0.12	<0.25	0.25	<0.5	0.5	>0.5	<1	1	2	>2	4	>4	8	<16	32	>64
Penicillin	9	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Oxacillin	0	0	0	9	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Erythromycin	0	0	0	0	0	9	0	0	0	0	0	0	0	0	0	0	0	0
Clindamycin	0	0	0	0	0	7	0	0	0	1	0	1	0	0	0	0	0	0
*Gentamicin**	0	0	0	0	0	0	0	0	7	0	1	0	1	0	0	0	0	0
Nitrofurantoin	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	9	0	0
Tetracycline	0	0	0	0	0	0	0	0	0	0	4	0	1	4	0	0	0	0
*Trimethoprim***	0	0	0	2	0	0	5	0	0	1	0	0	0	1	0	0	0	0

* No breakpoint for gentamicin is available for *Streptococcus* sp. from EUCAST (2020)
** Trimethoprim:sulfamethoxazole in the ratio 1:19. Breakpoints are expressed as the trimethoprim concentration.

Table 10. MIC for Streptococcus dysgalactiae, control group (1 horse, 5 isolates). Breakpoint values from EUCAST (2020) are marked with a vertical line.

µg/ml	*<0.03*	*0.06*	*0.12*	*<0.25*	*0.25*	*<0.5*	*0.5*	*>0.5*	*<1*	*1*	*2*	*>2*	*4*	*>4*	*8*	*<16*	*32*	*>64*
Penicillin	*5*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*
Oxacillin	*0*	*0*	*0*	*5*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*
Erythromycin	*0*	*0*	*0*	*0*	*0*	*5*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*
Clindamycin	*0*	*0*	*0*	*0*	*0*	*5*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*
*Gentamicin**	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*2*	*0*	*3*	*0*	*0*	*0*	*0*	*0*
Nitrofurantoin	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*5*	*0*	*0*
Tetracycline	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*3*	*0*	*2*	*0*	*0*	*0*	*0*	*0*
*Trimethoprim***	*0*	*0*	*0*	*5*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*

* No breakpoint for gentamicin is available for *Streptococcus* sp. from EUCAST (2020)
** Trimethoprim:sulfamethoxazole in the ratio 1:19. Breakpoints are expressed as the trimethoprim concentration.

Table 11. MIC for Enterococcus faecalis, control group (1 horse, 4 isolates). Breakpoint values from EUCAST (2020) are marked with a vertical line.

µg/ml	*0.06*	*0.12*	*0.25*	*>0.25*	*<0.5*	*0.5*	*>0.5*	*<1*	*1*	*>1*	*2*	*>2*	*4*	*>4*	*<8*	*8*	*>8*	*<16*	*32*	*>64*	*>128*
Vancomycin	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*4*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*
Teicoplanin	*0*	*0*	*0*	*0*	*4*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*
Ciprofloxacin	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*1*	*0*	*3*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*
Tigecycline	*1*	*2*	*1*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*
Linezolid	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*2*	*0*	*2*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*
Gentamicin	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*4*	*0*	*0*	*0*	*0*	*0*	*0*
Ampicillin	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*4*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*

Staphylococcus simulans

Four isolates of *Staphylococcus simulans (S. simulans)* were obtained from one horse; three isolates were found on D0 and one isolate on D3. Resistance was found against penicillin, oxacillin and fusidic acid (FA), but the isolates showed a normal susceptibility against the other antibiotics included in the panel (erythromycin, gentamicin and tetracycline) as shown in Table 5. *S. simulans* was not isolated from any mares in the control group; therefore, it was not possible to compare the antibiotic susceptibility between groups.

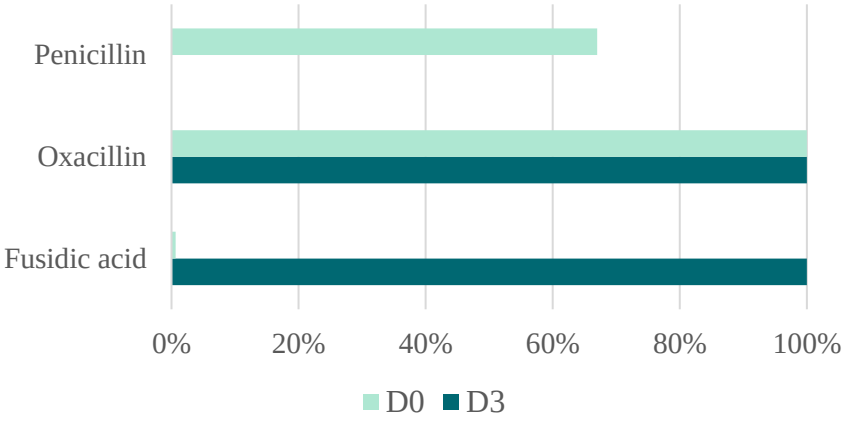


Figure 3. Resistance results of S. simulans.

The results showed variable resistance for the three antibiotics between D0 and D3, as shown in Figure 3. There were 67% resistant isolates on D0 compared to 0% on D3, against penicillin, 100% resistance on D0 and D3 against oxacillin, and 0,67% resistant isolates on D0 compared to 100% on D3 against FA. Two of the D0 isolates showed resistance to all three substances, while the other two isolates (one D0 and one D3) showed resistance to oxacillin and FA.

Streptococcus equi ssp. *zooepidemicus*

Eight isolates of *Streptococcus equi* ssp. *zooepidemicus (S. equi* ssp. *Zooepidemicus*) were obtained from two mares in the exposed group; five isolates came from D0 and three isolates from D3. These bacteria did not show any difference in resistance pattern between D0 and D3 to any of the substances included in the panel (Table 6).

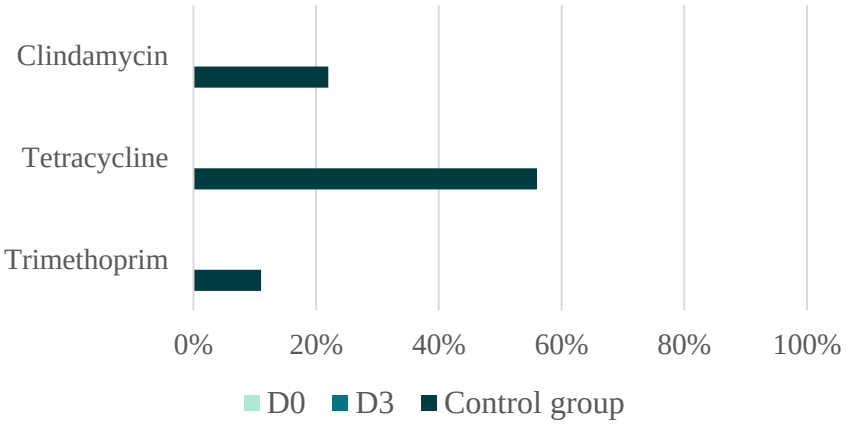


Figure 4. Resistance results of S. equi ssp. zooepidemicus. Resistant isolates were only obtained from the control group.

In the control group, consisting of five horses and nine isolates, resistance was found against clindamycin, tetracycline and trimethoprim in isolates from four of the five mares (Table 9). The variation in resistance among the isolates is shown in Figure 4. Only one of the isolates, belonging to a 19-year-old mare of Pony breed, showed resistance against all the three substances.

Streptococcus dysgalactiae

Ninety isolates of *Streptococcus dysgalactiae* (*S. dysgalactiae*) were obtained from 10 horses in the exposed group; 48 isolates from D0 and 42 isolates from D3. Resistance was found against erythromycin, nitrofurantoin and tetracycline, but the isolates were susceptible to all other substances included in the panel (Table 7). All ten mares in the exposed group had isolates resistant to tetracycline - in total, 82 out of 90 isolates (91%) were resistant to tetracycline. In the control group, out of five isolates from one mare, two isolates (40%) showed resistance against tetracycline but were susceptible to all other substances included in the panel (Table 10). None of the resistant isolates were resistant to more than two substances.

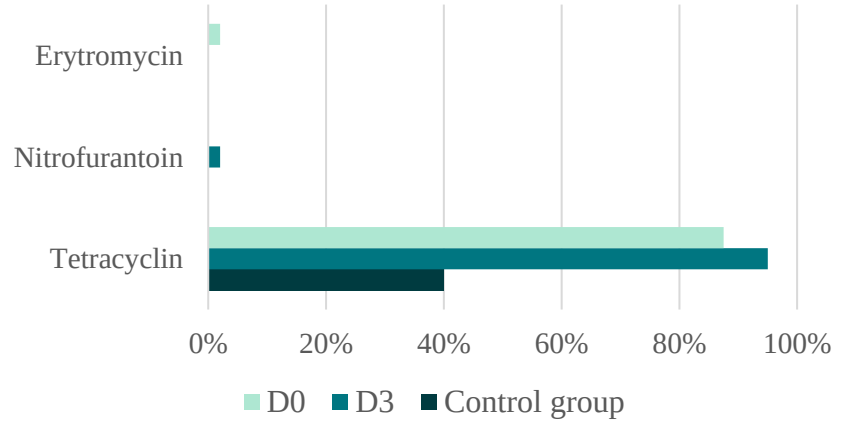


Figure 5. Resistance results of S. dysgalactiae.

Resistance to the different antibiotics varied between D0 and D3 isolates for *S. dysgalactiae*, as shown in Figure 5. One D0 isolate showed resistance against erythromycin and one isolate from D3 showed resistance against nitrofurantoin. In the exposed group, 87.5% of the isolates from D0 and 95% of the isolates from D3 showed resistance against tetracycline, compared to the control group where 40% of the isolates showed resistance against tetracycline but was susceptible to the other substances.

Enterococcus faecalis

Twenty isolates of *Enterococcus faecalis (E. faecalis)* came from two mares in the exposed group; six isolates from D0 and 14 isolates from D3. Four isolates were identified from one mare in the control group. No resistance was found in either group (Table 8 and 11).

Susceptibility to antibiotics

It was only possible to analyze the results for susceptibility testing for *S. simulans* and *S. dysgalactiae* since *S. equi* ssp. *zooepidemicus* and *E. faecalis* did not show any resistance in the exposed group.

The exposure to antibiotics in the insemination dose did not alter the resistance pattern of *S. simulans* to penicillin ($p = 1$), oxacillin ($p = 1$) and FA ($p = 1$), or *S. dysgalactiae* to erythromycin ($p = 1$), nitrofurantoin ($p = 0.47$) and tetracycline ($p = 0.28$) between D0 and D3.

The susceptibility of the bacteria at D3 was not different between mares that had been inseminated only once ($n = 20$), compared to those mares that had been inseminated up to five times ($n = 5$).

Discussion

The aim of this study was to investigate the normal vaginal bacterial flora of mares and the presence of any resistant bacteria, as well as whether there was any influence of antibiotics included in the semen extender INRA-96 on the flora, or on the pattern of AMR.

In total, 971 bacterial isolates were isolated from 35 out of 39 mares. No bacterial growth was obtained from three mares in the control group and one mare in the exposed group. Even though the predeterminate sampling procedure was followed as usual for these four mares, there might have been some methodical complication or an unknown event during the transport to the laboratory, such as a considerable change in temperature, that affected the ability to culture bacteria from these swabs. Ricketts *et al.* (1993) suggested that samples, in transport medium, should reach the laboratory within 2 days, even though longer viability of the bacteria is possible. In our study, most of the samples reached the laboratory within 2-4 days, but for a few samples it took as long as 7 days. This could possibly result in false-negative results of some bacteria.

The bacterial species obtained showed some similarities to those reported by Scott *et al.* (1971) and Singh (2009). We did not find any *Lactobacillus* spp. even though we used MRS-agar to optimize the culture conditions (149 isolates showed growth on MRS-agar). This was one of the bacteria we expected to isolate since it is found frequently in the vaginal flora of many species and it was a commonly isolated bacteria in the study by Fraga *et al.* (2007). However, even though our results showed some similarities with the scientific literature referred to in this study, we did not isolate *Klebsiella pneumoniae* ssp. *aerogenes*, *Enterococcus cecorum*, *Proteus* sp., *Enterobacter agglomerans* or *Streptococcus mitis* in contrast to Singh (2009). On the other hand, *E. coli* and *Enterococcus faecalis* were the second, and third most isolated bacteria in the study by Singh (2009), and coliform bacteria and beta-hemolytic streptococci, such as *S. equi* ssp. *zooepidemicus,* have been considered as frequently isolated bacteria from the vagina of mares (Scot *et al.*, 1971). On the other hand, Hinrichs *et al.* (1988) who collected vaginal samples using a sterile speculum and guarded culture instrument inserted through the speculum, obtained only 42% positive growth from samples of the vagina of 48 mares. Only two beta-hemolytic streptococci and no *E. coli* or *Streptococcus equi* ssp. *Zooepidemicus* could be collected from the vagina. In their study, they concluded that *E. coli* and beta-hemolytic streptococci did not appear to be a common bacterium in the clinically normal vagina of mares (Hinrichs *et al.*, 1988). If this is true for all populations of mare, it could imply that we had some contamination from the caudal

part of the reproductive tract such as vestibulum, introduced on the gloved hand while palpating the portio.

Some external factors that may have contributed to the differences in the species isolated in the present study, compared to the previous studies, could be the geographic location, housing conditions of the mares, used sampling technique (i.e. how and where in the genital tract the sample is taken) or identification method of bacterial species as well as the studied population of horses. We cannot exclude the risk of that the mares inseminated during the foal heat had a contaminated vaginal bacterial flora, since foaling is stated as one of the natural causes of contamination of the reproductive tract of mares (Pickett *et al*., 1999). Even though uterine contamination is usually eliminated before the foal heat, this might not be the case for vaginal contamination, due to a smaller inflammatory response compared to the uterine response post partum.

Since MALDI-TOF MS only recognizes bacteria that are included in the database, it is possible that some environmental bacteria that are not commonly known as pathogens are excluded from the database, hence giving the result "No identification". This was unfortunately the result for 6.3% of the bacterial isolates, which probably could have been avoided by using 16S rRNA and 18s rRNA gene sequencing as the method for bacterial identification instead. According to Singhal *et al*. (2015), 16S rRNA and 18s rRNA gene sequencing is considered as the gold standard for bacterial identification. However, due to constraints of cost and time, MALDI-TOF MS was used in the present study. To enhance the possibilities of identifying isolates showing "No identification" on MALDI-TOF MS after the first time they were tested, we added formic acid (FA) since this could help in the identification of gram positive bacteria (Singhal *et al.*, 2015). Other bacteria, such as *Nocardia* spp. and *Mycobacterium* spp. also need special solutions to enable their identification via MALDI-TOF MS (Singhal *et al.*, 2015), which were not used. Therefore, it is not possible to state if they were present or not.

From 21 mares in the exposed group, the same bacterial species could be isolated from both D0 and D3 and therefore could be compared for their susceptibility. Unfortunately, since MALDI-TOF MS only identifies the bacterial species, it is impossible to say if these isolates were of the same bacterial strain without performing a 16S rRNA and 18s rRNA gene sequencing. The species that were isolated on both D0 and D3 from 15 of the 21 mares were *Staphylococcus simulans, Streptococcus equi* ssp. *zooepidemicus, Streptococcus dysgalactiae* and *Enterococcus faecalis.* Since this study is a part of a larger PhD project, the results for *E. coli* will be incorporated on in a future report, including the 6 remaining mares.

Differences in the vaginal bacterial flora

There were considerable differences in the bacteria that could be isolated from individual mares, even though *E. coli* was the most frequently isolated bacterium in both groups. Since *E. coli* is a common environmental bacterium spread by fecal

contents and in soil, its presence in the vaginal bacteria flora regardless of the mares' geographic location, age, breed and sexual status, is not surprising. Even though the mares were housed in similar conditions during the study, some of the control horses were originally imported from other European countries such as the Netherlands and Ireland, whereas the breeding mares at the stud were largely Swedish-born. The length of time the mares had been housed in the environment also varied. Other aspects that might influence the bacterial flora are the ages and breeds of the mares and the possibility that the samples were taken on different days during estrus for the control group, where the mares were not checked for impending ovulation in the same way as for the breeding mares. It is also possible that some methodical variations might have affected the bacteriology, since contamination from the external reproductive organs or perianal region while taking the sample or a prolonged time between taking the sample and its arrival to the laboratory.

The seven most isolated bacteria (all groups) did differ between D0 and D3. This would mean that the exposure to antibiotics in semen extenders (or possibly something else in the inseminate, such as the semen itself or substances influencing the pH-value in the vagina) does have a significant effect of the bacterial species found in the vaginal bacterial flora in mares. There was also a difference between isolated bacteria in the control group and D0. It would have been better to use the same mare as her own control when comparing the bacterial results before and after insemination, but then a whole genome sequencing might be necessary to determine that it is the same bacterial strain that is compared. If it is true that there is a difference in bacterial species between D0 and D3, and control mares and D0, it would be interesting to know how a change in the composition of the bacterial flora after insemination might affect the natural defense-mechanism the normal vaginal flora. Only four of the 26 mares in the exposed group were maiden mares, and therefore most of the mares had been inseminated during previous breeding seasons before the D0 sample in this study was taken. This could explain the difference of bacterial species between the control group and D0. However, some mares in the control group were located at another stable; although the husbandry conditions were the same in both places, there might have been some differences in the microenvironment that contributed to differences in the vaginal flora among the control mares.

Another interesting aspect that needs further studies is whether different bacteria in the vaginal bacterial flora interact with each other, and if so, how this could be used as a biological treatment. This concept was studied by Fraga *et al.* (2007), who concluded that there are some lactic acid bacterial isolates that showed promising features for their use as probiotics in horses. Probiotics could then possibly be used as a drug free option to antibiotics when treating bacterial vaginoses, but this needs further studies.

Antimicrobial susceptibility patterns

There could be several explanations for the susceptibility patterns showing more resistant isolates from the control group, and sometimes also more resistant isolates

from D0 compared with D3, within the same bacterial species. One reason could theoretically be because of other, systemic treatment with antibiotics before the sample was taken. However, the mares were not treated with antibiotics for any reason during this study, except the two breeding mares that were systemically treated once with trimethoprim-sulfadiazine due to intrauterine fluid, but no more samples were obtained from these two mares, and they were not chosen for susceptibility testing. Another reason could be that resistant bacteria in the environment spread resistance genes by HGT to commensal bacteria (Wright, 2010), but this did not occur between D0 and D3. The number of inseminations, and thereby the repeated exposure to antibiotics in the semen extender, did not affect the resistance pattern within a specific bacterium when comparing results between mares that had only been inseminated once, compared to mares that had gone through multiple inseminations.

In *Staphylococcus simulans*, resistance was found against penicillin, oxacillin and FA. Two isolates showed resistance against all three substances, which could classify it as a MDR bacteria, but since penicillin and oxacillin belong to the same antibiotic category (beta-lactame antibiotics), this classification is not appropriate. Bacterial isolates resistant to beta-lactamases protects themselves by one of two mechanisms; i) formation of the enzyme penicillinase, which can break down penicillin and aminopenicillins, or ii) altering the pencillin-binding protein located in the bacterial cell wall (SVA, 2019). As the bacterium changes the penicillin-binding protein in the cell wall, all types of beta-lactam antibiotics (pencillins, aminopenicillins and cephalosporins) will become ineffective as they can no longer bind to the bacterium, which is called methicillin resistance. The resistance against fusidic acid in staphylococci can originate from natural mutants in normal populations of staphylococci or be transmitted from one bacterium to another by HGT (Turnidge & Collignon, 1999). An increasing resistance against FA for *Staphylococcus aureus* was reported from the UK in human bacteraemias and may reflect an extensive use of FA preparations in retail or hospital pharmacies (Livermore *et al.*, 2002).

The susceptibility results of *Streptococcus equi* ssp. *zooepidemicus* were in line with the resistance results reported from horses in Swedres-SVARM 2019 (2020), where *S. equi* ssp. *zooepidemicus* was susceptible to all antibiotics apart from clindamycin and trimethoprim-sulphamethoxazole. The results in Swedres-Svarm 2019 are mainly based on clinical isolates from the respiratory tract (69%) and abscesses (15%). One explanation for the result of resistance against tetracycline found in *S. equi* ssp. *zooepidemicus* (control group) could be that they have a low inherent susceptibility to tetracyclines (Swedres Swarm 2019, 2020). This might also be the case for *S. dysgalactiae*, since both of the species belong to the same bacterial genus and Lancefield group (C).

The statistical analysis comparing the susceptibility between D0 and D3 for *S. simulans* and *S. dysgalactiae* showed no significance of the antibiotics in the semen extender INRA-96 on the development of antimicrobial resistance. However, since antibiotic resistance genes are widespread in our environment, not only in pathogenic bacteria (Wright, 2010), there is always a risk that some of the commensal bacteria would pick up those genes by HGT. It would thus be possible to isolate

resistant bacteria, even though they have not been directly exposed to antibiotics, since natural selection of resistant bacteria would have occurred. Resistant bacteria may not contribute to the vaginal bacterial flora at any sampling time, since they often have a higher metabolic cost and therefore are usually out-competed by other bacteria (Andersson & Levin, 1999; Martinez, 2012). On the other hand, the bacteria that become resistant would have an advantage against susceptible bacteria if the population is exposed to antibiotics, as in this case with the semen extender. The observation of resistant bacteria from our samples (regardless of group) could, therefore, just be coincidence.

Other theories for the differences in resistance pattern in and between the sampled groups could be that the vaginal bacterial flora is mostly or totally killed by the exposure to the antibiotics in the semen extender, rather than developing resistance against the included substances. A new bacterial flora should then be rapidly introduced to the physiological niche, without an increasing number of resistant isolates since these bacteria have not been exposed to the antibiotics. Another theory is that only a few bacteria are affected by the exposure to the antibiotics in the semen extender, which minimizes the difference in susceptibility between D0 and D3.

It is difficult to say which possible scenario our results reflected. It might be necessary to use a more accurate identification technique when identifying bacterial species and strains, such as whole genome sequencing to ensure that the strains showing resistance on D0 and D3 are the same. Without this verification, it is not possible to know if the susceptibility of a strain has changed or whether a different strain of the same bacterium has appeared that is of a different susceptibility. It has been shown that antibiotics like gentamicin and polymixin B are detrimental to sperm function (Varner *et al.*, 1997; Aurich & Spergser, 2007), which decreases both the quality of semen and also the fertility rates. Also, it is of great importance that the semen extender containing antibiotics are handled correctly, since even factors like this can negatively affect the spermatozoan function (Dean *et al.*, 2012). It was shown that proliferation of antimicrobial resistance genes and gene transfer elements can be stimulated by short time storage of bacteria in temperatures below 20°C (Miller *et al.*, 2014). This is often the case since stallion semen is usually stored around 6°C, if it is not used for insemination soon after the semen collection. Therefore, it could be an advantage to use techniques like SLC, where we actually remove the bacterial fraction instead of decreasing it by using AMS. Although, SLC should not be used as a replacement of strict hygiene since the bacterial load seems to affect how effective SLC with Androcoll-E can remove the bacterial content (Morrell & Wallgren, 2014).

On the larger question of why this study is important, two of the main factors mentioned by WHO (2020) as contributing to the increasing spread of AMR are the possibility for the public to buy antibiotics without a prescription, and a general over-prescribing of antibiotics by veterinarians and health workers. In Sweden, there is a relatively stable situation of documented resistance in bacteria, probably due to good standard treatment guidelines; although the reported antibiotics sold for animal use in Sweden in 2019 (9.5 tonnes) excludes antibiotics for intrauterine (and intramammary) usage (Swedres-Svarm 2019, 2020). Since there are some

alternative options to adding AMS to semen extenders in the terms of reducing the bacterial amount in semen doses, I think we should take these options into consideration for future guidelines for AI in animal breeding.

Conclusions

Differences in the species of bacteria present in the vaginal flora were identified after exposure to antibiotics in semen extender. However, we did not detect a change in the antibiotic resistance pattern. Although, the susceptibility results showed a relatively stable situation for antimicrobial resistance among the vaginal bacterial flora. A few bacterial isolates showed AMR in the control group, which could be explained by factors such as HGT of resistance genes from environmental bacteria to the commensal vaginal flora. There are some possible improvements that can be made in further studies, such as using whole genome sequencing and bioinformants in the identification of bacteria and resistant genes, shortening the time of transport of the samples to the laboratory and sampling a larger population.

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Popular scientific abstract

In Sweden and many other countries, artificial insemination (AI) is frequently used for breeding horses. After collecting an ejaculate from the stallion, semen is introduced into the mare's uterus using a special kind of catheter. This is a useful technique for several reasons: there is less risk of injury than with natural mating for both horses and stud personnel, it is cheaper to send the genetic material (spermatozoa) from one stallion to several mares than to transport mares to the stallion, and there is less risk of spreading diseases between horse populations.

To enable the sperm samples to survive between collection from the stallion and its introduction into the mare's uterus, various substances (semen extender) are added to the ejaculate, including nutrients, buffering substances and antibiotics. The addition of antibiotic is to reduce the risk of the spermatozoa being affected by bacteria, either from the stallion or accidentally added from the environment (such as in the air, from semen collecting equipment, or from the person handling the semen). It also prevents against the spread of disease-causing bacteria that can otherwise be transmitted to the mare and cause infection and reduced fertility. It is not known which bacteria may have contaminated the ejaculate, and some antibiotics can have a detrimental effect on sperm quality; therefore, a mix of different antibiotic substances are usually added to the semen extender to be effective against a wide range of bacteria.

After insemination, the immune system of the mare will try to eradicate the foreign material (spermatozoa, the semen extender, bacteria) from the uterus. Beside activating the immune system in the uterus, mechanical drainage will be initiated, causing residual semen extender to come in contact with the bacteria in the mares' vagina. The objectives of this study were to investigate (i) how the antibiotics in semen extender affect the resistance pattern in the vaginal bacterial flora of mares, and (ii) if there are any resistant bacteria in the normal vaginal bacterial flora of mares.

Since bacteria have an ability to adapt to their environment, there is always a possibility that they may develop resistance to antibiotics in order to survive, or that they pick up resistance genes from other bacteria. As antibiotic resistance is a growing threat to global public health, it is important to know about sources that could increasing development of antibiotic resistance. By reducing these risk factors, we can reduce the risk of humans and animals being exposed to these resistant bacteria. Otherwise, there is a strong likelihood that bacterial diseases which until now have been treatable with antibiotics, may not be treatable at all in the future.

In this study we collected vaginal samples from 39 mares of various ages and breeds, housed in similar conditions in the same geographic area. The mares were separated into two groups; the exposed group consisting of 26 breeding mares and a control group consisting of 13 mares that has not been exposed to antibiotics in semen extenders (at least not in the previous ten years). The breeding mares were sampled twice: once before insemination and then again three days after insemination. The mares in the control group were only sampled once. All mares were sampled using the same procedure. The samples were then sent to a laboratory for culturing and identification. Samples from four mares (control sample or D0) produced no growth.

In total 971 bacterial isolates were obtained from 35 mares. The most common bacteria isolated from both groups were *E. coli*, which is a common bacterium in the environment usually spread by fecal content and soil. From 21 breeding mares, five different bacterial species were isolated in both the sample before (D0) and after insemination (D3). Four of these bacterial species were used for the antimicrobial susceptibility testing, to compare the resistance results of a specific bacterial species before and after insemination. The same species collected from the control group were also resistance-tested to check for any differences in resistance pattern. In total, 140 bacterial isolates were used for resistance testing. Resistance was found against 8 out of 15 antibiotics of the substances tested, with varying patterns and results between bacterial species.

However, when the results were compared, there was no evidence of an increasing development of antibiotic resistance in the bacterial vaginal flora, as a result of exposure to antibiotics in the semen extender. Resistant bacteria could be isolated from the vaginal flora in both groups, although, there was a difference in the bacterial species isolated before and after insemination and also between the groups. Despite this, we should still strive to reduce our prophylactic use of antibiotics, since the use and distribution of antibiotics world-wide is a known risk factor for increasing the development and spread of antibiotic resistance genes in the environment. Furthermore, there are some good working options to adding antibiotics to semen extenders, such as Single Layer Centrifugation, which in addition to physically removing the bacteria from semen, selects the most robust spermatozoa, thereby increasing sperm quality and fertility.

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