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Antimicrobial resistance of the microbiota in the reproductive tract of horses in conjunction with assisted reproduction

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Abstract

Antimicrobial resistance is a global challenge exacerbated by antibiotic misuse and inadequate infection control. This study explores the effects of antibiotics in semen extenders on the vaginal bacteria of the mare and investigates the separation of spermatozoa from bacteria in seminal plasma as an alternative to adding antibiotics to semen extenders. In addition, it examines the diversity of the seminal microbiota in healthy stallions across different countries. Antimicrobial resistance was influenced by breeding status and age of the mare, as well as environmental factors such as housing and location, and exposure to antibiotics in semen extenders. There were geographic differences in the diversity of the seminal microbiota, with implications for tailored antibiotic use. While phylum level composition of seminal microbiota remains consistent, genus level variations may stem from husbandry or individual differences. Bacterial load could be reduced by Single Layer Centrifugation with a low density colloid, without compromising sperm quality. This method has potential for circumventing the need for antibiotics in semen extenders, although further research is needed to confirm sperm fertilizing capacity.

Keywords: Antimicrobial resistance, Antibiotic misuse, Equine, Semen extenders, Vaginal bacteria, Seminal microbiota, Geographic diversity, Single Layer Centrifugation, Low density colloid

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Abstract

Antibiotikaresistens är en global utmaning som förvärrats av felaktigt användande av antibiotika och otillräcklig kontroll av infektioner. Denna studie undersöker effekterna av antibiotika i spädningsvätska för sperma på den bakteriella vaginalfloran hos sto och undersöker även avskiljande av spermier från bakterier som ett alternativ till att tillsätta antibiotika till spädningsvätska för sperma. Studien undersöker även sammansättningen av mikrobiotan hos friska hingstar i olika länder. Antibiotikaresistensen påverkades av reproduktionsstatus och ålder hos ston men även omgivningsfaktorer som hållningsform, plats och om stoet blivit exponerat för antibiotika i spädningsvätska. Sammansättningen av seminalplasmans mikrobiom uppvisade geografiska skillnader, något som kan vara av vikt för riktad antibiotikanvändning. Sammansättningen av seminalplasman på fylum-nivå var konstant men varierade på nivån släkte, eventuellt på grund av hållningsformer och individuella skillnader. Bakteriemängden kunde minskas med Single Layer Centrifugation (SLC) med en kolloid av låg densitet utan att påverka spermiernas kvalitet. Denna metod har potential för att ersätta tillsatserna av antibiotika i spädningsvätskor men ytterligare studier krävs för att säkerställa spermiernas befruktningsduglighet.

Nyckelord: Antibiotikaresistens, felaktig användning av antibiotika, Equin, spädningsvätska för sperma, vaginala bakterier, mikrobiota i speminalplasma, geografiska skillnader, Single Layer Centrifugation , kolloid med låg densitet

Dedication

To my family, my friends.

Contents

List of publications

This thesis is based on the work contained in the following papers, referred to by Roman numerals in the text:

I. Malaluang, et al. (2021). Antimicrobial resistance in equine reproduction (Literature review). Animals, 11 (11), 3035.

II. Malaluang, et al. (2022). Vaginal bacteria in mares and the occurrence of antimicrobial resistance. Microorganisms, 10 (11), 2204.

III. Malaluang, et al. (2023). Antimicrobial resistance in vaginal bacteria in inseminated mares. Pathogens, 12 (3), 375.

IV. Malaluang, et al. Bacterial Diversity in Stallion Semen Evaluated by 16S Sequencing (manuscript)

V. Malaluang, et al. Comparison of the effect of modified Single Layer Centrifugation and sperm washing on bacterial content and sperm quality in stallion semen (manuscript)

Papers I-III are reproduced with the permission of the publishers.

The contribution of Pongpreecha Malaluang to the papers included in this thesis was as follows:

I. Validation, data curation, writing original draft, review and editing

II. Validation, formal analysis, data curation, writing original draft, review and editing, and visualization

III. Validation, formal analysis, data curation, writing original draft, review and editing, and visualization

IV. Validation, data curation, writing-original draft, review and editing, and visualization

V. Validation, formal analysis, data curation, writing-original draft, review and editing, and visualization

Other papers not included in this thesis:

I. Kellerman, Cecilia, Pongpreecha Malaluang, Ingrid Hansson, Lena Eliasson Selling and Jane M. Morrell. (2022). Antibiotic resistance patterns in cervical microbes of gilts and sows. *Animals*, 12(1), 117.

II. Morrell, Jane M., Pongpreecha Malaluang, Theodoros Ntallaris and Anders Johannisson. (2022). Practical method for freezing buck semen. *Animals*, 12(3), 352.

III. Morrell, J. M., Pongpreecha Malaluang, Aleksandar Cojkic and Ingrid Hansson. (2022). Alternatives to Antibiotics in Semen Extenders Used in Artificial Insemination. *The Global Antimicrobial Resistance Epidemic: Innovative Approaches and Cutting-Edge Solutions*, 181.

IV. Åkerholm, Timjan, Pongpreecha Malaluang, Ingrid Hansson and Jane M. Morrell. (2023). Bacteria in the vagina during the estrous cycle in mares. *Theriogenology* (accepted).

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Abbreviations

%DFI	DNA fragmentation index
2SP	Sucrose phosphate buffer
AI	Artificial insemination
ALH	Lateral head displacement
ASVs	Amplicon sequence variants
AV	Artificial vagina
BCF	Beat cross frequency
CASA	Computer-assisted sperm analysis
CFUs	Colony forming units
cgMLST	Core genome multilocus sequence typing
CLSI	Clinical and Laboratory Standards Institute
D0	Day 0
D3	Day 3
DNA	Deoxyribonucleic acid
ECOFF	Epidemiological cut-off value
EUCAST	European Committee on Antimicrobial Susceptibility Testing
LIN	Linearity

MALDI-TOF MS	Matrix-assisted laser desorption ionization time of flight mass spectrometry
MIC	Minimum inhibitory concentration
MLST	Multilocus sequence typing
MRS agar	De Man, Rogosa, and Sharpe agar
MST	Minimum spanning tree
NGS	Next generation sequencing
PCR	Polymerase chain reaction
PM	Progressive motility
PPLO	Pleuropneumonia-like organisms
SCSA	Sperm Chromatin Structure Assay
SLC	Single Layer Centrifugation
SLU	Swedish University of Agricultural Sciences
sp. and spp.	Species (singular and plural, respectively)
subsp.	Subspecies
STR	Straightness
VAP	Velocity of the average path
VCL	Curvilinear velocity
VSL	Straight line velocity
WOB	Wobble

1. Introduction

1.1 Antimicrobial Resistance

The misuse and overuse of antibiotics, along with insufficient infection prevention and control measures, have led to a significant worldwide problem known as antimicrobial resistance (AMR) (Tang et al. 2017). It is considered to be one of the most significant challenges confronting humanity in the contemporary world (WHO 2021). Implementing approaches that encourage the conscientious utilization of antibiotics plays a pivotal role in combatting antibiotic resistance, particularly within the context of Sweden, where an admirable position has been maintained on the international stage when it comes to responsible antibiotic usage (Swedres-Svarm 2021).

Bacteria can exhibit innate resistance to various antibiotics or acquire it over time. Resistance may arise from genetic mutations or be a result of natural selection pressure, and it can occur in both pathogenic and non-pathogenic bacteria. Environmental bacteria harbor a diverse array of resistance genes. Exposure to antibiotics fosters the emergence of AMR as resistant bacteria gain a competitive edge over susceptible strains in the same environment. This selection process is also responsible for the contribution of topical antibiotic use to AMR. There are varying opinions on the extent of antibiotic exposure required for resistance to develop. A study focusing on *Clostridium perfringens* determined that even minimal amounts of antibiotics could lead to resistance (Johansson et al. 2004). Contrary to this theory, another study proposed that typically a higher antibiotic concentration is need to induce AMR than is usually observed in non-clinical settings, yet pollution events such as improper disposal of antibiotic-containing substances in the environment can still lead to its emergence

(Divala et al. 2018). The duration of exposure necessary to induce AMR is likely to vary between different antibiotics, but remains inadequately defined (Divala et al. 2018). Given that instances of environmental contamination, such as improper disposal of antimicrobials, can lead to AMR (Martínez & Rojo, 2011), it is crucial to dispose of substances containing antibiotics in an appropriate manner, such as through boiling or incineration, to prevent adverse effects (Anwar et al., 2020).

Bacteria can transfer genes for acquired resistance among themselves (Martínez & Rojo 2011), enabling them to flourish in an environment that would otherwise be toxic to them. Conjugation, transduction, and transformation (Martínez 2012) represent the primary horizontal modes of transmission in nature. In conjugation, genetic sequences within a plasmid are exchanged between bacteria in close contact through a tube-like structure called a pilus (Martínez 2012). Transduction involves the transfer of genetic material between bacteria by bacteriophages, although this mode of transmission is relatively inefficient, as it only infects closely related species of bacteria (Martínez 2012). Through transformation, bacterial cells absorb exogenous deoxyribonucleic acid (DNA) via a specific membrane channel and integrate it into their own DNA (Davies & Webb 1998). Remarkably, even dead bacteria can transfer resistance genes to other bacteria using this mechanism (Davies & Webb 1998).

1.2 Occurrence of Antimicrobial Resistance in the Vaginal Bacteria of Mares

Factors that increase the risk of AMR development in the microbiota of the reproductive tract include frequent use of antibiotics to treat infections (Kenney et al. 1975; Albihn et al. 2003) and their non-therapeutic application in semen extenders. Healthy stallions harbor bacteria on the mucosa of their distal reproductive tract, which are transferred to the ejaculate during semen collection (Rota et al. 2011), or the ejaculate might become contaminated from the environment during processing. Consequently, antibiotics are incorporated into semen extenders to preserve sperm quality, as specified by various national and international regulations, such as those outlined in the Commission Delegated Regulation (EU) 2020/686 (European Union 2020).

Prior research on AMR in the equine reproductive tract has largely focused on animals with fertility issues. Reports from multiple countries

since the 1980s, involved culturing bacteria from uterine swabs, uterine lavage, vaginal swabs, and clitoral swabs. The findings revealed antibiotic resistance in various bacteria such as *Enterococcus* spp., *Enterobacter* spp., *Escherichia coli*, *Gardnerella vaginalis*, *Klebsiella* spp., *Micrococcus* spp., *Proteus* spp., *Pseudomonas* spp., *Staphylococcus intermedius*, *Streptococcus* spp., *Streptococcus equinus*, *Streptococcus equi* subsp. *zooepidemicus*, and *Taylorella equigenitalis* (Malaluang et al. 2021). The mares included in these studies were examined by veterinarians because of fertility problems, and many had received various antibiotic treatments over extended periods. Only limited research has investigated AMR in the vaginal flora of healthy mares without fertility issues, and the influence of different environments or the estrous cycle on vaginal flora remains undetermined. Furthermore, the consequences of exposure of the vaginal bacteria to antibiotics in semen extenders on AMR, and whether these changes are temporary or long-lasting, remains unknown.

1.2.1 Antimicrobial Resistance in Vaginal Bacteria in Inseminated Mares

Artificial insemination (AI) is a commonly employed technique in contemporary animal reproduction practices (Pagl et al. 2006). Approximately 90% of newborn foals were conceived through the use of AI with chilled or frozen semen during 1990 to 2001 (Nath et al. 2010). In a study in Europe in which mares were categorised in 3 groups (sport, brood mares, and others), AI was predominantly employed for sport mares, with a rising trend since 2013. In 2017, sport mares constituted 67% of all mares inseminated, while there was a slight decline in AI in brood mares, from 37% in 2013 to 29% in 2017. The popularity of AI for other categories has notably decreased since 2013 (Kowalczyk et al. 2019). The utilization of cooled semen for equine AI has risen steadily in Europe, particularly among sport mares. On the other hand, there has been an increase in the use of frozen semen for brood mares in recent times. Among the mares not categorized as sport or brood mares, cooled semen remains the prevailing choice for AI (Kowalczyk et al. 2019).

Since antibiotics are added to semen extenders, as previously mentioned, the utilisation of cooled semen for AI involves considerable use of antibiotics. These antibiotics serve to prevent bacterial disease transmission to inseminated females and maintain sperm quality during storage (Morrell

& Wallgren 2014). Presence of resistance has been detected in isolates from the reproductive tract of the mare in several countries, including France (Dabernat et al. 1980), Sweden (Albihn et al. 2003), India (Berwal et al. 2006; Singh 2009), Italy (Frontoso et al. 2008; Nocera et al. 2017; Pisello et al. 2019), Germany (Goncagül & Seyrek-İntas 2013), the US (Davis et al. 2013; Ferrer & Palomares 2018), Slovakia (Benko et al. 2015), and Turkey (Goncagul et al. 2016). However, there have been limited comprehensive studies on AMR on the reproductive microflora of healthy horses, and no investigations have been conducted to assess the impact of antimicrobial substances in semen extenders.

1.3 Processing Stallion Semen for Artificial Insemination

The preservation of sperm quality at 5°C relies on various factors, such as proper dilution of seminal plasma (at a ratio of ≥3:1) using a suitable extender and gradual cooling under controlled conditions (Varner et al. 1988). Optimal maintenance of sperm motility, viability, membrane integrity, and fertility occurred when seminal plasma was diluted to 25% or less of its original volume and the total sperm concentration ranged from 25 to 50 × 10^6/mL (Varner et al. 1987; Jasko et al. 1991, 1992). The typical proportion of progressively motile spermatozoa in standard insemination dose for shipped semen is 1 billion (Loomis 2006), although this “standard” dose varies between countries. Centrifugation or collection of sperm-rich fractions using an open-ended artificial vagina (AV) (Tischner et al. 1974; Varner et al. 1987) is carried out to concentrate semen with a low sperm concentration, enabling effective removal of seminal plasma and replacement with semen extender. Centrifuging is a common procedure for semen with an initial total sperm concentration below 100 × 10^6/mL or progressively motile sperm concentration below 67 × 10^6/mL (Loomis 2006). In short, the semen is mixed with a semen extender at a 1:1 ratio and is centrifuged. Most of the supernatant is drawn off using a sterile syringe, leaving behind 10 mL of spermatozoa, extender, and seminal plasma. This remaining mixture is combined and further diluted with fresh extender to reach a total volume of 40 mL. Assuming a 75% recovery rate of the initial sperm count after centrifugation, the volume of extended semen per dose can be adjusted to achieve the desired number of spermatozoa (Loomis 2006).

Cryopreservation of stallion spermatozoa usually entails eliminating nearly all (at least 95%) of the seminal plasma via centrifugation. Afterwards, the sperm pellets are suspended in a cryoextender, and packaged in 0.5-mL straws or, less frequently, in 4- to 5-mL maxistraw (Loomis 2006). Removing the majority of the seminal plasma aids in mitigating its harmful impact on the viability of sperm cells when subjected to freezing conditions (Amann & Pickett 1987).

1.4 Bacterial Diversity in Stallion Semen

The external genitalia of the healthy stallion harbor numerous bacteria as part of their normal flora, including *Streptococcus dysgalactiae* ssp. *equisimilis*, *Bacillus* spp., *Staphylococcus aureus*, *Escherichia coli*, *Streptococcus equi* ssp. *zooepidemicus*, *Pseudomonas* spp., and *Klebsiella* spp. (Samper & Tibary 2006). These commensal bacteria naturally colonise the surface of the urethral fossa, penis, prepuce, and the urethra pre- and post-ejaculation, and they are transferred to the semen during ejaculation. When using an AV for semen collection, it is very difficult to avoid contamination of the semen with bacteria from the external genitalia (Ortega-Ferrusola et al. 2009; Rota et al. 2011). Moreover, bacteria present in semen after collection may originate from the environment (Pickett et al. 1999).

To prevent fertility problems in mares and maintain semen quality during storage, antibiotics are commonly added to semen to inhibit bacterial growth, and the temperature of the semen is lowered. However, the effectiveness of antibiotics may be limited if certain contaminating bacteria are present or if they are resistant to the antibiotics used (Guimarães et al. 2015). Therefore, it is crucial to identify the bacteria present in stallion semen to determine the appropriate antibiotics to be included in semen extenders.

Research investigating the bacteria found in semen from stallions without fertility issues was conducted in Italy (Corona & Cherchi 2009; Rota et al. 2011), Portugal (Guimarães et al. 2015), Spain (Ortega-Ferrusola et al. 2009; Varela et al. 2018; Quiñones-Pérez et al. 2021; Salas-Huetos et al. 2022) Germany (Pasing et al. 2013), Austria (Al-Kass et al. 2019), and Sweden (Al-Kass et al. 2020). The prevalence of bacteria differed across studies, possibly due to different methods of identification (Al-Kass et al. 2020), and other factors such as husbandry practices (Pasing et al. 2013).

Bacterial identification in stallion semen typically relied on conventional culture-based methods, which might not detect certain bacteria that are problematic to culture (Moretti et al. 2009). Some studies using culture-based techniques only identified a limited number of bacteria in stallion semen (Ortega-Ferrusola et al. 2009; Rota et al. 2011; Varela et al. 2018; Al-Kass et al. 2019). However, more recent studies using non-culture-based methods have explored the microbiome in healthy stallions (Al-Kass et al. 2020; Quiñones-Pérez et al. 2021; Quiñones-Pérez et al. 2022), revealing previously unidentified bacteria.

In recent years, studies on the human microbiome have progressed significantly through the utilization of next generation sequencing (NGS) technology (Kuczynski et al. 2012). This technique enables precise determination of the sequence of nucleotides within DNA (van der Straaten 2015). One of these non-culture-based methods, 16S (small subunit rRNA gene) sequencing, involves utilizing polymerase chain reaction (PCR) amplification of the 16S gene's hypervariable regions in bacteria to separate bacterial DNA from mammalian DNA for identification (Kozich et al. 2013). The sequencing process focuses on genes coding for the 16S region of bacteria, particularly for taxonomic definitions of bacterial species (Böttger 1989). Using this approach, over 90% of bacterial genera and 65%–83% of bacterial species can be identified (Mignard & Flandrois 2006). Understanding the bacterial diversity and potential factors affecting the microbiome can provide valuable insights into semen quality and fertility, as well as contributing to the development of better practices in AI and reproductive management.

1.5 Factors affecting the bacterial load in semen

Despite strict attention to hygiene during semen collection, bacteria are still present in almost all equine semen samples. The practice of washing the penis before collection to remove superficial dirt and debris is a subject of controversy as it may eliminate the normal skin flora, potentially leading to overgrowth by other bacteria (Clément et al. 1995). In any event, it is not likely that this procedure would eliminate bacteria from the urethral mucosa. Nonetheless, some authors have observed a reduction in bacterial presence in semen after penile washing (Neto et al. 2015). Variations observed in

bacterial contamination among studies could be attributed to the stallions' environment and husbandry (Pasing et al. 2013).

The presence of certain bacteria in semen seems not to be a concern for the mare unless significant quantities or specific types of bacteria are present. However, with the growing trend of AI using cooled semen transported to different locations (Aurich & Aurich 2006), there is the opportunity for microbes to proliferate during semen processing, at least while the temperature of the semen remains above 15 °C. As a result, the bacterial load at the time of AI may be substantially higher than immediately after semen collection.

1.6 Antimicrobial resistance in bacteria detected from semen

From 1999 to 2012, some bacteria were seen to exhibit an increase in resistance to specific antimicrobials over time, with variations in how different bacterial species reacted to various antimicrobial agents (Johns & Adams 2015). Bacterial growth was observed in bull (Zampieri et al. 2013) boar (Bresciani et al. 2014) and stallion semen (Guimarães et al. 2015) in the presence of antibiotics. Bacteria in bull semen were unaffected by gentamicin, tylosin, spectinomycin, and lincomycin, while ceftiofur/tylosin or ofloxacin prevented bacterial growth (Gloria et al. 2014). In a study conducted in Portugal (Guimarães et al. 2015), bacterial growth was observed in frozen stallion semen preserved with an amikacin-containing extender. Hernández-Avilés et al. (2018), showed that various antibiotics were effective against low levels of *Klebsiella pneumoniae* or *Pseudomonas aeruginosa* introduced into semen, but they were not effective against higher bacterial concentrations.

It is of significant importance to ensure that unused semen extender and semen doses are not disposed of via the drainage system, as they have the potential to impact environmental bacteria and subsequently pose a risk of transmitting AMR to humans or animals.

1.7 Processing methods of semen to reduce the bacterial load

Artificial insemination is widely utilized in horse breeding. Various breed organizations permit the use of either cooled or frozen spermatozoa to facilitate successful mare AI (Aurich 2008). The practice of preserving spermatozoa through cooled liquid storage for 24 to 48 hours is widely used (Wiebke et al. 2022), involving a significant decrease in sperm metabolism (Graham 2011) through the process of actively cooling extended spermatozoa to temperatures between 4 to 5 °C (Moran et al. 1992). An alternative, cryopreservation, has limitations, such as inducing varying levels of harm to sperm membranes (Yeste et al. 2015), which in turn impacts cryosurvival and diminishes fertilization potential. Furthermore, the optimal pregnancy rates using frozen semen are most likely attained when mares are inseminated either within 24 hours prior to ovulation or within 6 to 12 hours following ovulation (Martin et al. 1979; Torres-Boggino et al. 1984; Kloppe et al. 1988). Moreover, notable differences exist among stallions regarding their suitability for sperm cryopreservation, even when employing tailored protocols, as shown in studies by Vidament et al. (1997) and Loomis & Graham (2008). Thus, the use of frozen semen in equine AI is still limited. The possibility of preserving sperm cells above 15 °C for transportation exists (Batellier et al. 1998; Cuervo-Arango et al. 2015), but the practice has not proved popular due to lack of infrastructure for storing semen at this temperature. Nevertheless, the results from AI using these semen doses stored at elevated temperatures demonstrated less than optimal pregnancy rate.

Incorporating antimicrobial agents into stallion semen extenders was recognized as being essential for managing bacterial contamination and the potential spread of harmful microorganisms during the process of AI (Morrell & Wallgren 2014). Proposed alternatives to antibiotics in semen extenders have emerged, including the utilization of colloid centrifugation as suggested by Morrell & Wallgren (2014), which aims to diminish bacterial contamination in the ejaculate. The reduction or potential elimination of bacteria in semen samples could offer a means to manage bacterial contamination without excessive reliance on antimicrobial agents.

Colloid centrifugation was originally employed to select the most robust spermatozoa from an ejaculate. However, it was also found to be possible to separate spermatozoa from most of the bacteria in an ejaculate. The method

is straightforward and enables the viability of stallion spermatozoa to be improved without incorporating antibiotics in the semen extender (Morrell et al. 2014; Al-Kass et al. 2019). It is a practical option for stud farms, as it only necessitates access to a centrifuge with a swing-out rotor. In the Single Layer Centrifugation (SLC) technique originally described, extended semen is layered over a high density colloid in a centrifuge tube. After centrifugation at 300× g for 20 minutes, the sperm pellet is collected and transferred to fresh extender (Morrell & Nunes 2018). This method of sperm processing selects the most fertile spermatozoa in various species, including stallions, and also results in a significant reduction in bacterial count in the sample. Depending on the bacterial load and species, SLC was successful in removing 81% to > 90% of bacteria from stallion semen (Morrell et al. 2014). A study by Al-Kass et al. (2019) reported a similar outcome, where 25% of bacteria remained in the samples after SLC when antibiotics were included in the extender, while only 18% of bacteria remained after SLC without antibiotics.

1.7.1 Single Layer Centrifugation with a low density colloid

Although the separation of spermatozoa from bacteria using a high density colloid showed promising results for improving cryosurvival, some robust spermatozoa are lost in this procedure (Hoogewijs et al., 2011). Therefore, ongoing investigations are exploring the use of a low density colloid to separate boar spermatozoa from seminal plasma and its bacterial load, without specifically selecting for robust spermatozoa. It was possible to retrieve approximately 85% of the original load of boar spermatozoa using this method, and sperm quality was not adversely affected by the lack of selection for robustness (Morrell et al. 2019). Preliminary results from an AI trial indicated that fertility was similar in control and treated samples (Morrell et al., 2022). These results suggest that a similar approach for stallion semen might be possible.

2. Aims

The general aim of this thesis was to investigate the effect of antibiotics in semen extenders on the vaginal bacteria of mares and to determine whether spermatozoa can be separated from bacteria in seminal plasma by physical means instead of using antibiotics. In addition, the diversity of seminal microbiota of healthy stallions in different countries was examined.

To achieve these aims, the following studies were carried out.

Literature review: Reviewing existing literature on AMR in the reproductive tract of mares

Study I: To analyse the microbial composition of the vaginal flora and potential AMR in inseminated and non-inseminated mares. A second objective was to determine the best time to take samples of the vaginal microbiota after ovulation, which would be needed for the next study.

Study II: Compare AMR in bacteria from the vagina of mares before and after insemination

Study III: Investigate the bacterial diversity present in semen from healthy stallions by sampling individuals in three European countries

Study IV: Examine an alternative to adding antibiotics in semen extenders by assessing the effectiveness of SLC through a low density colloid in reducing bacterial contamination in stallion semen

3. Materials and methods

The articles and manuscripts included in this thesis provide a comprehensive overview of the materials and methods employed. A brief summary of the methods is provided in the following section.

3.1 Literature review

The published literature was reviewed to gain an overview of AMR in the reproductive tract of mares, highlighting the potential implications of antibiotic use and suggesting alternatives to mitigate the development of resistance. The keywords used in the literature search related to antibiotic resistance, equine, and female reproductive organs (vagina and uterus), excluding non-English publications, pre-1970 publications, and low-quality studies.

3.2 Location

The research conducted for this thesis was carried out at various institutions. These include the Swedish University of Agricultural Sciences (SLU) and SciLife Lab, Stockholm, in Sweden, as well as the Center for Artificial Insemination and Embryo Transfer, and the Institute of Microbiology, Department of Pathobiology, at the University for Veterinary Sciences in Vienna, Austria.

3.3 Study design

Study I: Swabs were taken from the vagina of non-inseminated mares at estrous and also from sham-inseminated mares at various time points to determine if the antibiotics in semen extenders affected the microbes present, and to determine the best time for sampling in a subsequent study (Figure 1).

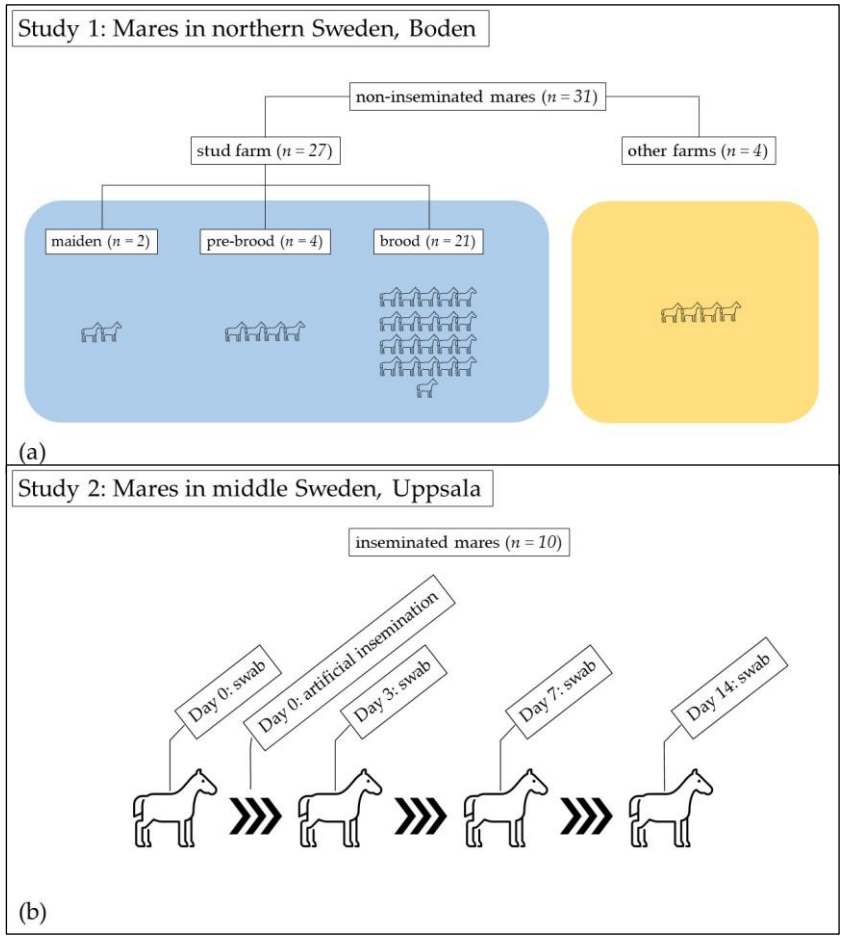

Figure 1. Experimental design for Study I: (a) 31 mares that were not inseminated were sampled on a single occasion, (b) 10 mares subjected to sham insemination were sampled on Day 0 (prior to insemination) and subsequently on days 3, 7, and 14 following insemination.

Study II: bacterial samples from the mare vagina were collected before and after AI, and the occurrence of AMR was determined, to evaluate the impact of antimicrobial substances in semen extenders on the mare vaginal microbiota (Figure 2).

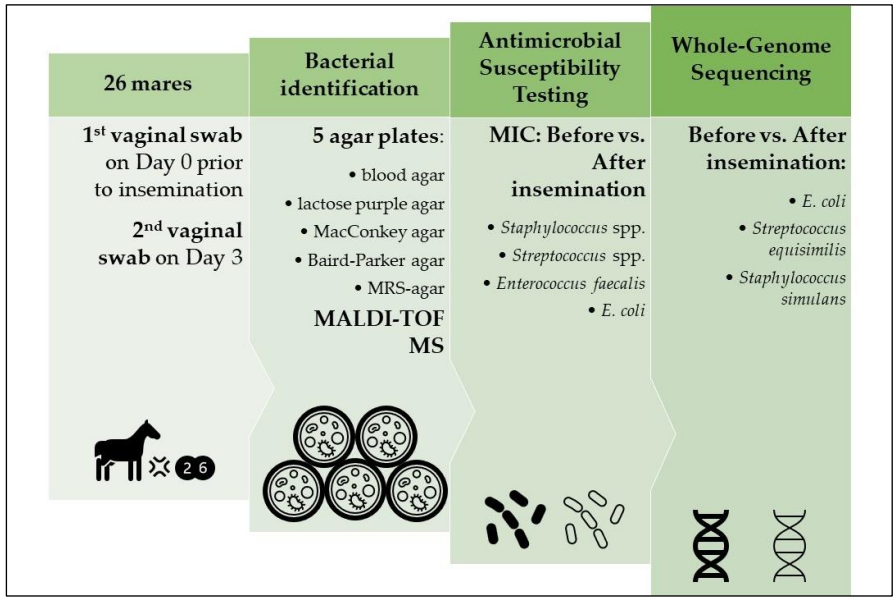

Figure 2. Experimental design for Study II: vaginal swabbing of 26 mares was done twice, once on day 0 before insemination, and the second time on day 3 after ovulation. Swabs were cultured and selected isolates were identified by Matrix-assisted laser desorption ionization time of flight mass spectrometry (MALDI-TOF MS). Antimicrobial susceptibility testing and whole-genome sequencing were conducted on strains identified as being present before and after insemination.

Study III: the bacterial diversity in semen from healthy stallions in three European countries was studied using 16S rRNA sequencing, as shown in Figure 3. Alpha diversity, relative ASV abundance, and Bray-Curtis pairwise analysis were made to compare the seminal bacteria in stallions from the three countries.

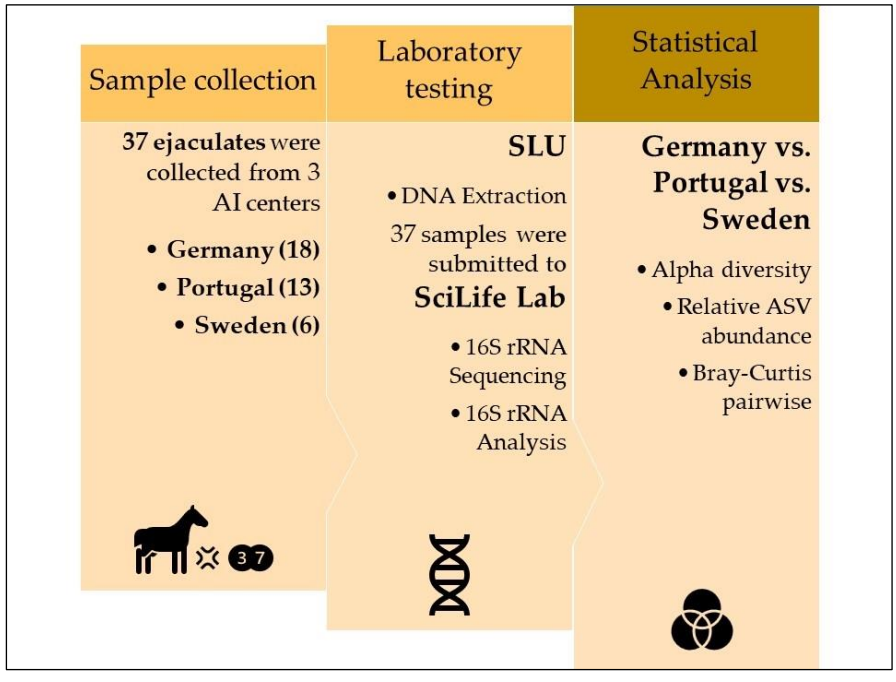

Figure 3. Experimental design for Study III: ejaculates from 37 stallions at three AI centers in Europe were collected, snap-frozen and sent to the laboratory at SLU for bacterial DNA extraction. Subsequently, 16S rRNA sequencing and bioinformatic analyses were performed.

Study IV: the bacterial content of stallion semen was compared after preparation by different methods: extension, washing or SLC through a low density colloid (Figure 4). Bacterial counts were compared in the samples prepared by different methods, and sperm characteristics were evaluated during storage for four days.

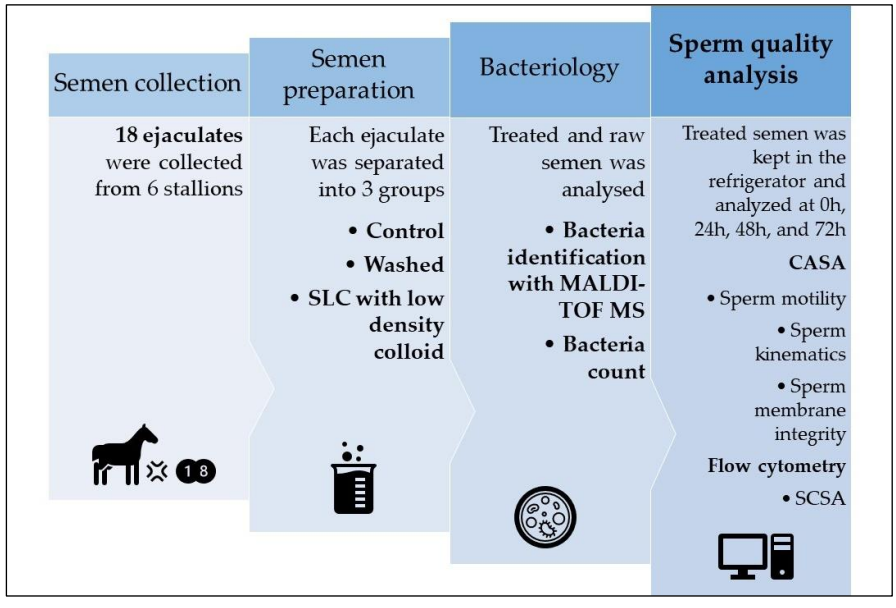

Figure 4. Experimental design for Study IV: 18 ejaculates from 6 stallions were collected and separated into 3 treatment groups: control, washed and SLC. Bacteriology and sperm quality analysis were performed to compare treatments.

3.4 Ethical approval

Study I and II: Ethical approval for vaginal swabbing of the mares was obtained in Sweden (reference number 5.8.18-15533/2018). The mares involved in the study were kept at SLU and private farms in Sweden, following standard husbandry practices appropriate for this species.

Study III and IV: No specific ethical permission was necessary as semen collection using an AV is a standard procedure commonly conducted at AI centers. Husbandry and care of the stallions complied with national and international regulations.

3.5 Animals and samples

Study I and II: Samples were collected during the breeding season in 2020 from 31 mares (2-20 years old). Vaginal swabs were taken using double-guarded occluded swabs to minimize contamination. The sampling site was approximately three centimeters from the vaginal fornix. Swabs were

preserved in Amie's agar gel with charcoal (Copan Diagnostics, Inc., Murrieta, CA, USA). Swabs from inseminated mares (Study I) were immediately plated out at the microbiology laboratory, while samples from non-inseminated mares (Study I) and mares in Study II were refrigerated until they could be sent to the laboratory on Monday-Wednesday.

Study III: The stallions were kept in separate boxes, each with fresh bedding made of straw (Portugal), straw or wood chippings (Germany), or wood shavings (in Sweden), which were replaced daily. They were able to go out into paddocks outdoors during the daytime.

Semen was collected from 38 stallions during 2021 using an AV following mounting of a dummy mare. The collected ejaculate was transferred to the laboratory in a sterile graduated tube. Strict aseptic measures were employed during semen collection to prevent contamination. A 1 mL aliquot of the raw ejaculate was stored in liquid nitrogen and later transferred to −80°C for storage and transport to the Laboratory at SLU.

Study IV: The stallions were kept at the Center for Artificial Insemination and Embryo Transfer, Vetmeduni Vienna, Austria, following standard husbandry procedures.

Ejaculates were collected from 6 stallions in November-December 2022 using a sterilized Hannover AV while the stallions mounted a phantom with an estrous mare nearby. Each ejaculate was divided into three parts and extended in EquiPlus without antibiotics (Minitüb, Tiefenbach, Germany), control, washed, and SLC samples. The sperm concentration was adjusted to approximately 25×10^6/mL before conducting sperm quality analysis.

3.6 Semen Preparation

Study IV: control samples were adjusted to a sperm concentration of approximately 25×10^6/mL with Equiplus without additional manipulation (= extended). To prepare the washed samples, EquiPlus was added in equal volumes, followed by centrifugation at 700 g for 12 minutes. After centrifugation, the supernatant was carefully removed, and the sperm pellet was resuspended with EquiPlus to achieve a sperm concentration of 25×10^6/mL. The SLC samples were prepared using sterile techniques, as described in previous studies (Morrell et al. 2014). A sterile plastic tube (Cytology Brush; Minitube, Celadice, Slovakia) was inserted through a hole in the lid of a 50 mL sterile tube (Minitube) containing 15 mL Equicoll.

Extended semen (15 mL) was gently pipetted on to the colloid, and the tube was centrifuged using a swing-out rotor at 300g for 20 minutes (Al-Kass et al. 2017). The sperm pellet was then collected, using a long Pasteur pipette passed through the tube insert, and resuspended with EquiPlus to a final volume of 15 mL, resulting in a sperm concentration of 25×10^6/mL.

3.7 Semen Quality Analysis

3.7.1 Sperm Concentration

Study IV: sperm concentration was determined using a Nucleocounter-SP 100 (Chemometec, Allerød, Denmark). In this method, 50µL of the sample were mixed with 5 mL of reagent S100 (Chemometec, Allerød, Denmark). The mixture was then loaded into a cassette containing propidium iodide (PI). The filled cassette was inserted into a fluorescence meter which measured the fluorescence from the PI-stained sperm and converted it to the corresponding sperm concentration.

3.7.2 Computer-Assisted Sperm Analysis (CASA)

Study IV: prior to motility analysis, the samples were allowed to reach room temperature. Sperm motility evaluation was conducted using an Olympus BX 51 microscope (Olympus, Tokyo, Japan) with a heated stage set at 38 °C, connected to a SpermVision analyzer (Minitüb GmbH, Tiefenbach, Germany). The SpermVision software program, adjusted explicitly for stallion spermatozoa, was utilized for the analysis. Eight fields were examined, with a minimum of 1000 spermatozoa assessed to determine sperm motility and kinematics.

3.7.3 Sperm Chromatin Structure Assay (SCSA)

Study IV: sperm samples were mixed with TNE buffer solution (0.01 M Tris-HCl, 1 mM EDTA, and 0.15 M sodium chloride, pH 7.4) and snap-frozen in liquid nitrogen, before storing at -80 °C. Samples were thawed on crushed ice for SCSA analysis as described (Al-Kass et al. 2017).

3.8 Bacteriological Analysis

Study I and II: Swab samples were plated on various agars, including blood agar (bovine or horse), lactose purple agar, MacConkey agar, Baird Parker agar, and De Man, Rogosa, and Sharpe (MRS) agar. Incubation was carried out at specific temperatures: 37.0 ± 1 °C anaerobically for 24 + 24 hours on one blood agar plate and aerobically at 37 ± 1 °C for 24 and 48 hours on the other plates (excluding MRS-agar). The MRS-agar plates were incubated at 25 °C for five days under anaerobic conditions. Bacterial colonies with distinct appearances were isolated and cultured on two blood agar plates (aerobic and anaerobic) at 37 °C for 24 to 48 hours to obtain pure cultures. The isolates were identified using MALDI-TOF MS, comparing their mass spectra with known bacterial strains in the database (Bruker Daltonics, Billerica, MA, USA). To preserve the isolates, cryotubes containing brain heart infusion (BHI) broth with 15% glycerol were stored at -70 °C for future antimicrobial susceptibility testing.

Study IV: each sample was diluted with 9 mL of sucrose phosphate buffer medium (2SP) (0.2 mol/L sucrose in 0.02 mol/L phosphate buffer, supplemented with 10% fetal calf serum) and serially diluted up to 1×10^{-8}. Triplicate plates were prepared using different agar types: Schaedler Agar with vitamin K1 and 5% sheep blood, Columbia Agar with 5% sheep blood (both from BBL™, BD Diagnostics, Schwechat, Austria), and pleuropneumonia-like organisms (PPLO) Agar (Difco™, BD Diagnostics, Schwechat, Austria) with 20% horse serum added (Gibco™, Thermo Fisher Scientific, Vienna, Austria). The Columbia Agar plates were incubated at 33 °C in ambient air, the PPLO Agar plates at 37 °C under microaerobic conditions, and the Schaedler Agar plates at 37 °C in an anaerobic jar (BD Diagnostics, Schwechat, Austria). Daily examination of the plates was conducted for up to 96 hours; the bacterial colonies were counted. The mean total colony counts per sample were then calculated.

3.9 Antimicrobial Susceptibility Testing

Study I and II: antibiotic susceptibility was assessed using *Thermo Scientific™ Sensititre™ STAFSTR* (for *Streptococcus* spp. and *Staphylococcus* spp.), *Thermo Scientific™ Sensititre™ EUVENC* (for *E. coli*), and *Thermo Scientific™ Sensititre™ EUVENSEC* (for *Enterococcus faecalis*) (Thermo Fisher Scientific, Waltham, MA, USA). Broth

microdilution method following Clinical and Laboratory Standards Institute (CLSI) standards was used to determine minimum inhibitory concentration (MIC) for *E. coli*, *Enterococcus faecalis*, *Staphylococcus* spp., and *Streptococcus* spp. European Committee on Antimicrobial Susceptibility Testing (EUCAST) provided the epidemiological cut-off value (ECOFF) values for susceptibility determination (Breakpoint tables for interpretation of MICs, Version 11.0, 2021, accessed on 7 November 2022 for Study I: and: www.eucast.org/mic_distributions_and_ecoffs, accessed on 20 February 2023 for Study II).

3.10 DNA Extraction

Study III: DNA extraction was conducted using a QIAamp DNA Mini Kit, and the manufacturer's protocol for purification of genomic DNA from cells. A total of 10 µL of semen was used for the extraction process. Samples were centrifuged, and only the pelleted cells were utilized. The purity and concentration of the DNA were evaluated using a NanoDrop 8000 Spectrophotometer (Thermo Scientific, Waltham (HQ), MA, USA). A 260/280 ratio of 1.7 to 1.9 was considered to be satisfactory DNA purity. The DNA samples were then stored at -80 °C until further preparation.

3.11 16S rRNA Sequencing

Study III: bacterial DNA content in the 16S region was prepared for Illumina sequencing using a two-step amplification protocol. In the first step, a reaction volume of 21 µL was used, including 4 µL of the sample, 10 µL of KAPA HiFi HotStart ReadyMix (Roche), 0.2 µL of BSA (Thermo Scientific), 2 µL of Primer mix (containing 341F and 805R forward and reverse primers at a concentration of 7.5 µM), and 4.8 µL of ultra-pure water. For the second step, the reaction volume was 20 µL and consisted of 6 µL of purified DNA template from the first PCR step, 10 µL of KAPA HiFi HotStart ReadyMix, and 4 µL of indexing primer mix (i5 and i7 indexing primers at a concentration of 2.5 µM). The PCR setups and bead cleanups were performed in duplicate using the Agilent NGS workstation Bravo (Agilent Technologies, USA) in a 96-well plate format. As the bacterial DNA amounts varied among the samples, a primer test was conducted in the first PCR step to determine the appropriate amount for each case. Sample

concentration measurements were estimated using a Qubit 3.0 fluorometer with the High Sensitivity DNA kit. The final bead cleanup was performed after the second PCR using MagSi-NGS prep plus (Tataa). This step involved the removal of free primers and purification of the amplicon by binding the DNA to magnetic beads, followed by washing and releasing the DNA in an elution buffer (Qiagen). The quality of the adapter-ligated libraries was assessed using the Caliper GX LabChip GX/HT DNA high sensitivity kit (PerkinElmer).

3.11.1 16S rRNA Analysis

Study III: analysis of 16S rRNA sequencing data was performed with the Nextflow pipeline ampliseq v1.1.2 (https://github.com/nf-core/ampliseq). Initially, the quality of the sequencing data was assessed using FastQC (Andrews 2010), followed by the removal of primer sequences from the reads using cutadapt v2.7 (Martin 2011). Sequencing reads were then subjected to denoising, dereplication, and filtering of chimeric sequences using DADA2 (Callahan et al. 2016). The resulting processed sequences were used to obtain Amplicon Sequence Variants (ASVs). Taxonomic classification of these ASVs, ranging from phylum to species level, was performed by clustering at 99% similarity using the SILVA v132 database (Quast et al. 2013) by applying I Bayes classifier (Bolyen et al. 2019). Any ASVs classified as Mitochondria or Chloroplast were subsequently excluded.

3.12 Whole-Genome Sequencing

Study I and II: isolates were sub-cultured twice on horse blood agar plates to obtain pure cultures before DNA extraction. The DNA was extracted using the EZ1 DNA Tissue Kit (Qiagen, Hilden, Germany) with modified protocols for Gram-negative and Gram-positive bacteria. Sequencing libraries were constructed using the Nextera XT DNA Library Preparation Kit (Illumina, San Diego, CA, USA); their quality was assessed using the HS DNA ScreenTape Analysis D1000 (Agilent Technologies, Inc., Santa Clara, CA, USA). The WGS was performed on the Illumina NextSeq 500 system (Illumina Inc., San Diego, CA, USA) with 2 × 150-bp paired-end reads. Sequence reads were analyzed using SeqSphere+ software (Ridom GmbH, Münster, Germany). Genome assembly was conducted using SKESA

(Souvorov et al. 2018), and multilocus sequence typing (MLST) profiles were allocated for *E. coli* and *S. dysgalactiae* using established schemes (Jolley et al. 2018; Zhou et al. 2020). Core genome multilocus sequence typing (cgMLST) analysis was performed for *E. coli*, *S. dysgalactiae*, and *S. simulans*. Minimum spanning tree (MST) based on cgMLST data were created to investigate genetic relationships. Genomes were screened for antimicrobial resistance-associated genes and point mutations using AMRFinderPlus (Feldgarden et al. 2019) and ResFinder 4.1 (Camacho et al. 2009; Zankari et al. 2017; Bortolaia et al. 2020).

3.13 Statistical Analysis

Study I and II: the Chi-squared test was used to compare the proportions of resistant bacteria between non-inseminated and inseminated mares. In cases where there were few observations for specific cells, Fisher's Exact test was employed. A p-value <0.05 was considered statistically significant.

Study III: Microbiome data analysis involved the calculation of alpha and beta diversity using the R package Phyloseq v1.44.0 (McMurdie & Holmes 2013). To assess the significance of bacterial diversity and richness within the samples, alpha diversity was evaluated using one-way ANOVA with a false discovery rate corrected p-value threshold of <0.05. The significance of species evenness (Pielou) was determined using the Kruskal-Wallis test. For beta diversity, overall and pairwise PERMANOVA tests were conducted, and p-values were Bonferroni corrected to <0.05 for significance.

Study IV: data analysis was conducted using repeated measures ANOVA in the R Software (version 4.3.0). The Tukey test was employed for multiple comparisons. In the analysis, stallions and ejaculates were considered as random factors, while treatments and time were treated as variables. Only treatments were considered variables in the colony forming units (CFUs) analysis. Diagnostic plots were used to assess normality. The results are given as Least Squares Means ± Standard Error. Statistical significance was considered at p-value <0.05.

4. Results

A summary of the results for this thesis is given below. The full details are presented in the article reprints and manuscripts at the end.

Literature review

Several articles report AMR in bacteria isolated from the equine reproductive tract in various countries (Figure 5). The material examined mostly consisted of swabs from the uterus, vagina and clitoris; AMR was commonly detected in this material. However, mares with reproductive problems were sampled more frequently than healthy mares. In some countries, such as the United States of America and Italy, a change in AMR was observed over time.

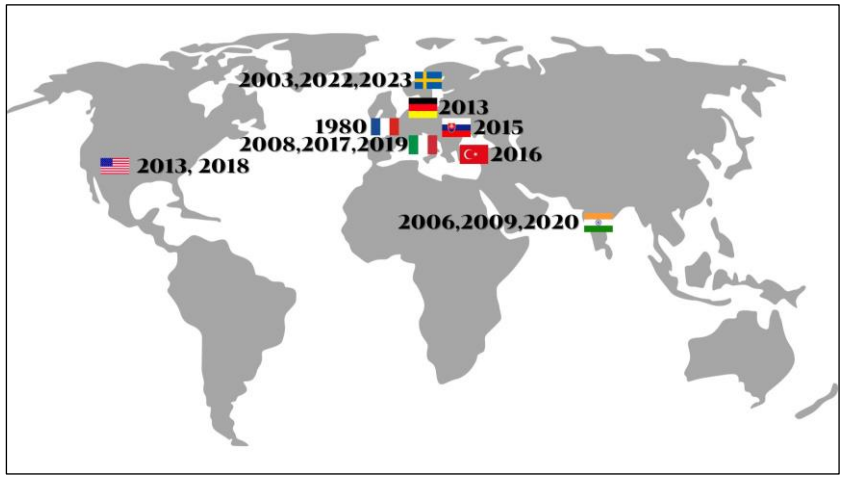

Figure 5. A global map showcasing the nations where cases of AMR have been documented within the reproductive systems of equids, as follows: France (Dabernat et al. 1980); Germany (Goncagül & Seyrek-İntas 2013); India (Berwal et al. 2006), (Singh 2009), (Balamurugan et al. 2020); Italy (Frontoso et al. 2008), (Nocera et al. 2017), (Pisello et al. 2019); Slovakia (Benko et al. 2015); Sweden (Albihn et al. 2003), (Malaluang et al. 2022), (Malaluang et al. 2023); Turkey (Goncagul et al. 2016); US (Davis et al. 2013), (Ferrer & Palomares 2018).

4.1 Study I

In total, 41 bacterial species were identified, with *Escherichia coli* being the dominant species (40%). The microbiota varied among different sub-groups of mares and was more diverse in the mares from Boden compared to Uppsala (Table 1).

Of the *E. coli* isolates, 24 showed resistance to at least one of four tested antibiotics. Differences in antibiotic resistance were observed between inseminated and non-inseminated mares, with trimethoprim ($p = 0.00003$) and chloramphenicol ($p = 0.0309$) showing higher resistance in inseminated mares and vice versa in sulfamethoxazole ($p = 0.0273$).

Out of 61 *Streptococcus* spp. isolates, two were multidrug-resistant. Some differences in resistance were found between inseminated and non-inseminated mares for *Streptococcus* spp., with resistance to erythromycin being significantly higher in non-inseminated mares ($p = 0.013$).

All *Staphylococcus* spp. isolates were susceptible to some antibiotics, but resistance was observed in a few cases. *Enterococcus faecalis* isolates were susceptible to all antibiotics tested.

Table 1. Bacterial species identified from the vaginal flora of mares in two regions in Sweden: Boden and Uppsala.

Mares in Northern Sweden, Boden	
Gram-positive (n = 14)	***Corynebacterium casei, Enterococcus casseliflavus, Enterococcus faecalis, Enterococcus mundtii, Staphylococcus aureus, Staphylococcus capitis, Staphylococcus gallinarum, Staphylococcus simulans, Staphylococcus vitulinus, Streptococcus equinus, Streptococcus equisimilis, Streptococcus hyovaginalis, Streptococcus thoraltensis, and Streptococcus zooepidemicus***
Gram-negative (n = 13)	***Acinetobacter johnsonii, Acinetobacter lwoffii, Acinetobacter schindleri, Acinetobacter* sp.*, Aerococcus viridans, Arthrobacter gandavensis, Enterobacter kobei, Escherichia coli, Kluyvera intermedia, Lelliottia amnigena, Pseudomonas fulva, Rahnella aquatilis, and Serratia marcescens***
Mares in middle Sweden, Uppsala	
Gram-positive (n = 16)	***Actinomyces urogenitalis, Archanobacterium hippocoleae, Micrococcus luteus, Rothia dentocariosa, Rothia mucilaginosa, Staphylococcus capitis, Staphylococcus epidermidis, Staphylococcus equorum, Staphylococcus haemolyticus, Streptococcus canis, Streptococcus equisimilis, Streptococcus parasanguinis, Streptococcus pluranimalium, Streptococcus thoraltensis, Streptococcus vestibularis, and Streptococcus zooepidemicus***
Gram-negative (n = 2)	***Bacteroides thetaiotaomicron and Escherichia coli***

The *E. coli* isolates were studied using WGS to determine their genetic relatedness. Three clusters of highly related isolates were identified, suggesting possible transmission within the herd. In addition, specific genes responsible for antibiotic resistance, such as *dfrA14* for trimethoprim resistance and *mdf(A)* for chloramphenicol resistance, were identified in *E. coli* isolates. In Table 2, a higher number of isolates was observed on Day 3 compared to the other post-ovulation time points during the study. Consequently, Day 3 was selected for the next study, involving a larger investigation into changes in antibiotic resistance after exposure to antibiotics in semen extenders.

Table 2. Antimicrobial resistance of bacterial isolates obtained from the vagina of 10 mares assessed both before and after insemination.

	Bacteria	ID of isolate	Resistance
Day 0	*S. equisimilis*	P5	Tet
	Streptococcus spp.	P11	Ery, Tet
	Staphylococcus spp.	P21	Pen, Oxa, Fus, Ery
	Staphylococcus spp.	P2, P143, P146	Pen
	Staphylococcus spp.	P10	Ery
Day 3	*E. coli*	P136	Tri, Chl
	E. coli	P159, P160	Chl
	E. coli	P133	Tri
	S. equisimilis	P57	Tet
	S. zooepidemicus	P158	Tet
	Streptococcus spp.	P109	Ery, Cli, Tet, T/S
	Staphylococcus spp.	P198	Fus, Gen
	Staphylococcus spp.	P156	Gen
	Staphylococcus spp.	P76	Pen, Oxa
Day 7	*E. coli*	P177, P178, P179, P180 P181, P192, PM12	Tri
	E. coli	P85	Col
	Staphylococcus spp.	P207	Pen
Day 14	*E. coli*	P229	Tri, Chl
	E. coli	P225	Tri
	S. zooepidemicus	P148	Ery, Cli, Tet
	S. zooepidemicus	P126, P151, PM5	Tet
	Streptococcus spp.	P217, PM6	Ery, Cli, Tet, T/S
	Staphylococcus spp.	P245	Pen

Note: Tri = trimethoprim, Chl = chloramphenicol, Col = colistin, Tet = tetracycline, Pen = penicillin, Ery = erythromycin, Cli = clindamycin, T/S = trimethoprim/sulfamethoxazole, Oxa = oxacillin, Fus = fusidic acid, and Gen = gentamicin.

4.2 Study II

In total, 985 isolates from 32 bacterial species were isolated from the 26 mares (Table 3). *Escherichia coli* was the most prevalent species, followed by *Streptococcus* spp. and *Acinetobacter* spp. Resistance of *E. coli* to trimethoprim, chloramphenicol, and tetracycline increased from Day 0 (D0)

to Day 3 (D3) after exposure to semen extenders via artificial insemination, but no significant changes were observed for other antibiotics. No association was found between semen extender exposure and resistance in *Staphylococcus simulans* and *Streptococcus equisimilis*.

Table 3. Bacteria obtained from cranial vaginal of 26 Swedish mares isolated both before (D0) and three days after insemination (D3). The percentages (%) provided represent the relative proportion of all identified bacterial species.

	D0	D3	(%)	No. of Mares
Gram-Negative				
Acinetobacter bohemicus	−	1	0.1	1
Acinetobacter kookii	−	2	0.2	1
Acinetobacter lwoffii	21	30	5.2	12
Acinetobacter schindleri	46	26	7.3	13
Acinetobacter spp.	2	−	0.2	2
Escherichia coli	179	341	52.8	23
Klebsiella oxytoca	−	21	2.1	1
Pantoea agglomerans	−	3	0.3	1
Rahnella aquatilis	1	1	0.2	2
Serratia marcescens	11	−	1.1	1
Gram-Positive				
Aerococcus viridans	6	−	0.6	1
Arthrobacter gandavensis	3	−	0.3	1
Corynebacterium callunae	−	5	0.5	2
Corynebacterium casei	3	−	0.3	1
Enterococcus casseliflarus	−	15	1.5	4
Enterococcus faecalis	6	40	4.7	4
Enterococcus mundtii	−	1	0.1	1
Lactococcus raffnolactis	−	2	0.2	1
Paenibacillus amylolyticus	−	2	0.2	2
Staphylococcus aureus	1	−	0.1	1

	D0	D3	(%)	No. of Mares
Staphylococcus capitis	4	5	0.9	3
Staphylococcus haemolyticus	−	2	0.2	2
Staphylococcus schleiferi	−	10	1	3
Staphylococcus simulans	3	1	0.4	1
Staphylococcus vitulinus	4	2	0.6	2
Streptococcus canis	−	2	0.2	2
Streptococcus equisimilis	48	90	14	18
Streptococcus equinus	1	3	0.4	2
Streptococcus gallolyticus	−	4	0.4	1
Streptococcus hyovaginalis	1	−	0.1	1
Streptococcus thoraltensis	1	−	0.1	1
Streptococcus zooepidemicus	8	27	3.6	9
Total	349	636	100	

Among 520 *E. coli* isolates, 284 showed resistance to at least one antibiotic, with 29 being multidrug-resistant. Significant increases in resistance were observed for trimethoprim ($p = 0.0006$), chloramphenicol ($p = 0.012$), and tetracycline ($p = 0.03$) after exposure to semen extenders. Some resistance was found against sulfamethoxazole, ampicillin, azithromycin, and tigecycline, but there was no change in resistance following AI. All *E. coli* isolates remained susceptible to certain antibiotics. *Streptococcus dysgalactiae* subsp. *equisimilis* isolates did not show a change in resistance after semen extender exposure. Various isolates were resistant to three antibiotics, while remaining susceptible to others. Similar observations were made for *Enterococcus faecalis* and *Streptococcus equi* subsp. *zooepidemicus* isolates.

Staphylococcus simulans isolates displayed resistance to oxacillin, penicillin, fusidic acid, and trimethoprim/sulfamethoxazole. One isolate was multidrug-resistant. Exposure to antibiotics via AI did not influence

resistance. All *Staphylococcus simulans* isolates remained susceptible to erythromycin, gentamicin, and tetracycline.

Phenotypic resistance was assessed by comparing isolates obtained from individual mares before and after exposure to antibiotics, all within the confines of the same cluster. This refers to isolates belonging to similar strains. In the case of *E. coli*, increased resistance to sulfamethoxazole (Cluster 1), trimethoprim (Clusters 2 and 10), chloramphenicol (Clusters 2, 6, and 14), and tetracycline (Cluster 6) was observed.

Whole-genome sequencing was utilized to analyze *E. coli*, *Streptococcus equisimilis*, and *Staphylococcus simulans* isolates. Genetic clustering suggested possible transmission within the herd for some strains.

Resistance genes were identified in *E. coli*, including *sul1*, *sul2*, *dfrA1*, *dfrA14*, *tet(A)*, *marR_S3N*, *mdf(A)*, and *blaEC* genes. *Streptococcus equisimilis* isolates possessed the *lsaC* gene for erythromycin resistance. *Staphylococcus simulans* isolates had the *blaZ* gene responsible for penicillin and oxacillin resistance.

4.3 Study III

The study investigated the microbiome in semen from stallions in three countries: Germany, Portugal, and Sweden. The bacterial DNA concentration ranged from 3.87 to 243.83 ng/µL. A total of 1,908 identifiable ASVs were found, and bacterial diversity varied significantly among the countries (Shannon Index, $p = 0.017$). However, there were no significant differences in richness (observed ASVs, $p > 0.05$) and community evenness (Pielou, $p > 0.05$) among the three countries.

Nineteen bacterial phyla were identified in the semen samples from 37 stallions, with the most frequently observed being *Bacteroidota (Bacteriodetes)*, *Bacillota (Firmicutes)*, *Actinomycetota (Actinobacteria)*, *Synergistota (Synergistetes)*, *Pseudomonadota (Proteobacteria)*, *Spirochaetota (Spirochaetes)*, Candidate Phyla Radiation *(Patescibacteria)*, *Mycoplasmatota (Tenericutes)*, *Fusobacteriota (Fusobacteria)*, and *Campylobacterota (Epsilonbacteraeota)*. The occurrence of the twenty most abundant bacterial genera among the 37 stallions is depicted in Figure 6, with *Peptoniphilus*, *Proteiniphilum*, *Fastidiosipila*, *Corynebacterium 1*, *Petrimonas*, *Corynebacterium*, *W5053*, *Pyramidobacter*, *Ezakiella*, and *Lawsonella* being the most frequently observed.

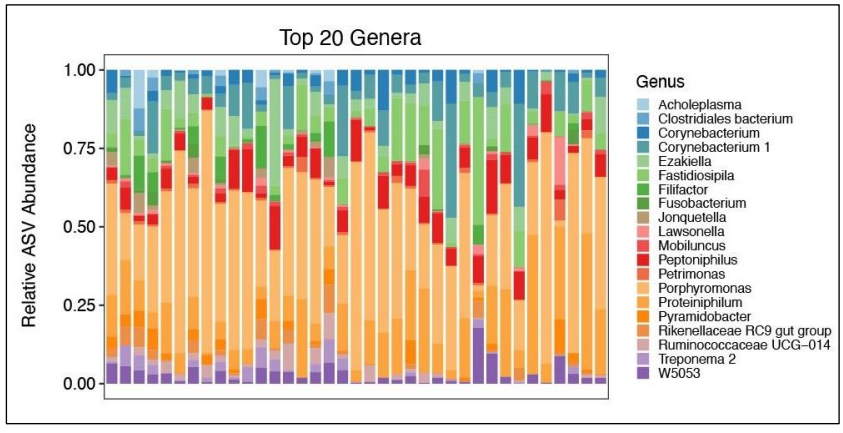

Figure 6. Distribution of the 20 most prevalent bacterial genera in the semen samples of 37 stallions, as determined by 16S rRNA sequencing.

Bacteriodetes, *Firmicutes*, and *Actinobacteria* were dominant, representing around 80% to 95% of the relative ASV abundance. Figure 7 illustrates the distribution of genera in each country. In Germany, the three dominant genera were *Peptoniphilus, Proteiniphilum*, and *Corynebacterium 1,* accounting for approximately 40% of the relative ASV abundance. In Portugal and Sweden, *Peptoniphilus*, *Proteiniphilum*, and *Fastidiosipila* were the dominant genera, constituting about 70% of the relative ASV abundance.

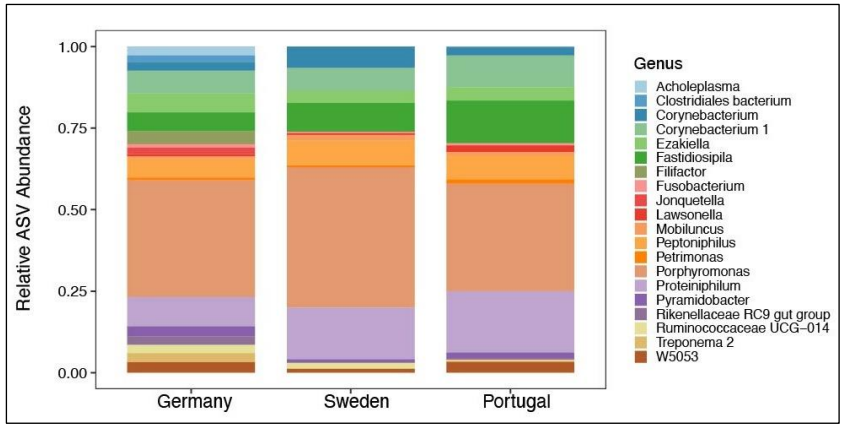

Figure 7. Mean relative abundance of genera between stallions in Germany, Sweden, and Portugal.

The stallion semen community structure (beta diversity) was analyzed using PERMANOVA tests with 10,000 permutations, revealing significant differences ($p < 0.05$) between Germany and the other two countries based on Bray-Curtis measurements. However, there was no significant difference ($p > 0.05$) between Sweden and Portugal.

4.4 Study IV

The bacterial count in extended semen samples was reduced compared to the raw ejaculate (extended = $1.6\times10^6\pm0.67\times10^6$; raw = $76\times10^6\pm20\times10^6$, $p = 0.0018$). Figure 8 shows the total bacterial count in each of the treatment groups. The highest bacterial count was found in the extended control, followed by the washed samples, and the lowest count was observed in the SLC samples. Significant differences in bacterial counts were observed across all sample groups ($p < 0.0001$).

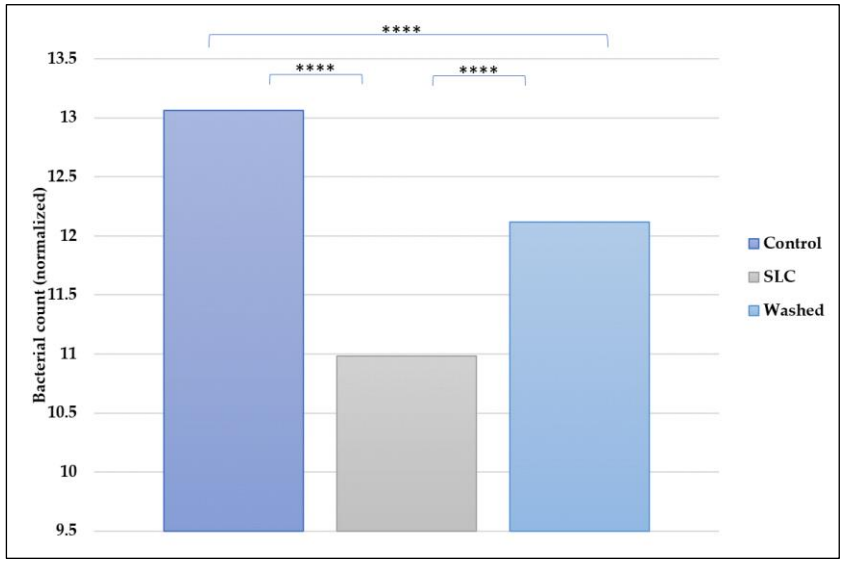

Figure 8. Total bacterial colony count (normalized) per treatment group at time 0. The values presented are Least Square Means (n = 18). The statistical significance is denoted as **** $P < 0.0001$.

Thirteen bacterial species were isolated from the semen samples (Table 4). Among them, *Finegoldia magna* and *Peptoniphilus* sp. were consistently present in all semen samples. The SLC and washing method successfully

separated sperm from six and five bacterial species respectively, including *Brevibacterium paucivorans*, *Campylobacter sputorum*, *Corynebacterium* sp. A, *Mobiluncus porci*, *Pantoea agglomerans*, and *Tessaracoccus* sp. (washing method only partially separated *Tessaracoccus* sp.). However, neither the low density SLC nor washing were effective in reducing the presence of *Corynebacterium* sp. B, *Cutibacterium avidum*, *Finegoldia magna*, *Mycoplasma subdolum*, *Peptoniphilus* sp., and *Proteiniphilum* sp.

Table 4. Reduction (%) in individual bacterial species achieved by different sperm preparation methods.

	Semen treatment method			
Bacterial species	**Raw**	**Extended**	**Washed**	**SLC**
Brevibacterium paucivorans	**17**	**0**	**0**	**0**
Campylobacter sputorum	**17**	**0**	**0**	**0**
***Corynebacterium* sp. A**	**100**	**67**	**0**	**0**
***Corynebacterium* sp. B**	**33**	**33**	**33**	**33**
Cutibacterium avidum	**78**	**78**	**78**	**78**
Finegoldia magna	**100**	**100**	**100**	**100**
Mobiluncus porci	**33**	**0**	**0**	**0**
Mycoplasma subdolum	**67**	**67**	**67**	**67**
Pantoea agglomerans	**33**	**0**	**0**	**0**
***Peptoniphilus* sp.**	**100**	**100**	**100**	**100**
***Proteiniphilum* sp.**	**33**	**33**	**33**	**33**
Staphlococcus xylosus	**72**	**11**	**6**	**6**
***Tessaracoccus* sp.**	**50**	**44**	**6**	**0**

Differences in sperm motility were observed between the control and washed samples, and between the extended and SLC samples after 72 hours (Figure 9). There were also significant differences in some kinematic parameters between the SLC and extended samples at different time points. The SLC samples showed lower values for VAP, VCL, VSL, and ALH compared to the extended samples (Table 5). However, no significant differences were found in membrane integrity (%DFI - DNA fragmentation index) among the extended, washed, and SLC groups (Figure 10).

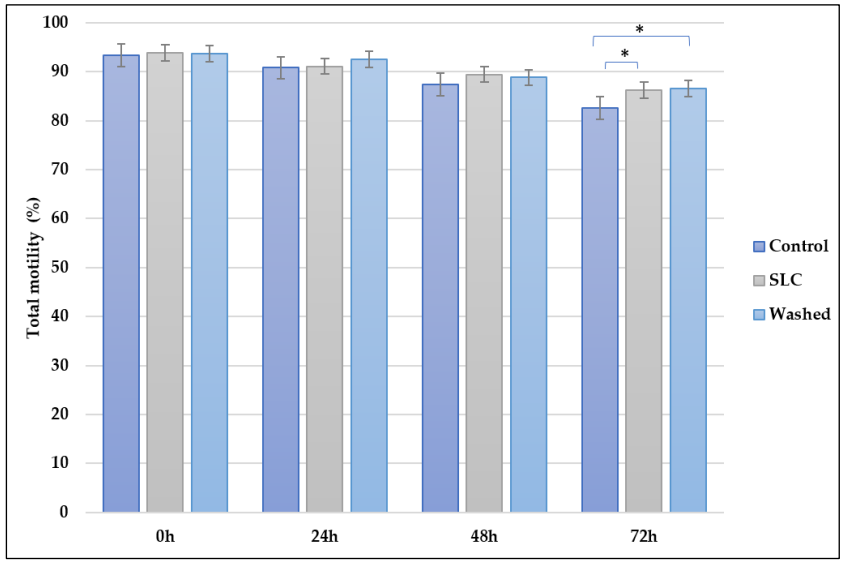

Figure 9. Total motility in control, washed and SLC samples during storage for 72 hours at 6°C. The reported values are Least Square Means ± SE (n = 18). Statistical significance is indicated by * P < 0.05. Note: control = extended semen.

Table 5. Sperm kinematics evaluated for washed, SLC, and control groups without antibiotics over a period of 0 to 72 hours. The reported values represent the Least Squares Means ± Standard Error, with a sample size of n=18.

Time		Washed	Control	SLC
0h	PM%	87.93 ± 0.59	87.48 ± 0.68	88.56 ± 0.68
	VAP (µm/s)	96.47 ± 3.70 [a]	103.54 ± 4.21 [a,b]	92.33 ± 2.86 [b]
	VCL (µm/s)	176.55 ± 5.10	183.48 ± 6.55 [a]	169.93 ± 4.09 [a]
	VSL (µm/s)	83.48 ± 3.27	88.27 ± 3.24	81.17 ± 2.56
	STR%	0.86 ± 0.01	0.85 ± 0.01 [a]	0.88 ± 0.01 [a]
	LIN%	0.47 ± 0.01	0.48 ± 0.01	0.47 ± 0.01
	WOB%	0.54 ± 0.01	0.56 ± 0.01	0.54 ± 0.01
	ALH (µm)	3.89 ± 0.12	3.96 ± 0.15	3.66 ± 0.13
	BCF (Hz)	34.59 ± 0.92 [a]	36.37 ± 0.87 [a]	35.51 ± 0.92

Time		Washed	Control	SLC
	PM%	83.64 ± 0.89	81.18 ± 1.77	83.63 ± 1.16
	VAP (µm/s)	80.75 ± 2.92 [a]	99.78 ± 3.82 [a,b]	87.82 ± 4.36 [b]
	VCL (µm/s)	163.40 ± 5.20 [a]	194.72 ± 6.21 [a,b]	174.11 ± 7.79 [b]
	VSL (µm/s)	68.56 ± 2.52 [a]	81.43 ± 2.92 [a,b]	73.60 ± 3.21 [b]
24h	STR%	0.84 ± 0.01	0.82 ± 0.01 [a]	0.84 ± 0.01 [a]
	LIN%	0.41 ± 0.01	0.41 ± 0.01	0.42 ± 0.01
	WOB%	0.49 ± 0.01 [a]	0.51 ± 0.01 [a]	0.50 ± 0.01
	ALH (µm)	3.99 ± 0.10	4.17 ± 0.13 [a]	3.80 ± 0.15 [a]
	BCF (Hz)	29.27 ± 0.91 [a,b]	32.94 ± 0.47 [a]	33.12 ± 0.97 [b]
	PM%	77.53 ± 1.13	72.72 ± 2.42 [a]	76.49 ± 2.31 [a]
	VAP (µm/s)	75.92 ± 2.47 [a,b]	101.19 ± 3.58 [a,c]	85.31 ± 3.59 [b,c]
	VCL (µm/s)	160.63 ± 4.09 [a,b]	200.34 ± 5.90 [a,c]	176.98 ± 7.23 [b,c]
	VSL (µm/s)	61.68 ± 2.18 [a,b]	77.05 ± 2.15 [a,c]	69.19 ± 2.41 [b,c]
48h	STR%	0.81 ± 0.01	0.77 ± 0.02 [a]	0.81 ± 0.01 [a]
	LIN%	0.38 ± 0.01	0.38 ± 0.01	0.39 ± 0.01
	WOB%	0.47 ± 0.01 [a]	0.50 ± 0.01 [a,b]	0.48 ± 0.01 [b]
	ALH (µm)	4.29 ± 0.12	4.78 ± 0.19 [a]	4.11 ± 0.13 [a]
	BCF (Hz)	26.61 ± 0.79 [a,b]	30.16 ± 0.79 [a]	31.02 ± 0.96 [b]
	PM%	70.00 ± 1.61	66.15 ± 2.52	69.96 ± 2.37
	VAP (µm/s)	76.03 ± 3.31 [a]	90.70 ± 4.53 [a,b]	78.41 ± 3.64 [b]
	VCL (µm/s)	159.93 ± 5.90 [a]	185.18 ± 6.78 [a,b]	164.01 ± 7.01 [b]
	VSL (µm/s)	59.83 ± 2.62 [a]	69.68 ± 2.97 [a]	62.42 ± 2.26
72h	STR%	0.78 ± 0.01	0.77 ± 0.01 [a]	0.80 ± 0.01 [a]
	LIN%	0.37 ± 0.01	0.37 ± 0.01	0.38 ± 0.01
	WOB%	0.47 ± 0.01	0.48 ± 0.01	0.47 ± 0.01
	ALH (µm)	4.80 ± 0.16 [a]	4.73 ± 0.17 [b]	4.16 ± 0.20 [a,b]
	BCF (Hz)	24.53 ± 0.65 [a,b]	27.73 ± 0.82 [a]	29.77 ± 0.85 [b]

Note: statistical differences between columns are denoted by similar letters within rows (a, b, c) at $P < 0.05$. The control group consists of extended semen. Abbreviations used are as follows: PM (progressive motility), VAP (velocity of the average path), VCL (curvilinear velocity), VSL (straight line velocity), STR (straightness), LIN (linearity), WOB (wobble), ALH (lateral head displacement), and BCF (beat cross frequency).

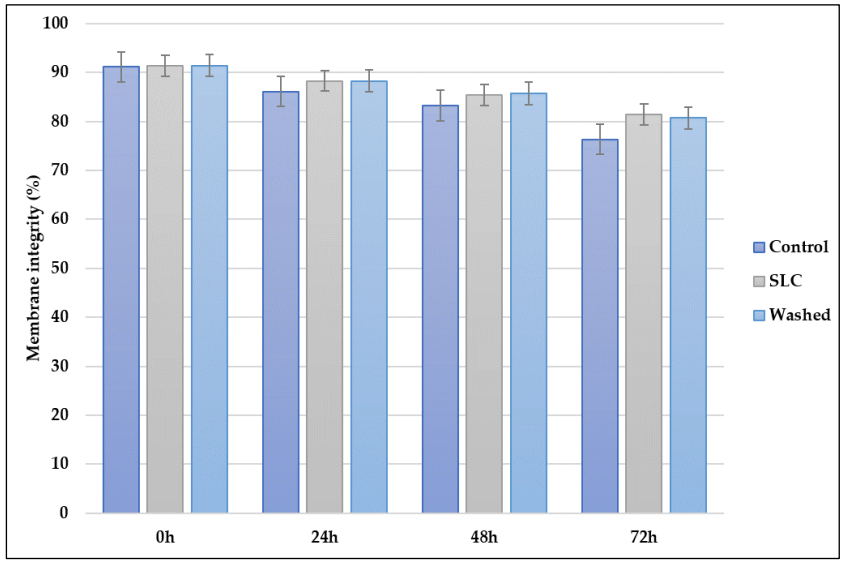

Figure 10. Membrane integrity in control, washed, and SLC samples during a 72-hour storage at 6°C. Values are Least Square Means ± SE (n = 18). Note: control = extended semen.

5. Discussion

The objective of this thesis was to explore how antibiotics in semen extenders influence the vaginal bacteria of mares. Furthermore, some of the factors affecting the appearance of bacteria in stallion semen were determined, and a possible alternative to the use of antibiotics to inhibit bacteria was investigated.

The bacteria isolated from vaginal swabs predominantly comprised *E. coli*, in agreement with previous studies in Korea (19.8%) (Choi et al. 2007) and India (21.7%) (Singh 2009). While most research on the bacteria in the reproductive tract of mares focused on the uterus (Malaluang et al. 2021), the studies in this thesis examined vaginal bacteria. The prevalence of *E. coli* is understandable due to its common occurrence in the intestine and the environment, and its rapid generational turnover. Disparities in the species isolated among studies could stem from different environments, housing/husbandry, agar plates, incubator settings, and identification methods. Notably, Study I revealed that the location of the mare influenced the vaginal flora, since distinct bacterial compositions were found in specific locations. Among 41 species of bacteria, only 5 were consistent across the different sites in Sweden: *E. coli*, *S. capitis*, *S. equisimilis*, *S. thoraltensis*, and *S. zooepidemicus*. Vaginal flora diversity depended on mare location, specifically differing among stud farms. These findings underscore the importance of knowing the variations in the "normal" microbiota of each individual before diagnosing a potential disturbance.

5.1 Antimicrobial resistance

Various environmental factors, such as housing management, age, prior medical history, staffing, and geographic location, could potentially

influence the microbiota of stallion semen, as evidenced in this study. Antimicrobial resistance originates from resistance genes, often present in environmental bacteria and possibly transferred to the reproductive system (Wright 2010). Low-level exposure to antimicrobials, including antibiotics in semen extenders as in Study II, might contribute to the development of AMR (Johansson et al. 2004). Therefore, it is important to acknowledge that improper disposal of antibiotic-containing substances in the environment can contribute to the development of antibiotic resistance, as mentioned by Divala et al. 2018. The duration of exposure required to potentially induce AMR is likely to differ for various antibiotics (Divala et al. 2018), as demonstrated by the resistance of *Staphylococcus* spp. from inseminated mares in Study I to penicillin and gentamicin, both of which were present in the semen extender, suggesting the possibility of AMR development following insemination. In Study I, inseminated mares were exposed to antibiotics in semen extender, while non-inseminated mares might have encountered antibiotics through unknown therapeutic use or gene transmission between environmental and vaginal bacteria. Antibiotic resistance varied among mares with different backgrounds.

Bacteria can exchange acquired resistance genes, allowing them to thrive in otherwise toxic environments (Martínez & Rojo 2011). Conjugation, transduction, and transformation are the primary natural methods of horizontal gene transfer (Martínez 2012), potentially facilitating the transfer of resistance genes observed in Study I and II isolates through all these modes.

In Study I, a greater number of resistant isolates was found on Day 3 (D3) than on the other days tested, prompting its selection for Study II on antibiotic resistance changes due to exposure from semen extenders.

5.2 Occurrence of Antimicrobial Resistance in the Vaginal Bacteria of Mares

The development of antimicrobial resistance within the microbiota of the reproductive tract observed in Studies I and II could be attributed to several factors. These factors include the use of antibiotics for treatment of infection (Kenney et al. 1975; Albihn et al. 2003), as well as their non-therapeutic application, such as in semen extenders. However, the exclusion criteria for mares in our study included antibiotic treatment within the previous 6

months. Additional possibilities for the acquisition of AMR could involve the transmission of resistance from bacteria present in stallion semen. Stallions typically host bacteria on the mucosa of their distal reproductive tract, which can be transferred to the ejaculate during semen collection (Rota et al. 2011). Alternatively, the ejaculate may become contaminated from the environment during processing.

5.2.1 Antimicrobial Resistance in Vaginal Bacteria in Inseminated Mares

Several reports (Malaluang et al. 2021) have focused on the presence of AMR in bacteria isolated from diverse samples of the reproductive tract of horses in various countries, particularly in cases related to clinical disease or fertility problems. In contrast to their findings, bacteria obtained from the vaginal samples of healthy mares in Studies I and II exhibited susceptibility to most of the tested antibiotics. Furthermore, instances of susceptibility to all tested antibiotics were observed in *Enterococcus faecalis* in Studies I and II, as well as in *Streptococcus equi* subsp. *zooepidemicus* in Study II, in line with general observations in Swedish veterinary medicine (Swedres-Svarm 2021). However, Study II reported the first case of tigecycline resistance in *E. coli*, which might have acquired a resistance gene during the experiment or emerged as the dominant strain after all susceptible isolates in the semen extender had been eliminated.

5.3 Bacterial Diversity in Stallion Semen

The diversity of bacteria in Study III exhibited distinct differences. Notably, there were no differences in the overall structure of bacterial communities (beta diversity) between Portugal and Sweden. Although the phylum-level composition of stallion semen microbiota was comparable across the three European nations, significant disparities emerged at the genus level, with stallions in Germany displaying the highest diversity. Stallions in Portugal and Sweden showed greater similarity to each other in their seminal microbiome, in comparison to Germany. These findings underscore the importance of knowing the specific geographic seminal microbiome, to tailor antibiotic use effectively in semen extenders. The prominent phyla identified in our samples – *Bacteroidota (Bacteriodetes)*, *Bacillota (Firmicutes)*, *Actinomycetota (Actinobacteria)*, *Synergistota*

(Synergistetes), Pseudomonadota (Proteobacteria)– align with a prior study (Quiñones-Pérez et al. 2021). However, variations appeared at the genus level, as has been described earlier (Al-Kass et al. 2020), potentially influenced by climatic variations over different years or individual distinctions, further impacted by diverse analytical methodologies. Nonetheless, *Corynebacterium* consistently surfaced in stallion semen in studies using either culture-based or non-culture based detection methods.

Pasing et al. (2013) conducted a study on microorganisms on stallion genital mucosa and in semen from February to August. An increase in *Staphylococcus vitulinus* occurred in May, which was attributed to environmental factors, bedding quality changes, or increased staff workload during the breeding season. A recent study on bull semen microbial load found abstinence length (1-3 days) affected bacterial loads, with the lowest load being seen when semen collections were made daily (Cojkic et al. 2023). This finding indicates the need to explore bedding, husbandry, and management effects on stallion semen microbial load to reduce antibiotic use in extenders. Hygiene practices also influence semen microbial content, e.g., possibly washing the stallion's penis before collection, multiple false mounts, or attempting to discard the pre-sperm fraction, as practised for other species. These practices are not common in stallion semen collection. Semen collection and handling must be carried out with rigorous focus on maintaining hygiene (Althouse 2008). The effect of factors such as washing the penis, false mounts, pre-sperm fraction were not within the scope of the current study but could be relevant for extensive studies on the control of seminal bacterial content.

5.4 Factors affecting the bacterial load in semen

The observed variations in bacterial contamination across different studies in Study III could be linked to several factors, including the stallions' environment, the husbandry practices on the stud (Pasing et al. 2013), and other factors such as the number of intromissions into the AV required to achieve ejaculation. To mitigate these variations, we selected stallions from similar environments and husbandry practices.

5.5 Single Layer Centrifugation with a low density colloid

In Study IV, SLC using a low density colloid led to a notable decrease in bacterial count within stallion semen samples. In a previous investigation involving stallion semen, a high density colloid SLC could successfully eliminate around 90% of the bacterial content (Morrell et al. 2014) and selected the most robust spermatozoa. Use of a low density colloid in the present study reduced bacterial load without negatively impacting sperm motility, velocities, membrane and DNA integrity when compared to both washed samples and control samples (extended semen). These findings hold considerable potential for minimizing the need for antibiotics in semen extenders. Nonetheless, additional investigations are required to establish the conditions in which antibiotics can be excluded, especially in light of the differences in seminal microbiome among stallions in different countries identified in the previous study. However, a fertility trial is needed to ensure that sperm fertilizing ability is not affected.

6. Conclusions

The thesis highlights the presence of diverse bacteria in vaginal swabs from mares, with *E. coli* as the predominant species in both study I and II. Unlike earlier studies that concentrated on uterine bacteria, this study focused on the vaginal microbiota in mares. The prevalence of *E. coli* is likely due to its intestinal occurrence and rapid generation time. Differences in isolated species across studies attributed to various factors such as environmental conditions, housing and husbandry, growth media, incubation settings, and identification techniques. Notably, the study highlights the impact of mare location on vaginal flora, revealing distinct bacterial compositions particularly within specific groups, such as on stud farms.

The study on equine vaginal bacteria in both paper I and II demonstrated a complex landscape of microbiota with diverse antibiotic susceptibility. Changes in susceptibility were identified, potentially due to prior antibiotic treatments. *E. coli* resistance varied with breeding status, age, and previous insemination. Development of antibiotic resistance appeared to be a complex phenomenon, potentially involving gene transfer and survival advantages. Consistent resistance trends were observed for *E. coli* with an increase in resistance after insemination with the antibiotic-containing extender, while *Streptococcus* spp. resistance varied. The study emphasized the influence of environment, medical history, and antimicrobial exposure on resistance, highlighting the intricate nature of antibiotic resistance development across diverse mare populations.

The distinct differences in seminal microbiome between stallions in different countries is notable and deserves further investigation. The implications of these findings highlight the necessity for understanding

geographic variation in the seminal microbiome for effective antibiotic use in semen preservation. The presence of common bacterial phyla is in line with previous research, although genus-level differences suggest potential influences of climate and individual factors on the microbiome. Variables such as the length of abstinence and the hygiene practices on the stud farm also impact semen microbial content and warrant further investigation in an effort to reduce bacterial load.

Utilizing a low density colloid in SLC of stallion semen produced a significant reduction in bacterial count. This reduction was achieved without compromising crucial sperm characteristics such as motility, velocity, and chromatin integrity when compared to washed and control samples. These findings suggest a potential avenue for reducing antibiotic dependence in semen extenders. However, further research is necessary to determine optimal conditions for antibiotic-free protocols, and a fertility trial is recommended to determine that sperm fertility is not adversely affected in treated sperm samples.

7. Future considerations

The findings from the research outlined in this thesis suggest several intriguing directions for future investigations, three of which are detailed below.

1. Factors affecting the vaginal microbiota

Variations in the vaginal microbiota were observed, influenced by the geographical location of the mares. Exploring the factors that might impact this diversity, such as environmental elements (feed, water, paddock, bedding, and soil type) and seasonal and climatic conditions, could yield intriguing insights. Consideration of husbandry factors, including bedding change frequency, paddock access, and mare-related factors such as the number of foals, insemination intervals, overall health, and nutritional status, would also be essential.

2. Factors affecting the seminal microbiota

Again, the study's findings revealed variations among individuals but substantial differences between stallion groups from different countries. The period of abstinence from certain semen collection may be important, but the type of bedding and its frequency of changing might also significantly influence the bacterial community present. Comparing the microbiota and microbiome, specifically examining the bacteria inhabiting the reproductive tract versus the DNA present, would be helpful in determining which antibiotics might be essential in semen extenders.

3. Fertility of stallion spermatozoa after passing through a low density colloid

Conducting an artificial insemination trial is crucial to ascertain if the passage of sperm through the low density colloid could impairs their fertilizing capacity. Extrapolating from the current in vitro trial, this fertility study is expected to pose no issues for the mare. The mare's reproductive tract can effectively handle the presence of bacteria in the uterus during natural mating, and the bacterial concentration remaining after centrifugation with a low density colloid is significantly lower than naturally occurs in stallion semen.

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Popular science summary

The world of the equine microbiota comes into focus through this study that examines bacterial populations in the reproductive tract of mares and stallions, shedding light on vital aspects of antibiotic resistance and reproductive health.

In a ground-breaking exploration, we investigated the complex world of vaginal bacteria in mares. Unlike previous investigations that concentrated on uterine bacteria, this study turns the spotlight on the vaginal microbiota. Notably, *E. coli* stands out as a major player in both study I and II, probably due to its presence in the intestines and its rapid turnover. However, variations in bacterial species across studies arise from multiple factors such as environmental conditions, housing, and lab techniques. Noteworthy findings also reveal how the location of mares affects vaginal flora, unveiling distinct bacterial compositions within specific groups of mares, such as those found on a particular stud farm.

Antibiotic resistance takes center stage in this study, revealing a complex web of susceptibility and resistance in the vaginal bacteria of the mare. Changes in susceptibility might arise from past antibiotic treatments, while resistance in *E. coli* shows connections to insemination status, age, and breeding history. Antibiotic resistance development is linked to gene transfer and survival advantages. Resistance in *Streptococcus* spp. seems to vary due to environmental influences, emphasizing the complexity of antibiotic resistance across diverse populations of mares.

The research on stallion semen spans three European countries, uncovering intriguing disparities in bacterial diversity. Although Portugal

and Sweden share similar bacterial structures, Germany stands apart. While larger microbial categories align, differences emerge at the genus level, possibly due to climate and individual factors. This insight highlights the significance of comprehending geographic variations in the seminal microbiome for effective control of bacteria during semen preservation.

A promising alternative to antibiotics comes in the form of SLC using a low density colloid. This innovation significantly reduces bacterial counts in stallion semen without compromising sperm quality, potentially reducing the need for antibiotics in semen extenders. However, fine-tuning these protocols and confirming maintained fertility in treated sperm samples through fertility trials is imperative before this procedure cab be widely adopted.

Populärvetenskaplig sammanfattning

Hästens mikrobiota studeras i detalj i denna studie som undersöker bakteriepopulationer i reproduktionsorganen hos ston och hingstar. Avhandlingen belyser viktiga aspekter av antibiotikaresistens och reproduktive hälsa.

Till skillnad från tidigare undersökningar som har gjorts på bakterier i livmodern undersökte denna studie den komplexa bakteriefloran i vagina hos ston.

Bakterien E. coli är tydligt representerad i både studie I och II, förmodligen på grund av dess förekomst i mag-tarmsystemet och dess snabba omsättning. Det gick dock att se att variationerna i vilka bakterier som återfanns i de olika studierna kommer sig av många olika faktorer som omgivning, stallmiljö och laboratorietekniker. Resultaten visar också på att den geografiska lokalisationen av ston påverkar deras vaginalflora då specifika grupper av ston vid specifika gårdar hade olika sammansättning av bakteriefloror.

Antibiotikaresistens är huvudfokus in denna studie som uppvisar en komplex bild av existensen av både verksamma antibiotika och antibiotikaresistens bland vaginalbakterier hos ston. Förändringen i känslighet hos antibiotika skulle kunna komma från tidigare antibiotikabehandlingar samtidigt som resistens bland E. coli uppvisade ett samband till inseminationsstatus, ålder och reproduktionsbakgrund. Utvecklandet av antibiotikaresistens är kopplat till genöverföringar och förbättrade möjligheter till överlevnad. Resistens hos bakteriegruppen Streptococcus verkar variera på grund av omgivningsfaktorer, något som

betonar komplexiteten för antibiotikaresistens över olika populationer av ston.

Forskningen på hingstsperma sträcker sig över tre europeiska länder och påvisar intressanta skillnader bland bakteriesammansättningen. Portugal och Sverige har liknande bakteriella sammansättningar men Tyskland har en avvikande bild. Huvudsakliga mikrobiella kategorier är liknande men skillnader framkommer vid nivån släkte, eventuellt på grund av klimat- och individuella faktorer. Denna observation lyfter vikten av att förstå de geografiska skillnaderna i seminalmikrobiomet för att effektivt kunna kontrollera bakterierna när spermier ska bevaras.

Ett lovande alternativ till antibiotikatillsats finns i form av Single Layer Centrifugation med en kolloid med låg densitet. Denna innovation minskar mängden bakterier i hingstsperma utan att påverka spermiekvaliteten och har potential att minska behovet av antibiotika i spädningsvätskor. Det är dock viktigt att dessa protokoll finjusteras och att bibehållen fertilitet för de behandlade spermieproverna bekräftas med vidare fertilitetsstudier.

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animals

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Review

Antimicrobial Resistance in Equine Reproduction

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Simple Summary: Bacteria can develop resistance to antibiotics, resulting in the appearance of infections that are difficult or impossible to treat. This ability enables bacteria to survive in hostile environments and can result from exposure to even small amounts of antibiotic substances. Bacteria are present in the reproductive tract of the horse; they can develop resistance to antibiotics, because the animal has been treated for an infection, or due to insemination with a semen dose that contains antibiotics. Bacteria colonize the membrane lining the male reproductive tract and are transferred to the semen during collection. They can cause sperm quality to deteriorate during storage or may cause an infection in the mare. Therefore, antibiotics are added to the semen dose, according to legislation. However, these antibiotics may contribute to the development of resistance. Current recommendations are that antibiotics should only be used to treat bacterial infections and where the sensitivity of the bacterium to the antibiotic has first been established. Therefore, adding antibiotics to semen extenders does not fit these recommendations. In this review, we examine the effects of bacteria in semen and in the inseminated mare, and possible alternatives to their use.

Abstract: Bacteria develop resistance to antibiotics following low-level "background" exposure to antimicrobial agents as well as from exposure at therapeutic levels during treatment for bacterial infections. In this review, we look specifically at antimicrobial resistance (AMR) in the equine reproductive tract and its possible origin, focusing particularly on antibiotics in semen extenders used in preparing semen doses for artificial insemination. Our review of the literature indicated that AMR in the equine uterus and vagina were reported worldwide in the last 20 years, in locations as diverse as Europe, India, and the United States. Bacteria colonizing the mucosa of the reproductive tract are transferred to semen during collection; further contamination of the semen may occur during processing, despite strict attention to hygiene at critical control points. These bacteria compete with spermatozoa for nutrients in the semen extender, producing metabolic byproducts and toxins that have a detrimental effect on sperm quality. Potential pathogens such as *Klebsiella pneumoniae*, *Escherichia coli*, and *Pseudomonas aeruginosa* may occasionally cause fertility issues in inseminated mares. Antibiotics are added during semen processing, according to legislation, to impede the growth of these microorganisms but may have a detrimental effect on sperm quality, depending on the antimicrobial agent and concentration used. However, this addition of antibiotics is counter to current recommendations on the prudent use of antibiotics, which recommend that antibiotics should be used only for therapeutic purposes and after establishing bacterial sensitivity. There is some evidence of resistance among bacteria found in semen samples. Potential alternatives to the addition of antibiotics are considered, especially physical removal separation of spermatozoa from bacteria. Suggestions for further research with colloid centrifugation are provided.

Keywords: antibiotics; resistance mechanisms; sperm quality; uterine health; semen extenders; prudent use of antimicrobials

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1. Introduction

The World Health Organisation (WHO) described antibiotic resistance as being one of the greatest challenges facing humankind in the modern world [1]. There are already many instances where bacterial infections, such as tuberculosis, pneumonia, food-borne diseases, or gonorrhoea, do not respond to treatment with currently available antibiotics [2]. No new classes of antimicrobial agents have been developed in the last few decades, and there is no incentive for pharmaceutical companies to invest to produce new ones [3]. Even low-level usage of antibiotics, such as in topical applications, can contribute to the appearance of antimicrobial resistance (AMR). Therapeutic treatment of both animals and humans contributes to background resistance, i.e., maintaining resistance within bacteria in the environment. Thus, in an effort to curb the development of AMR, antibiotics should be used only for therapeutic purposes and preferably after sensitivity testing of the bacteria present [4].

Although AMR in horses has been reported previously, the subject of AMR in the reproductive tract of horses has not been described in detail. The widespread use of antibiotics in semen extenders used to prepare semen doses for artificial insemination (AI) could represent an important cause of AMR in brood mares, and therefore deserves particular attention. This application represents a hidden use of antibiotics that may not be completely justified. Therefore, this review presents an overview of AMR in the reproductive tract of the mare. First, we will consider how bacteria develop resistance, since this is crucial to any attempt to hinder AMR, followed by a description of how bacteria colonize the reproductive tract of the mare. In the next section, we describe how these bacteria are exposed to antibiotics and the effects of the antibiotics on sperm quality and on the mare. Finally, suggestions for alternatives to antibiotics to delay the development of AMR are presented. The search terms are shown in Supplementary file.

2. Development of Antimicrobial Resistance

Bacteria may have natural resistance to different types of antibiotics, or they acquire it. Resistance can be due to genetic mutations or due to selection pressure in nature and can arise in both pathogenic and non-pathogenic bacteria. A variety of resistance genes occur in environmental bacteria [5]. Exposure to antibiotics facilitates the development of AMR, since resistant bacteria have an advantage over other strains in the same environment. This selection also explains why topical use of antibiotics contributes to AMR. Opinions differ on how much exposure to antibiotics is needed for resistance to develop. A study of *Clostridium perfringens* concluded that even small amounts of antibiotics could result in resistance [6]. Another study, however, suggested that a higher concentration of antibiotics is required to induce AMR than is usually seen in a non-clinical environment, although it can arise due to pollution events [7], such as incorrect disposal of antibiotic-containing substances into the environment. The duration of exposure needed to induce AMR is likely to be different for different antibiotics, but is poorly defined [7].

Genes for acquired resistance can be transferred between bacteria [8], allowing the bacteria to thrive in an otherwise toxic environment. The main horizontal means of transmission in nature are by conjugation, transduction, and transformation [9], as shown in Figure 1. During conjugation, transfer of the genetic sequence in a plasmid occurs between in-contact bacteria through a tube-like structure known as a pilus. Some, mainly Gram-positive, bacteria can conjugate via transponders [9]. In transduction, bacteriophages transfer genetic material between bacteria, although this is a relatively ineffective mode of transmission, infecting only closely related species of bacteria [9]. With transformation, exogenous DNA is absorbed into the bacterial cell through a specific membrane channel and incorporated into the bacterial DNA [10]. Even dead bacteria can pass on resistance genes to other bacteria by this method [10].

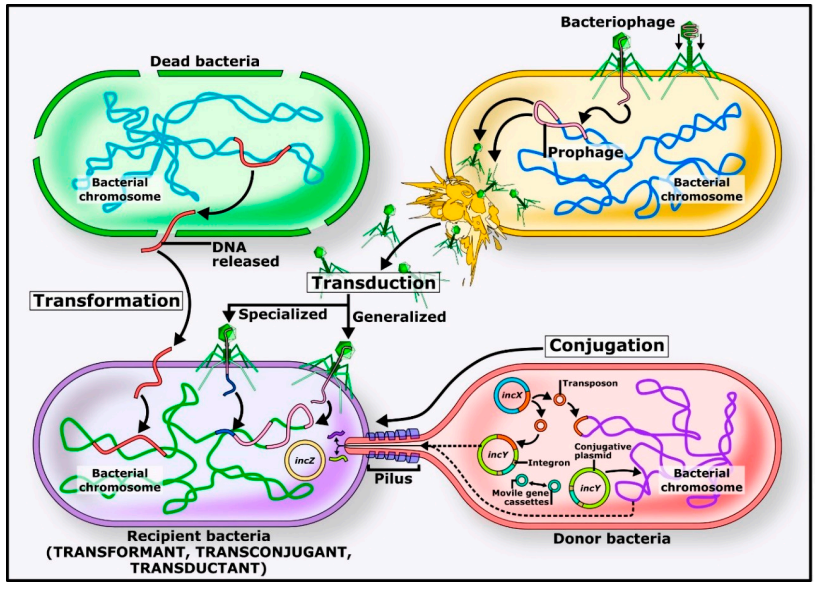

Figure 1. Mechanisms of horizontal gene transfer in bacteria [10]. Note: The main routes of transmission of genetic material between bacteria are transformation, transduction and conjugation. Genes can pass between related and non-related species by these routes. These mechanisms of transmission allow bacteria to evolve to survive.

Once acquired, resistance genes are transmitted vertically during cell division. Enterococci are able to transfer AMR to less resistant strains [11], and *Aeromonas* spp. can pass resistance genes to unrelated bacteria [12]. Transfer of plasmids enables other DNA, e.g., obtained via transformation, to be passed on to subsequent generations. Therefore, use of one antimicrobial agent can lead to the transfer of resistance against other antimicrobials within the population [13].

Acquisition of AMR has a fitness cost [14], since bacteria with resistance genes start to produce novel genetic elements at a higher metabolic cost than the non-resistant cell. In the absence of selection pressure, the genes for resistance will be lost due to competition from bacteria without the gene [9]. Thus, resistance patterns within any given bacterial population are evolving constantly, corresponding to selection in response to changes in the environment.

An isolate is considered to be resistant if it has a higher minimum inhibitory concentration (MIC) than the corresponding wild-type strain [15]. Different categories of resistance occur: bacteria with acquired resistance to antimicrobial substances from three or more antibiotic classes are known as multi-drug resistant; if susceptible to one or two antimicrobial substances, they are defined as extensively drug-resistant; bacteria that are non-susceptible to all agents in all antimicrobial classes are pan-drug-resistant [16]. Examples of the latter are strains of *Klebsiella (K) pneumoniae* and *Acinetobacter* spp. [16], both of which are reported to be common constituents in the vaginal flora of the mare [17].

Risk factors for the development of AMR in the flora of the reproductive tract include repeated use of antibiotics, e.g., against infections, but also their non-therapeutic use in semen extenders [18,19]. Bacteria are found on the mucosa of the distal reproductive tract of healthy stallions and are transferred to the ejaculate during semen collection [20] or may contaminate the ejaculate from the environment during processing. Adding a semen extender not only supplies nutrients and buffers to keep the spermatozoa alive but also

provides an excellent medium for the growth of microorganisms. Therefore, antibiotics are added to semen extenders to prevent deterioration of sperm quality to be used are specified in various regulations, e.g., as stipulated by European Council Directive 92/65/EEC [21]. In addition, strict hygiene measures are taken to reduce bacterial contamination during semen collection and artificial insemination (AI) and stallions should not be used for both natural mating and semen collection for AI during the same time period [22]. Bacteria in sperm samples will be discussed in detail in Section 4.

3. Bacteria in the Equine Uterus

Although the healthy equine uterus was previously considered to be sterile, it is now known to possess its own flora [23]. These observations have arisen from the use of bacterial DNA sequencing (16S sequencing), enabling bacteria to be identified that would not appear on culture [24]. Some mares have poor conformation of the vulva or perineal region [25], predisposing them to additional bacterial contamination of the reproductive tract. Such poor conformation can be inherited or occur with age. Injury to the cervix and/or vagina may predispose to bacterial ingress, whereas poor uterine clearance predisposes to persistence of bacteria within the uterus. Bacteria in the caudal reproductive tract (vagina and cervix) may be carried into the uterus during mating or AI, or during gynecological examination despite strict hygienic measures [26]. The effect of bacteria in semen will be considered in detail in Section 5.

Occasionally, the bacteria introduced during mating or insemination are pathogenic. Thus, *Streptococcus* (*Str.) equi* ssp. *zooepidemicus*, *K pneumoniae*, *Escherichia* (*E.*) *coli* and *Pseudomonas (Ps.) aeruginosa* have been found in the urethra and prepuce of stallions after mating (51.1%, 28.9%, 17.8% and 2.2%, respectively) [27]. These bacteria were also isolated from mares bred by these stallions (90.9%, 1.3%, 1.3%, and 1.3%, respectively), indicating that the stallion was the source of some of the bacteria. There is a well-defined response in the reproductive tract of the mare to deal with bacteria (and other debris) deposited during mating [28]. However, this response may be overwhelmed if the bacterial load is very high or if specific bacteria are present, which are known pathogens. These bacteria are *Taylorella equigenitalis* (the causative agent of contagious equine metritis, CEM), *Klebsiella pneumoniae*, and *Ps aeruginosa* [29]. In addition, *Str. equisimilis*, *Str. zooepidemicus*, *E. coli, Bacillus* spp. [30,31], *Mycoplasma (M.) equigenitalium, M. subdolum*, and *Acholeplasma* spp. have been isolated from infertile mares or from mares diagnosed with endometritis or abortion [32,33], although these organisms may be facultative pathogens rather than obligate pathogens.

An inflammatory response occurs following mating, involving cytokines and complement [34]. Its function is to remove bacteria, seminal plasma, and excess spermatozoa from the uterus [25]. Fluid and inflammatory cells are normally voided within 24–48 h after mating. However, in some mares, there is retention of fluid and inflammatory cells for prolonged periods, which affects ciliary function and leads to the development of acute endometritis, with bacteria adhering to the endometrium. Vascular degeneration occurs and uterine drainage is disrupted, reducing venous return to capillary beds [34] and disturbing hormone delivery to the endometrium. The presence of lymphocytes and plasma cells for prolonged periods leads to chronic degeneration in the endometrium. It is not known whether bacteria are the primary initiators of these events or are secondary invaders of the affected tissue [35].

4. Antimicrobial Resistance in the Mare's Reproductive Tract

The bacterial flora of the mare's reproductive tract is exposed to antibiotics via several routes: after systemic administration, via local application and in semen extenders used in AI. The drivers for the development of AMR are summarized in Figure 2.

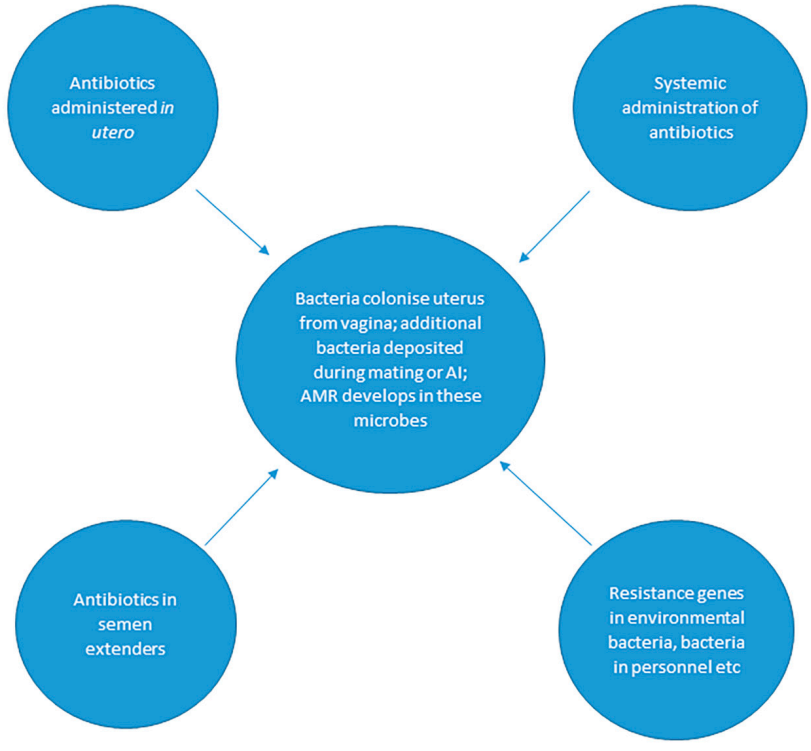

Figure 2. Sources of AMR in the microbial flora of the equine reproductive tract. Note that AMR can develop by any of the mechanisms described in Section 2.

Some antibiotics are found in the uterus after systemic administration; for example, ceftiofur administered systemically passed from the bloodstream to the uterus [36]. The penetration of antibiotics into uterine tissue and secretions depends on the pharmacodynamics of the particular compound, the dose and route of administration, and duration of exposure. Concentrations of sulfadiazine-trimethoprim administered orally were reported to exceed MIC in the endometrium of healthy mares [37]. Enrofloxacilin given by the intravenous route was effective against uterine infections, i.e., achieved effective concentrations in uterine tissue [38], whereas it was not recommended for uterine lavage due to tissue irritation [39]. Regarding transfer from the uterus to the bloodstream, ciprofloxacin administered by the intrauterine route achieved MICs in uterine tissue in healthy mares but with minimal systemic absorption [40]. In contrast, neomycin administered by the intrauterine route was transferred to the blood stream, although the uptake depended on the stage of the oestrous cycle and whether or not infection was present [41]. It should be noted that most of the studies that have been performed on transfer of antibiotics to the uterus after systemic administration or between the uterus and the rest of the body after intrauterine administration have been carried out in healthy animals [35]; the pharmacodynamics may be different in the presence of pathology. Studies in cattle report contradictory findings regarding penetration of antibiotics into the uterus following systemic administration. Thus, ceftiofur administration to cows with postpartum metritis did not influence the number or type of bacteria present in the uterus but did reduce the number of days of elevated body

temperature in these animals [42]. Oxytetracycline administered intramuscularly to cows with metritis resulted in penetration of the antibiotic into uterine secretions, but levels were below MIC values [43]. Therefore, apparently some antibiotics in the insemination dose can pass from the uterus to the bloodstream. Local application includes uterine administration, e.g., for endometritis, but also occurs via inseminated semen, in which antibiotics are added to the semen extender. Either local or systemic administration of antibiotics could therefore lead to development of AMR in the bacterial flora of the reproductive tract via the mechanisms already described, even through low-level exposure.

Several articles from various countries report AMR in bacteria from the equine reproductive tract; the bacteria were isolated from uterine lavage or swabs from the uterus, vagina, or clitoris. The reports indicate that AMR is widespread in the bacteria isolated from this material, regardless of the reproductive status of mares, although mares with reproductive problems, such as failure to conceive, were more commonly sampled than those with normal fertility. The findings of the various reports are summarized in Table 1. Longitudinal studies in some countries indicate that the AMR pattern has changed over time, e.g., in the United States [26] and Italy [44]. However, it is not clear whether the increase in reporting is due to an actual increase in occurrence or merely a reflection of wider testing.

Table 1. Summary of antimicrobial resistance patterns in the equine reproductive tracts in various countries.

Country and Source	Material Used and Susceptibility/Resistance Results
France [45]	*Taylorella equigenitalis* isolates from cervical swabs of mares with acute endometritis or cervicitis; the isolates were resistant to streptomycin, clindamycin, lincomycin, and metronidazole.
Sweden [19]	135 bacterial isolates from uterine swabs from mares with fertility problems; ß-haemolytic *Streptococcus* was resistant to gentamicin, neomycin, oxytetracycline, and trimethoprim-sulfamethoxazole out of the 11 tested antibiotics. *E. coli* was resistant to 9 of 10 tested antibiotics, including ampicillin, cephalothin, chloramphenicol, gentamicin, neomycin, nitrofurantoin, oxytetracycline, streptomycin, and trimethoprim-sulfamethoxazole.
India [46]	Bacteria from uterine flushes from three repeat breeder mares included *Escherichia coli*, *Klebsiella* spp. and *Micrococcus* spp. susceptible to amoxicillin, chloramphenicol, doxycycline and gentamicin and resistant to cloxacillin, metronidazole, penicillin, and sulphadiazine.
Italy [47]	Isolates from uterus of mares with fertility problems: *Str. group C* were only susceptible to amoxicillin/clavulanic acid at 82.7%. *E. coli* showed high susceptibility to a significant number of drugs.
India [17]	Bacteria in vaginal swabs of infertile and healthy mares showed resistance. All isolates belonging to *Streptococcus* spp. were highly resistant to amoxiclav, ampicillin, carbenicillin, cefotaxime, cephalexin, enrofloxacin, clindamycin, cloxacillin, co-trimoxazole, co-trimazine, erythromycin, gentamicin, oxacillin, and tetracycline. *Enterococcus* spp. and *E. coli* isolates from infertile mares were resistant to ß-lactam antibiotics and imipenem. *Enterococcus* spp. were highly resistant to ampicillin, carbenicillin, cefdinir, cefotaxime, cephalexin, chloramphenicol, enrofloxacin, clindamycin, cloxacillin, co-trim-oxazole, co-trimazine, erythromycin, gentamicin, norfloxacin, oxacillin, and vancomycin.
US [26]	Uterine swab collected at pre-breeding examination or infertility investigation. *E. coli* was highly resistant to ampicillin and trimethoprim-sulfonamide, *S. equi* subsp. *zooepidemicus* was highly resistant to oxytetracycline and bacteria belonging to *Enterobacteriaceae* were highly resistant to ampicillin, cefazolin, penicillin, and polymyxin B.
Germany [48]	Isolates from the uterus of mares with fertility problems showed that ß-hemolytic *streptococci* were resistant to colistin, whereas all *E. coli* strains were resistant to penicillin and erythromycin.
Slovakia [49]	Bacterial pathogens in equine cervical swabs in English thoroughbred mares taken during the foal heat cycle. β-haemolytic *streptococci* and *K* spp. showed high resistance to penicillin. *E. coli, Pseudomonas* spp. were highly resistant to penicillin and sulfisoxazole, and *Proteus* spp. were highly resistance to penicillin, tetracycline and sulfisoxazole.

Table 1. *Cont.*

Country and Source	Material Used and Susceptibility/Resistance Results
Turkey [50]	Endometrial swabs taken from mares with pneumovagina and normal mares. *E. coli* was resistant to penicillin. *S. equi* subsp. *zooepidemicus* was highly resistant to tetracycline and colistin. *Staphylococcus intermedius* was resistant to penicillin, tetracycline, erythromycin, gentamicin, and colistin. *Str. equinus* was highly resistance to enrofloxacin, gentamicin, and colistin. *Ent. faecium* was resistant to ceftiofur and enrofloxacin. *Gardnerella vaginalis* was highly resistant to gentamicin, and sulfamethoxazole/trimethoprim.
Italy [51]	*Ent. casseliflavus* isolates from a mare with endometritis were resistant or intermediate to 18 of the 23 tested antibiotics, including amikacin, kanamycin, neomycin, streptomycin, imipenem, meropenem, ceftiofur, ceftriaxone, ciprofloxacin, enrofloxacin, norfloxacin, clindamycin, erythromycin, amoxicillin-clavulanic acid, ampicillin, colistin sulfate, rifampicin, and trimethoprim-sulfamethoxazole.
US [52]	Gram-positive bacteria from mares with postpartum metritis were highly resistant to ampicillin, clarithromycin, clindamycin, erythromycin, oxacillin, penicillin, rifampin, ticarcillin, and trimethoprim/sulfonamides. *Str. zooepidemicus* was highly resistant to amikacin, enrofloxacin, and orbifloxacin.
Italy [44]	Isolates from uterine swabs of mares suffering from endometritis. *E. coli* was highly resistant to ampicillin, cefquinome, cefazolin, ceftiofur, penicillin, rifampin, and thiamphenicol. *Str. zooepidemicus* was highly resistant to amikacin, cefazolin, ceftiofur, enrofloxacin, gentamicin, and marbofloxacin.
India [53]	Isolates from cervical swabs of mares presented at the clinic. *E. coli* was sensitive to ofloxacin, azithromycin, gentamicin and amikacin, and resistance to tetracycline, cefotaxime, amoxicillin+clavulanate, and amikacin. β-hemolytic *streptococci* had a high sensitivity to cefotaxime, amoxicillin+clavulanate and azithromycin, and high resistance against tetracycline, amikacin, and gentamicin. Both species, plus *Staphylococcus* spp., showed resistance to tetracycline.

5. Bacteria in Semen

Bacteria from the skin and from the animal´s environment colonize the mucosa of the distal reproductive tract and are transferred to the semen during ejaculation [54]. The majority of these bacteria are non-pathogenic but may be facultative pathogens or pathogens, as previously described (Section 3). Therefore, semen collection should be conducted with strict attention to hygiene. However, washing the penis prior to semen collection to remove superficial dirt and debris is controversial, since it may remove the normal skin flora, predisposing it to overgrowth by other bacteria [55]. In any case, this procedure is unlikely to remove bacteria from the mucosa. However, some authors have reported fewer bacteria in semen following penile washing [56]. Differences between studies regarding bacterial contamination may reflect the environment in which the stallions are kept [57], in addition to factors such as the number of intromissions into the artificial vagina required before ejaculation is completed.

As explained previously, the presence of some bacteria in semen is not a problem for the mare per se, unless large numbers or specific types of bacteria are present. However, AI is increasingly performed with cooled semen transported to other premises [58], allowing additional time for microbes to grow as the semen is being processed and cooled. Therefore, the bacterial load may be considerably greater by the time of AI than immediately after semen collection.

5.1. Effects of Bacteria on Sperm Quality

Bacteria compete with spermatozoa for nutrients in the extender, producing metabolic byproducts and toxins that have a detrimental effect on sperm quality. Bennett [59] reported a negative association between bacteria in stallion semen and both sperm quality and fertility. The bacterial load of *Klebsiella* spp. and *Pseudomonas* spp. in semen was negatively correlated with sperm membrane integrity [60], and *Klebsiella* spp. were correlated with the proportion of dead spermatozoa. Lateral sperm head displacement post-thaw was negatively correlated with the presence of non-β-haemolytic *streptococci* [60]. Other

researchers reported that the microbial flora present in stallion semen was not linked to sperm quality [57].

In other species, bacteria belonging to the family *Enterobacteriaceae* in ram semen were associated with reduced sperm quality during storage at 15 °C [61]. The presence of bacteria was associated with reduced motility in human semen samples; another microorganism, the yeast *Candida albicans*, was found in azoospermic semen [62].

5.2. Effects of Antibiotics in Semen Extenders on Bacteria

Various combinations of antibiotics (potassium penicillin G-amikacin disulfate, ticarcillin disodium-potassium clavulanate, piperacillin sodium/tazobactam sodium, or meropenem) were moderately effective against low doses of *K. pneumoniae* or *Ps. aeruginosa* inoculated into semen. However, these antimicrobials did not inhibit growth when higher numbers of the bacteria were inoculated [63]. The presence of biofilms, such as in some cases of chronic endometritis, may render antibiotics ineffective.

5.3. Effects of Antibiotics in Semen Extenders on Sperm Quality

An extensive range of antimicrobial substances is used in semen extenders for various species [64]. These latter authors summarized the antibiotics used for stallion sperm samples, including amikacin, cefquinome, ceftiofur, clavulanic acid, gentamicin, meropenem, penicillin, and ticarcillin. However, antibiotics can have a negative effect on sperm quality, as summarized in Table 2.

Table 2. Effects of antibiotics in semen extenders on sperm quality.

Reference	Antibiotics	Effect on Sperm Quality
[65]	Amikacin, gentamicin, potassium and sodium penicillin, polymixin B, streptomycin, and ticarcillin	Total motility, progressive motility and rapid motility were lower when polymixin B was added than for the other antibiotics
[66]	Gentamicin	Adverse effect on sperm motility and velocity. These authors concluded that the presence of gentamicin could affect sperm function during cooled storage
[67]	Llincomycin and spectinomycin	Sperm DNA fragmentation index was greater in the samples containing these antibiotics than in those without. Sperm motility, membrane integrity and mitochondrial membrane potential were not different between semen samples with and without lincomycin and spectinomycin
[64]	Potassium penicillin G-amikacin disulfate, ticarcillin disodium-potassium clavulanate, piperacillin sodium/tazobactam sodium, or meropenem	Slight differences were detected for sperm motility and kinematics and in chromatin integrity when various antibiotic combinations were used
[68]	Adding amikacin sulfate and potassium penicillin G to INRA-96® extender (already contains penicillin, gentamicin and amphoteracin-B)	Reported to increase the antimicrobial effect without having an adverse effect on sperm motility

6. Effects of Antibiotics in Semen Extenders on the Mare

The uterine microbiome is exposed to antibiotics each time the mare is inseminated. The liquid portion of the inseminate is expelled by backflow in the hours following insemination, exposing bacteria in the vagina and the environment to the antibiotics in the semen extenders. A study was conducted in Sweden to determine the effect of this exposure on the vaginal flora. Some changes in resistance patterns of vaginal bacteria were observed following exposure to antibiotics in a semen extender. Approximately half of the 1036 isolates identified were *E. coli*, and 13.3% were *Str. dysgalactiae*. There was an increase in resistance of *E. coli* to trimethoprim and chloramphenicol after AI, although there was no change in resistance to tigecycline or ampicillin. The resistance patterns of *Str. dysgalactiae* and *Staphylococcus (St.) simulans* isolates did not change after AI. *Str. dysgalactiae* isolates

were resistant to erythromycin, nitrofurantoin, and tetracycline, whereas some *St. simulans* isolates were resistant to penicillin, oxacillin, and fusidic acid. None of the *Enterococcus faecalis* isolates were resistant to any of the antibiotics [69].

Apart from the effect on the vaginal flora of the mare, the personnel handling the semen doses may be exposed to antibiotics, although the risk is likely to be small if normal hygienic measures are taken. However, it is important that the remains of semen extenders or unused insemination doses be disposed of properly, not by pouring down the drain [46], to avoid further contamination of the environment and exposure of environmental bacteria to antimicrobials. Proper disposal involves boiling to destroy the antibiotic activity or placing in waste for burning [70].

7. Alternatives to Addition of Conventional Antibiotics to Semen Extenders

7.1. Antimicrobial Peptides

Antimicrobial peptides are produced by the immune system of some mammals as a defense against bacteria, as reviewed by Vickram et al. [71]. They are active against a range of microorganisms, depending on structural differences [72]. A unifying feature is that they are amphipathic and have a cationic charge; these features could help to explain their selective action on negatively charged lipids in bacterial membranes [73]. Although their use has not been reported in semen extenders for stallion semen, a cationic peptide derived from semenogelin was proposed as an antimicrobial agent for addition to extenders for human semen [74]. Another peptide, GL13K, was reported to be active against a biofilm containing *Ps. aeruginosa* [75]. A cyclic hexapeptide was considered to be a potential candidate as an antimicrobial agent for boar semen, as it apparently did not affect pregnancy rates in AI when used in combination with a low dose of gentamicin, in contrast to other peptides that negatively affected sperm membrane integrity [76]. More recently, magainin derivatives and cyclic hexapeptides were investigated for inclusion in boar semen extenders [77]. However, opinion is divided as to whether these antimicrobial peptides could provide activity against all microbes without provoking resistance [71].

7.2. Nanoparticles

The use of iron oxide (Fe_3O_4) nanoparticles during boar semen processing produced a slight antibiotic effect with no adverse effects on sperm characteristics [78]. However, the effect on stallion spermatozoa has not been reported.

7.3. Physical Removal of Bacteria

Microfiltration of boar seminal plasma through a syringe prefilter was reported to reduce bacterial load when sperm doses for artificial insemination were reconstructed with this seminal plasma [79]. However, such a method might not be practical for preparing large volumes of semen, such as whole stallion ejaculates, since it involves both centrifugation and filtering. As yet, there have not been any documented reports of its use for stallion semen.

Colloid centrifugation was used to separate spermatozoa from bacteria in an ejaculate. This technique is a relatively simple procedure and prolongs the survival of stallion spermatozoa without adding antibiotics in the semen extender [67,80]; it is practical for use on the stud farm, only requiring access to a centrifuge with a swing-out rotor. In single layer centrifugation (SLC), a colloid formulation with high density is poured into a centrifugation tube, and extended semen is carefully pipetted on top. The preparation is then centrifuged at 300× *g* for 20 min before removing the supernatant and most of the colloid. The sperm pellet is harvested using a sterile pipette [81]. It was shown that semen quality is improved in various species after SLC, including stallions, and the number of bacteria in the sample is considerably reduced. The SLC was effective in removing 81% to >90% of bacteria from stallion semen, depending on bacterial load and species [80]. Similar results were found in a study by Al-Kass et al. [67], although interestingly, they found that 25% of the bacteria remained in the samples after SLC if antibiotics were present in

the extender, whereas only 18% of the bacteria remained after SLC where no antibiotics were included. Further studies are underway using a low-density colloid to separate boar spermatozoa from seminal plasma with its bacterial load, without selecting for robust spermatozoa [82]; this method has not been tested with stallion semen yet.

8. Conclusions

Bacteria are present in the uterus of the mare; AMR in the flora of the mare´s reproductive tract is reported in many countries and involves resistance against several antimicrobial agents. It is not known whether AMR arises primarily from local antibiotic treatment or whether systemic administration also plays a significant role. Since certain antibiotics can appear in the uterus after systemic administration, it is likely that this route of administration does play a part in the development of AMR in the equine reproductive tract. However, a major route of exposure of the bacteria in this location is via antibiotics in semen extenders used in insemination doses. Thus, it is clear that the recommendations concerning prudent use of antimicrobials are also appropriate for any intra-uterine application of antibiotics, including AI. Since AMR can be spread by low-level usage of antibiotics, as well as by therapeutic administration, it would be advisable to avoid use of antibiotics whenever possible. The addition of antibiotics to semen extenders is a situation where alternative (physical) methods are available to remove bacterial contamination, thus obviating the need for antibiotics in this context. Colloid centrifugation is a practical method for reducing the bacterial load in semen samples and can be carried out effectively using equipment that is already present on many studs. The addition of antibiotics to semen extenders may not be the only way to impede bacterial growth, but further studies, including large-scale AI trials, are needed to ensure that fertility is not compromised by any remaining bacteria in insemination doses. Antimicrobial peptides or iron oxide nanoparticles could also offer useful alternatives to the addition of antibiotics, but testing of these compounds with stallion semen is necessary before their use in this species can be recommended.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/ani11113035/s1, Supplementary file: Literature searches.

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Vaginal Bacteria in Mares and the Occurrence of Antimicrobial Resistance

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Abstract: Antibiotics are added to semen extenders in insemination doses but their effect on the vaginal microbiota of the inseminated female is unknown. The objectives of this study were to define the equine vaginal microbiota and its antimicrobial resistance, and to determine whether it changes after exposure to antibiotics in semen extenders. Vaginal swabs were taken prior to sham-insemination (day 0), and again on days 3, 7, and 14 after insemination. Isolated bacteria were identified by MALDI-TOF and tested for antimicrobial susceptibility by microdilution. The bacteria isolated from the vagina differed according to reproductive status (brood mare or maiden mare), location (north or middle of Sweden), and the stage of the estrous cycle. Five bacterial species were frequently isolated from mares in both locations: *Escherichia coli*, *Staphylococcus capitis*, *Streptococcus equisimilis*, *Streptococcus thoraltensis*, and *Streptococcus zooepidemicus*. Overall, vaginal bacteria isolated from inseminated mares showed higher antibiotic resistance than from non-inseminated mares, suggesting a possible link between exposure to antibiotics in the semen extender and the appearance of antimicrobial resistance. The whole-genome sequencing of *E. coli* isolates from inseminated mares revealed some genes which are known to confer antimicrobial resistance; however, some instances of resistance in these isolates were not characteristic of induced AMR.

Keywords: vaginal bacteria; mares; AMR

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1. Introduction

Misuse and overuse of antimicrobials, especially in preventing infections or for growth promotion, are accelerating antimicrobial resistance (AMR), which has been described as one of the world's biggest public health concerns [1]. The more prudent use of antibiotics has been advocated to minimize the development of resistance [2]. Thus, antibiotics should only be used for therapeutic purposes. However, this is not always the case as, for example, antimicrobials are added to insemination doses to prevent disease transmission and the deterioration of sperm quality during storage. Their use is specified by national and international requirements for the sale of semen obtained from production animals, including horses [3].

Previous studies concerning AMR in the equine reproductive tract primarily refer to animals with fertility problems and have been conducted in many countries since the 1980s, such as France, Germany, India, Italy, Slovakia, Sweden, Turkey, and the US, as reviewed by Malaluang et al. [4]. These studies cultured bacteria from uterine swabs, uterine lavage, vaginal swabs, and clitoral swabs. Resistance to antibiotics was detected in *Enterococcus* spp., *Enterobacter* spp., *Escherichia coli*, *Gardnerella vaginalis*, *Klebsiella* spp., *Micrococcus* spp., *Proteus* spp., *Pseudomonas* spp., *Staphylococcus intermedius*, *Streptococcus* spp., *Streptococcus equinus*, *Streptococcus equi* subsp. *Zooepidemicus*, and *Taylorella equigenitalis*, as reviewed

recently [4]. The mares included in these studies were examined by the veterinarian because of fertility problems, and many had already been treated with various antibiotics over prolonged periods. Few studies have been performed regarding antimicrobial resistance in the vaginal flora of healthy mares without fertility issues, and the impact of different environments or the estrous cycle on vaginal flora has not been determined. Moreover, it is unknown whether the exposure of vaginal bacteria to antibiotics in semen extenders can alter antimicrobial resistance patterns and whether such changes are transient or long-lasting. Therefore, the objectives of the current study were (i) to describe the vaginal flora of two populations of mares in Sweden, using samples from brood mares and non-breeding mares; and (ii) to conduct a pilot study to determine suitable sampling times for a larger study on the possible changes in antimicrobial resistance after exposure to antibiotics in semen extenders.

2. Materials and Methods

2.1. Non-Inseminated Mares

Study 1 was a cross-sectional observational study: Swab samples were taken from the vagina at estrous in healthy mares. The study mares (n = 31) included both brood mares (n = 21) from a stud farm in Boden, in northern Sweden, which had not been inseminated since the last breeding season, as well as mares (n = 10) that had never been inseminated or had not been inseminated within the previous ten years (Figure 1a). These mares had unknown therapeutic profiles. In total, 6 of these last 10 mares were kept at the same stud farm as the 21 brood mares; 2 of these were new brood mares. The remaining four mares were housed at nearby farms. The horses were all housed under similar conditions and were of different breeds, including Swedish Standard Trotter, North-Swedish Trotter, and Arabian Thoroughbred. Their ages ranged from 2 to 20 years old, with a mean age of 9 ± 4 years. These mares were sampled once, as close as possible to the time of ovulation.

2.2. Inseminated Mares

Study 2 was a longitudinal experimental study: Swab samples were taken from the equine vagina following insemination with a semen extender (Figure 1b). The barren mares (n = 10) included in this study were housed at the Faculty for Veterinary Medicine and Animal Science, Swedish University of Agricultural Sciences, Uppsala. The inclusion criterion was that they had never been inseminated or had not been inseminated within the previous ten years (Figure 1b). These mares had not received antibiotics in the last six months. The mares were identified to be in standing heat from their behavior towards the stallion and were judged to be in the immediate pre-ovulatory phase based on ultrasound examination. They were sham-inseminated with a semen extender (INRA-96; IMV Technologies, L'Aigle, France). This extender contained the antimicrobials penicillin and gentamicin, as well as an antifungal, amphotericin B. Swabs of the vagina were taken before the semen extender was deposited in the uterus (day 0; D0), followed by sampling three (D3), seven (D7), and fourteen days later (D14). The D3 group included five mares which were swabbed on day five (D5) instead of D3. Samples taken at D0 are also included in the results from non-inseminated mares.

Ethical approval for swabbing was available prior to the study (number 5.8.18-15533/2018).

2.3. Sampling Technique

With the mares standing in examination stocks, the tail was wrapped before cleaning the perineal area and vulva with soap and lukewarm water. The vulva was dried with bleached paper and the procedure was repeated until the area was visibly clean. Sample contamination was minimized using a rectal glove, liquid paraffin, and a double-guarded occluded swab. The sampling site was approximately three centimeters distal to the vaginal fornix in the cranial vagina.

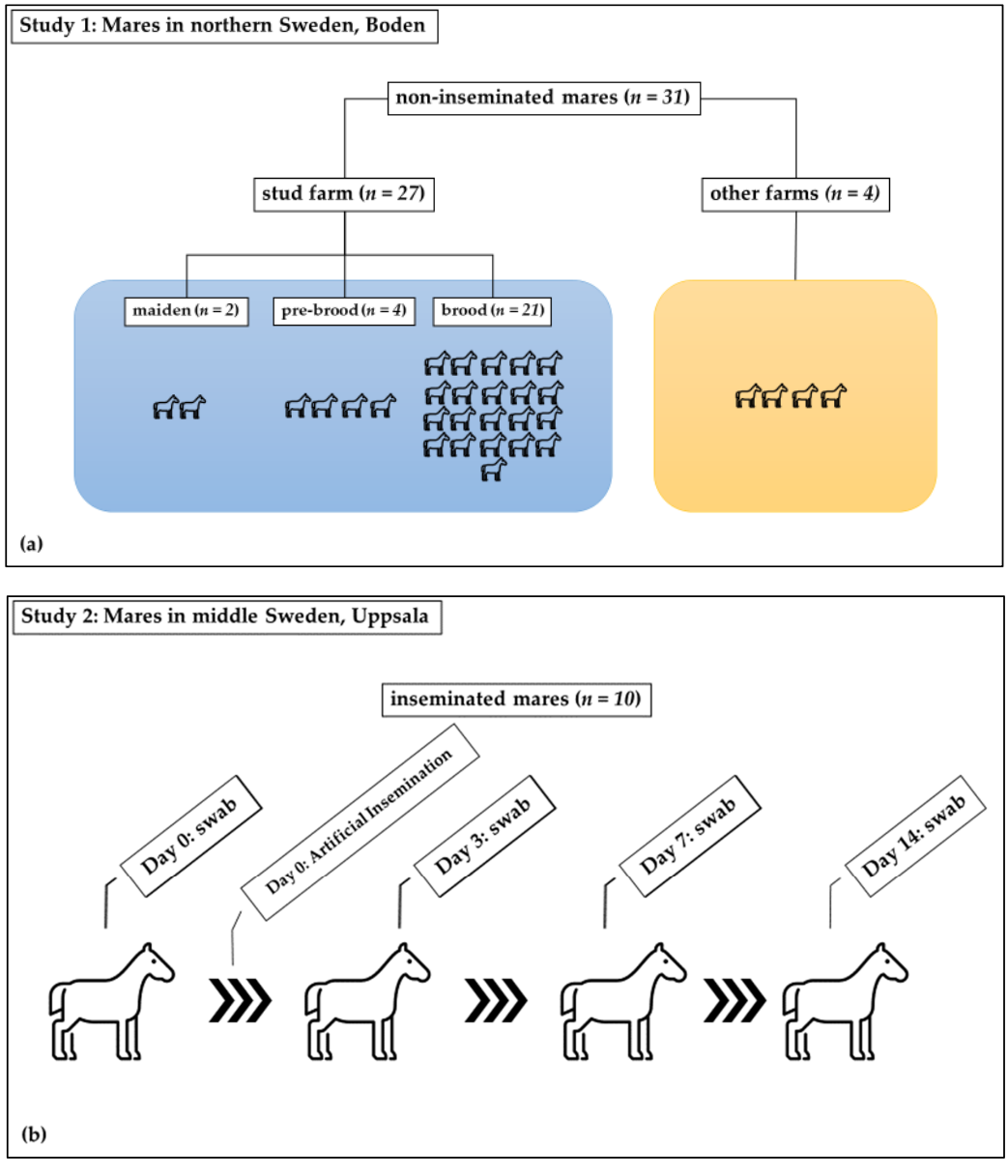

Figure 1. Distribution of mares in study groups: (**a**) Study 1: Non-inseminated mares were sampled once; (**b**) Study 2: Sham-inseminated mares were sampled on Day 0 (before insemination) and on days 3, 7, and 14 after insemination.

The swabs were transferred directly after sampling into Amie's agar gel with charcoal (Copan Diagnostics, Inc., Murrieta, CA, USA). The swabs from inseminated mares were

immediately transferred to the laboratory at SLU, where the analysis was initiated the same day. The samples from non-inseminated mares were stored at refrigerator temperature to prevent bacterial overgrowth until they were sent to the laboratory on Monday-Wednesday. The bacteriological analysis was initiated on the day of arrival at the lab.

2.4. Bacteriological Analysis

The swab samples were plated directly onto two blood agars (either bovine or horse) agars, lactose purple agar, MacConkey agar, Baird Parker agar, and De Man, Rogosa, and Sharpe (MRS) agar. One blood agar plate was incubated anaerobically at 37.0 ± 1 °C for 24 + 24 h, the other plates, except MRS-agar, were incubated aerobically at 37± 1 °C. Bacterial growth was recorded after 24 and 48 h incubation for all plates except the MRS-agar plates that were incubated anaerobically at 25 °C for five days before examination. Bacterial colonies with different macromorphology on the initial agar plates were noted and re-cultured on two blood agar plates (for aerobic and anaerobic culture conditions) and incubated for 24 to 48 h in a 37 °C incubator to obtain a pure culture. The isolates were identified at the species level by Matrix-Assisted Laser Desorption Ionization Time of Flight Mass Spectrometry (MALDI-TOF MS). The mass spectra of the bacterial isolates were compared automatically with those of known bacterial strains in the database (Bruker Daltonics, Billerica, MA, USA). All isolates were preserved in cryotubes with brain heart infusion (BHI) broth with 15% glycerol at −70 °C for subsequent antimicrobial susceptibility testing.

2.5. Antimicrobial Susceptibility Testing

Study 1: testing for antimicrobial resistance was performed on up to 22 selected isolates (mean 9) identified from all samples from the non-inseminated mares. In study 2, the testing was performed on 5 to 20 selected bacterial species (mean 10) identified from all sampling times (D0, D3, D7, and D14) from the same mare to compare antimicrobial resistance before and after sham-insemination. Susceptibility to selected antibiotic substances was assessed with *Thermo Scientific*™ *Sensititre* ™ *STAFSTR* (for *Streptococcus* spp. and *Staphylococcus* spp.), *Thermo Scientific*™ *Sensititre*™ *EUVENC* (for *Escherichia coli*), and *Thermo Scientific*™ *Sensititre*™ *EUVENSEC* (for *Enterococcus faecalis*) (Thermo Fisher Scientific, Waltham, MA, USA). Antibiotic minimum inhibitory concentration (MIC) was determined for *Escherichia coli*, *Enterococcus faecalis*, *Staphylococcus* spp., and *Streptococcus* spp. using broth microdilution following the standards of the Clinical and Laboratory Standards Institute [5]. Epidemiological cut-off (ECOFF) values for determining susceptibility were obtained from the European Committee on Antimicrobial Susceptibility Testing (EUCAST), (Breakpoint tables for interpretation of MICs, Version 11.0, 2021. http://www.eucast.org, accessed on 7 November 2022). The ECOFF values classify isolates with acquired reduced susceptibility as 'non-wild type'. In this paper, non-wild type isolates are called 'resistant', in agreement with the Swedish Veterinary Antibiotic Resistance Monitoring report [6]. Multidrug resistance was defined as resistance to three or more antibiotic classes. For example, resistance to ciprofloxacin and nalidixic acid was considered resistance to one antibiotic class (quinolones).

2.6. Whole-Genome Sequencing

Since we could not be certain that isolates from any mare showing phenotypic resistance at any time during testing were of the same strain that showed phenotypic susceptibility at any other time, whole-genome sequencing (WGS) was performed. Only *E. coli* was subjected to WGS as the number of isolates of the other bacterial species was limited. All *E. coli* isolates from the individual mares with at least one isolate phenotypically resistant to any antibiotics at any time point were selected for WGS. The WGS was performed on 21 *E. coli* isolates in total, showing increased or decreased resistance to antibiotics according to the antimicrobial susceptibility testing. "Increased resistance" means that the proportion of bacteria from an individual mare showing resistance was higher later in the sampling

sequence, whereas "decreased resistance "means that the proportion of bacteria showing resistance was lower later in the sampling sequence.

All isolates were re-cultured twice from single colonies on horse blood agar plates for 24 h at 37 °C in an aerobic atmosphere to ensure a pure culture, prior to DNA extraction using the EZ1 DNA Tissue Kit (Qiagen, Hilden, Germany) and the bacterial protocol on the EZ1 Advanced XL instrument (Qiagen) according to the manufacturer's instructions. The DNA was eluted in a total volume of 100 µL, and the concentration was measured using the Qubit ds DNA High Sensitivity Assay Kit (Invitrogen, Carlsbad, CA, USA) on the Qubit® 2.0 Fluorometer (Invitrogen). According to the manufacturer's instructions, sequencing libraries were prepared using the Nextera XT DNA Library Preparation Kit (Illumina Inc., San Diego, CA, USA). The quality of the libraries was checked using the High Sensitivity DNA ScreenTape Analysis D1000 (Agilent Technologies, Inc., Santa Clara, CA, USA) on the 4150 TapeStation System (Agilent Technologies, Inc.). Quantification was done by the Qubit ds DNA High Sensitivity Assay Kit (Invitrogen) on the Qubit® 2.0 Fluorometer (Invitrogen). The whole-genome sequencing was performed using the NextSeq 500/550 Mid Output kit V2.5 with 2 × 150-bp paired-end reads (Illumina Inc.) on a NextSeq 500 system (Illumina Inc.) at SLU.

Obtained sequences were analyzed using the Ridom SeqSphere + v7.0.5 software (Ridom GmbH, Münster, Germany). Genome assembly was performed de novo using SKESA (Souvorov et al., 2018) through a pipeline script in Ridom SeqSphere+ (Ridom GmbH). Core genome MLST (cgMLST) analysis was done using the E. *coli* cgMLST task template v1.0 in Ridom SeqSphere+ (Ridom, GmbH) containing 2513 loci. A minimum spanning tree (MST) based on cgMLST data was generated in Ridom SeqSphere+ (Ridom, GmbH) to investigate the relationship between isolates. Default parameters were used for generating the MST and missing alleles were ignored in the pairwise comparisons. A cluster distance threshold with a maximum of 10 cgMLST target differences was used to indicate the relationship, which was the default in the software. AMRFinderPlus [7] and ResFinder 4.1 [8–10] were used to detect the genes and point mutations associated with antimicrobial resistance. AMRFinderPlus was run via Ridom SeqSphere+ (Ridom, GmbH).

2.7. Statistical Analysis

Differences in the proportions of resistant bacteria between non-inseminated and inseminated mares were analyzed using the Chi-squared test, or Fisher's Exact test if there were few observations for individual cells. A *p*-value of 0.05 or less was considered statistically significant.

3. Results

3.1. Bacterial Isolation

In study 1 (31 non-inseminated mares), 485 isolates of 28 different species of bacteria were detected. In total, 1 to 25 isolates (mean 15) were identified from the 31 non-inseminated mares.

In study 2 (10 sham-inseminated mares), 65 isolates were identified from 19 bacteria species. In total, 1 to 14 isolates (mean 4) were identified from mares on D0, 4 to 11 isolates (mean 7) were identified from mares on D3, 3 to 10 isolates (mean 5) were identified from mares on D7, and 2 to 9 isolates (mean 5) were identified from mares on D14.

A total of 41 different species of bacteria were detected. Five bacterial species were isolated from mares at both sites in Sweden, *Escherichia coli*, *Staphylococcus capitis*, *Streptococcus equisimilis*, *Streptococcus thoraltensis*, and *Streptococcus zooepidemicus*, with *E. coli* being dominant (40%). Barren and brood mares had different bacterial species; only two bacteria species, *Escherichia coli* and *Streptococcus zooepidemicus*, were common to all mares regardless of their reproductive status (Figure 2).

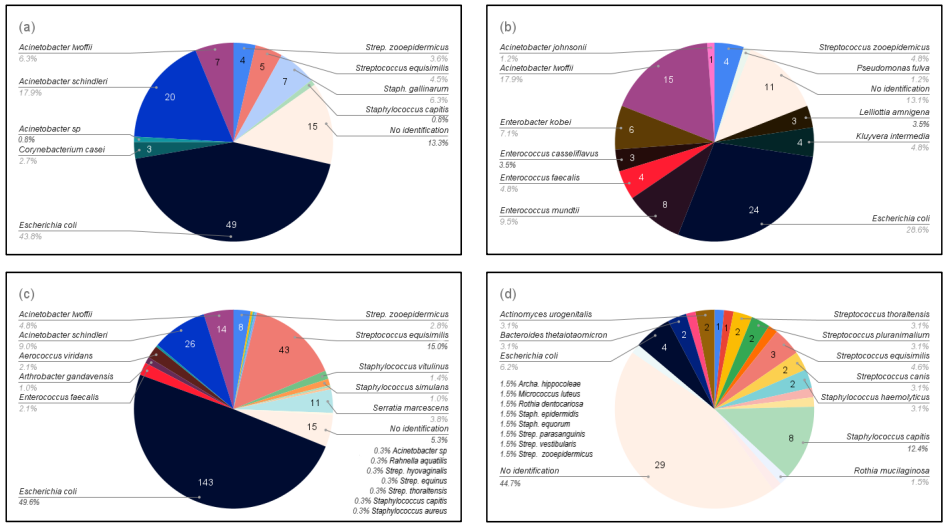

Figure 2. Bacteria isolated from the four groups of mares according to reproductive status: (**a**) Maiden mares in the stud farm in Boden (study 1); (**b**) Maiden mares in other farms in Boden (study 1); (**c**) Broodmares in the stud farm in Boden (study 1); and (**d**) Barren mares in Uppsala, including samples both before and after sham-insemination with semen extenders (study 2).

A distinct bacterial flora was found in specific sub-groups of mares (Table 1), with a greater diversity of bacteria being found in the mares in Boden. In addition, more species of Gram-negative bacteria were isolated from the mares in Boden than from the mares in Uppsala.

Table 1. Species of bacteria isolated from the vaginal flora of mares in two locations in Sweden, Boden and Uppsala.

Mares in Northern Sweden, Boden	
Gram-positive (n = 14)	*Corynebacterium casei*, *Enterococcus casseliflavus*, *Enterococcus faecalis*, *Enterococcus mundtii*, *Staphylococcus aureus*, *Staphylococcus capitis*, *Staphylococcus gallinarum*, *Staphylococcus simulans*, *Staphylococcus vitulinus*, *Streptococcus equinus*, *Streptococcus equisimilis*, *Streptococcus hyovaginalis*, *Streptococcus thoraltensis*, and *Streptococcus zooepidemicus*
Gram-negative (n = 13)	*Acinetobacter johnsonii*, *Acinetobacter lwoffii*, *Acinetobacter schindleri*, *Acinetobacter sp.*, *Aerococcus viridans*, *Arthrobacter gandavensis*, *Enterobacter kobei*, *Escherichia coli*, *Kluyvera intermedia*, *Lelliottia amnigena*, *Pseudomonas fulva*, *Rahnella aquatilis*, and *Serratia marcescens*
Mares in middle Sweden, Uppsala	
Gram-positive (n = 16)	*Actinomyces urogenitalis*, *Archanobacterium hippocoleae*, *Micrococcus luteus*, *Rothia dentocariosa*, *Rothia mucilaginosa*, *Staphylococcus capitis*, *Staphylococcus epidermidis*, *Staphylococcus equorum*, *Staphylococcus haemolyticus*, *Streptococcus canis*, *Streptococcus equisimilis*, *Streptococcus parasanguinis*, *Streptococcus pluranimalium*, *Streptococcus thoraltensis*, *Streptococcus vestibularis*, and *Streptococcus zooepidemicus*
Gram-negative (n = 2)	*Bacteroides thetaiotaomicron* and *Escherichia coli*

The numbers of isolates observed on D0, D3, D7, and D14 were 66, 89, 59, and 63, respectively, with a higher number of isolates being observed on D3 than at the other time point ($p < 0.0001$).

3.2. Antimicrobial Susceptibility

Resistance to at least one of the four antibiotics, chloramphenicol, colistin, sulfamethoxazole, and trimethoprim, was found for 24 *E. coli* isolates. There were significant differences between inseminated and non-inseminated mares, with *E. coli* from inseminated mares showing a higher resistance to trimethoprim ($p = 0.00003$) and chloramphenicol ($p = 0.0309$). In contrast, *E. coli* isolates from non-inseminated mares showed a higher resistance to sulfamethoxazole ($p = 0.0273$). However, the resistance of E. coli isolates to colistin was not significantly different between inseminated and non-inseminated mares. (Table 2).

Resistance to one to five of the eight tested antibiotics was found in 61 *Streptococcus* spp. isolates, of which, 2 isolates were multidrug resistant (resistant to at least three classes of antibiotics). In this case, no ECOFFs were given in the EUCAST for cefoxitin, enrofloxacin, fusidic acid, and gentamicin. There was a difference in resistance between inseminated and non-inseminated mares, with resistance to erythromycin being higher in non-inseminated mares ($p = 0.013$), whereas some *Streptococcus* spp. isolates were resistant to clindamycin, penicillin, tetracycline, and trimethoprim/sulfamethoxazole. However, no difference was found between inseminated and non-inseminated mares for resistance to clindamycin, penicillin, tetracycline, and trimethoprim/sulfamethoxazole (Table 3).

All *Staphylococcus* spp. isolates were susceptible to fusidic acid, tetracycline, and trimethoprim/sulfamethoxazole. There was no EUCAST cut-off MIC value for cefalotin, cefoxitin, clindamycin, enrofloxacin, and nitrofurantoin, meaning that an assessment of resistance could not be performed. Eight of twenty *Staphylococcus* spp. isolates were resistant to one to four of the seven tested antibiotics, including erythromycin, gentamicin, oxacillin, and penicillin; two *Staphylococcus* isolates from the inseminated mares were classified as multi-resistant (Table 4).

All *Enterococcus faecalis* isolates were susceptible to all six antibiotics tested, including ampicillin, ciprofloxacin, gentamicin, linezolid, teicoplanin, and tigecycline. There was no EUCAST cut-off MIC value for chloramphenicol, daptomycin, erythromycin, tetracycline, vancomycin, and quinupristin/dalfopristin, which means that an assessment of resistance could not be performed (Table 5).

The proportion of antimicrobial-resistant isolates of bacteria from inseminated and non-inseminated mares is shown in Table 6. In both groups of mares, most isolates were susceptible, or only a few isolates (below 50%) showed resistance, to the antibiotics tested. However, *S. equisimilis* and *S. zooepidemicus* from inseminated mares were often resistant to tetracycline, and 10 percent of *Staphylococcus* spp. from inseminated mares were resistant to oxacillin. In non-inseminated mares, resistance to sulfamethoxazole was often found among *E. coli* isolates and to erythromycin in *S. zooepidemicus*.

In inseminated mares, some bacterial species were shown to be resistant following exposure to antibiotics in the semen extender (Table 7). Thus, *E. coli* isolates were observed.

Table 2. Distribution of MICs (mg/L) and resistance of the 79 isolates of *Escherichia coli* from the cranial vagina of seven inseminated (I) and five non-inseminated (NI) mares. The results are shown as the percentage of isolates at different MIC values.

		Res	<0.015	0.03	0.06	0.12	0.25	0.5	1	2	4	8	16	32	64	128	256	512	>1024
Ampicillin	I (n = 34)	0								8.8	76.5	14.7							
	NI (n = 45)	0								15.6	71.1	13.3							
Azithromycin	I (n = 34)	0								2.9	44.1	53.0							
	NI (n = 45)	0								4.4	13.3	64.5	17.8						
Cefotaxime	I (n = 34)	0					100												
	NI (n = 45)	0					100												
Ceftazidime	I (n = 34)	0						100											
	NI (n = 45)	0						100											
Ciprofloxacin	I (n = 34)	0	85.3	14.7															
	NI (n = 45)	0	93.3	6.7															
Chloramphenicol *	I (n = 34)	11.8										88.2	11.8						
	NI (n = 45)	0										100							
Colistin	I (n = 34)	2.9							97.1		2.9								
	NI (n = 45)	0							100										
Gentamicin	I (n = 34)	0						58.8	41.2										
	NI (n = 45)	0						91.1	8.9										

Table 2. *Cont.*

		Res	<0.015	0.03	0.06	0.12	0.25	0.5	1	2	4	8	16	32	64	128	256	512	>1024
Meropenem	I (n = 34)	0		100															
	NI (n = 45)	0		100															
Nalidixic acid	I (n = 34)	0									100								
	NI (n = 45)	0									97.8	2.2							
Sulfamethoxazole *	I (n = 34)	32.4										2.9	14.7	47.1	2.9				32.4
	NI (n = 45)	60.0											2.2			4.4	17.8	15.6	60.0
Tetracycline	I (n = 34)	0								70.6	11.8	17.6							
	NI (n = 45)	0								84.4	15.6								
Tigecycline	I (n = 34)	0					100												
	NI (n = 45)	0					100												
Trimethoprim *	I (n = 34)	32.4					2.9	55.9	8.8				2.9	29.5					
	NI (n = 45)	0					48.9	51.1											

White fields denote the range of dilutions tested for each antibiotic and bold vertical lines indicate the cut-off values used to define resistance. Grey fields denote the range outside of the dilutions tested. MICs equal to or lower than the lowest concentration tested are given as the lowest tested concentration. * Denotes a significant difference between inseminated and non-inseminated mares.

Table 3. Distribution of MICs (mg/L) and resistance of the 100 isolates of *Streptococcus* spp. from the cranial vagina of inseminated (I) and non-inseminated (NI) mares. The results are shown as the percentage of isolates at different MIC values. *Streptococcus* spp. from inseminated mares (n = 10), *Streptococcus equi* subsp. *zooepidemicus* from inseminated mares (n = 21) and non-inseminated mares (n = 13), and *Streptococcus equisimilis* from inseminated mares (n = 8) and non-inseminated mares (n = 48).

		Res	**<0.03**	**0.06**	**0.12**	**0.25**	**0.5**	**1**	**2**	**4**	**8**	**16**	**32**	**>64**
Cefalotin	I (n = 39)	0						100.0						
	NI (n = 61)	0						100.0						
Cefoxitin	I (n = 39)	-				5.1	7.6	71.8	10.3	2.6	2.6			
	NI (n = 61)	-				1.7	18.0	80.3						
Clindamycin	I (n = 39)	12.8					87.2	5.1	7.7					
	NI (n = 61)	3.2					96.8	1.6	1.6					
Enrofloxacin	I (n = 39)	-					23.1	76.9						
	NI (n = 61)	-					19.7	80.3						
Erythromycin *	I (n = 39)	12.8					87.2		12.8					
	NI (n = 61)	1.6					98.4	1.6						
Fusidic acid	I (n = 39)	-					53.8		46.2					
	NI (n = 61)	-					3.3		96.7					
Gentamicin	I (n = 39)	-							10.3	89.7				
	NI (n = 61)	-						19.7	8.2	72.1				
Nitrofurantoin	I (n = 39)	0										76.9	23.1	
	NI (n = 61)	0										85.2	14.8	
Oxacillin	I (n = 39)	0				100.0								
	NI (n = 61)	0				100.0								
Penicillin	I (n = 39)	5.2	87.2	7.6	5.2									
	NI (n = 61)	0	100.0											
Tetracycline	I (n = 39)	56.3				10.3	2.6	7.7	23.1	56.3				
	NI (n = 61)	77.0							23.0	77.0				
Trimethoprim/	I (n = 39)	7.7				84.6	7.7			7.7				
Sulfamethoxazole	NI (n = 61)	1.6				88.6	8.2	1.6		1.6				

White fields denote the range of dilutions tested for each antibiotic and bold vertical lines indicate the cut-off values used to define resistance. Grey fields denote the range outside of the dilutions tested. MICs equal to or lower than the lowest concentration tested are given as the lowest tested concentration. - = no cut-off values available. * Denotes a significant difference between inseminated and non-inseminated mares.

Table 4. Distribution of MICs (mg/L) and resistance of the 20 isolates of *Staphylococcus* spp. from the cranial vagina of inseminated mares (I). The results are shown as the percentage of isolates at different MIC values. (8 *Staphylococcus* capitis, 2 *Staphylococcus* epidermidis, 1 *Staphylococcus* equorum, 8 *Staphylococcus* haemolyticus, and 1 *Staphylococcus* warneri).

		Res	**<0.03**	**0.06**	**0.12**	**0.25**	**0.5**	**1**	**2**	**4**	**8**	**16**	**32**	**>64**
Cefalotin	I (n = 20)	-						95.0	5.0					
Cefoxitin	I (n = 20)	-				5.0	5.0	50.0	30.0	10.0				
Clindamycin	I (n = 20)	-					95.0		5.0					
Enrofloxacin	I (n = 20)	-				95.0	5.0							
Erythromycin	I (n = 20)	10					85.0	5.0		10.0				
Fusidic acid	I (n = 20)	10					90.0		10.0					
Gentamicin	I (n = 20)	10						90.0		10.0				
Nitrofurantoin	I (n = 20)	-										100.0		
Oxacillin	I (n = 20)	10				90.0		10.0						
Penicillin	I (n = 20)	35	55.0	5.0	5.0	5.0	15.0	15.0						
Tetracycline	I (n = 20)	0				45.0	35.0	10.0	10.0					
T/S	I (n = 20)	0				70.0	25.0		5.0					

T/S = Trimethoprim/Sulfamethoxazole (only Trimethoprim concentration). White fields denote the range of dilutions tested for each antibiotic and bold vertical lines indicate the cut-off values used to define resistance. Grey fields denote the range outside of the dilutions tested. MICs equal to or lower than the lowest concentration tested are given as the lowest tested concentration. - = no cut-off values available.

Thus, E. *coli* isolates were observed to be resistance to trimethoprim (32.4%), chloramphenicol (11.8%), and colistin (2.9%). *S. zooepidemicus* isolates showed resistance to tetracycline (23.8%), clindamycin (14.3%), and erythromycin (9.5%). *S. gallolyticus* isolates had resistance to erythromycin (75.0%), clindamycin (75.0%), tetracycline (75.0%), and trimethoprim/sulfamethoxazole (75.0%). *S. capitis* isolates were observed to be resistant to gentamicin (25.0%). *S. haemolyticus* isolates showed resistance to fusidic acid (14.3%) and gentamicin (14.3%). However, some bacteria were shown to be resistant to antibiotics both before and after exposure to the semen extender. *S. equisimilis* isolates were observed to be resistant to tetracycline (50.0% and 16.7% before and after exposure to the semen extender, respectively). One of the *Staphylococcus* spp., the isolate that was resistant to oxacillin, was sampled before and another three days after insemination. *S. haemolyticus* isolates were also resistant to penicillin before and after exposure (100.0% and 28.6%, respectively). Moreover, some bacteria were shown to be resistant only before exposure to antibiotics in the semen extender, e.g., in *S. capitis* resistance to penicillin (50.0%) was found, whereas, in *S. epidermidis*, resistance to penicillin (100.0%), fusidic acid (100.0%) and erythromycin (100.0%) was observed. It was not possible to detect changes in antimicrobial resistance because no identical bacteria were found in individual mares at all time points. Furthermore, it was not possible to determine visually if the same isolates were being tested. Therefore, the *E. coli* isolates were selected to be subjected to WSG.

Table 5. Distribution of MICs (mg/L) and resistance of the four isolates of *Enterococcus faecalis* from the cranial vagina of non-inseminated mares (NI). The results are shown as the percentage of isolates at different MIC values.

		Res	<0.03	0.06	0.12	0.25	0.5	1	2	4	8	16	32	64	128	256	512	1024
Ampicillin	NI (n = 4)	0						100.0										
Chloramphenicol	NI (n = 4)	-									100.0							
Ciprofloxacin	NI (n = 4)	0						25.0	75.0									
Daptomycin	NI (n = 4)	-						75.0	25.0									
Erythromycin	NI (n = 4)	-						100.0										
Gentamicin	NI (n = 4)	0									100.0							
Linezolid	NI (n = 4)	0							50.0	50.0								
Q/D	NI (n = 4)	-										100.0						
Teicoplanin	NI (n = 4)	0					100.0											
Tetracycline	NI (n = 4)	-						75.0	25.0									
Tigecycline	NI (n = 4)	0		25.0	50.0	25.0												
Vancomycin	NI (n = 4)	-							100.0									

Q/D = Quinupristin/Dalfopristin. White fields denote the range of dilutions tested for each antibiotic and bold vertical lines indicate the cut-off values used to define resistance. Grey fields denote the range outside of the dilutions tested. MICs equal to or lower than the lowest concentration tested are given as the lowest tested concentration. - = no cut-off values available.

Table 6. Percentage of antimicrobial-resistant isolates of *Escherichia coli*, *Streptococcus* spp., *Streptococcus equi* subsp. *zooepidemicus*, *Streptococcus equisimilis*, *Staphylococcus* spp., and *Enterococcus faecalis* from the vaginas of healthy mares.

Antimicrobial	*E. coli*		*Streptococcus* spp.	*Streptococcus zooepidemicus*		*Streptococcus equisimilis*		*Staphylococcus* spp.	*E. faecalis*
	I (n = 34)	NI (n = 45)	I (n = 10)	I (n = 21)	NI (n = 8)	I (n = 8)	NI (n = 48)	I (n = 20)	NI (n = 4)
Trimethoprim	32	0	NA	NA	NA	NA	NA	NA	NA
Tetracycline	NA	NA	40	76	25	63	88	NA	NA
Chloramphenicol	12	0	NA	NA	NA	NA	NA	NA	NA
Colistin	3	0	NA	NA	NA	NA	NA	NA	NA
Gentamicin	0	0	NA	NA	NA	NA	NA	10	0
Sulfamethoxazole	32	60	NA	NA	NA	NA	NA	NA	NA
Penicillin	NA	NA	10	0	0	0	0	35	NA
Oxacillin	0	0	0	0	0	0	0	10	NA
Fusidic acid	NA	NA	NA	NA	NA	NA	NA	10	NA
Erythromycin	NA	NA	40	10	100	0	2	5	NA
Clindamycin	NA	NA	30	14	25	0	0	NA	NA
T/S	NA	NA	30	0	13	0	0	0	NA

T/S = Trimethoprim/Sulfamethoxazole, I = inseminated mare, NI = non-inseminated mare, NA = not applicable since the isolate was not tested for the antimicrobial substance or there were no ECOFFs given.

Table 7. Antimicrobial resistance of bacteria isolates from the vagina of 10 mares before and after insemination.

Bacteria	Day 0		Day 3		Day 7		Day 14	
	Isolate ID	Resistance	Isolate ID	Resistance	Isolate ID	Resistance	Isolate ID	Resistance
E. coli			P136	Tri and Chl	P177, P178, P179, P180, P181, P192, and PM12	Tri	P229	Tri and Chl
			P159 and P160	Chl	P85	Col	P225	Tri
			P133	Tri				
S. equisimilis	P5	Tet	P57	Tet				
S. zooepidemicus			P158	Tet			P148	Ery, Cli, and Tet
							P126, P151, and PM5	Tet
Streptococcus spp.	P11	Ery and Tet	P109	Ery, Cli, Tet, and T/S			P217 and PM6	Ery, Cli, Tet, and T/S
Staphylococcus spp.	P21	Pen, Oxa, Fus, and Ery	P198	Fus and Gen	P207	Pen	P245	Pen
	P2, P143, and P146	Pen	P156	Gen				
	P10	Ery	P76	Pen and Oxa				

Tri = trimethoprim, Chl = chloramphenicol, Col = colistin, Tet = tetracycline, Pen = penicillin, Ery = erythromycin, Cli = clindamycin, T/S = trimetoprim/sulfamethoxazol, Oxa = oxacillin, Fus = fusidic acid, and Gen = gentamicin.

3.3. Whole-Genome Sequencing

The purpose of the WGS was to determine whether *E. coli* isolates of different antimicrobial susceptibility sampled at different time points were likely to be of the same strain or not. Bacterial species other than *E. coli* were not subjected to WGS due to the limitation in the number of isolates detected at any time point.

Twenty-one *E. coli* isolates from inseminated mares at four time points, before (D0) and after (D3/D5, D7, and D14) insemination, could be differentiated into three clusters by

cgMLST analysis (Figure 3). There was, at most, one allele's difference between isolates within each cluster, and therefore isolates within each cluster were considered to be highly genetically related, i.e., from the same strain. Clusters 1 and 3 consisted of isolates from one mare each, whereas cluster 2 consisted of isolates from two different mares. The finding of genetically related isolates in two different mares indicates a possible spread within the herd.

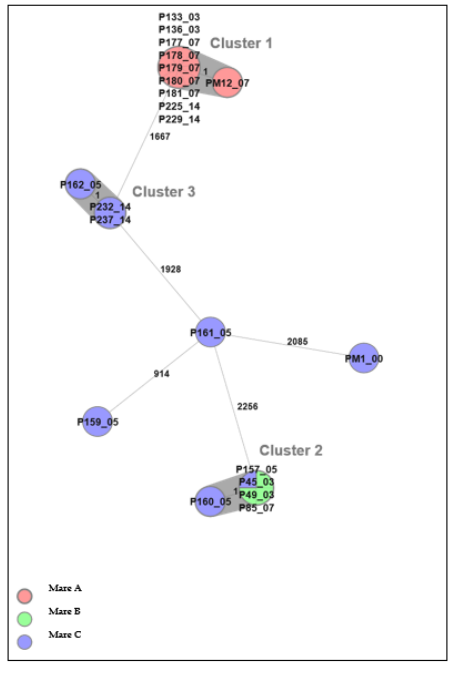

Figure 3. A minimum spanning tree (MST) was generated for the 21 *E. coli* isolates from the three mares in Uppsala, based on core genome multi-locus sequence typing (cgMLST) data. The text labels in circles denote the isolate ID followed by sampling day. Different colors denote individual mares. The grey background indicates identified clusters (genetically related isolates). Numbers next to the lines represent allelic differences, but the line length between isolates is not proportional to the numbers.

Cluster 1 comprised ten isolates from mare A, of which all were phenotypically resistant to trimethoprim. In addition, two of the isolates were phenotypically resistant to chloramphenicol (P136_03 and P229_14); five belonged to cluster 2, of which three were phenotypically sensitive to all tested antibiotics (P45_03, P49_03, and P157_05), and two (P85_07 and P160_05) were phenotypically resistant to colistin and chloramphenicol, respectively. The colistin-resistant *E. coli* was isolated from mare B and the chloramphenicol-resistant isolate from mare C. The three isolates in cluster 3, P162_05, P232_14, and P237_14 (from mare C), were all phenotypically sensitive to all tested antibiotics.

3.4. AMR Genes

All *E. coli* isolates phenotypically resistant to trimethoprim had gene *dfrA14* which is responsible for trimethoprim resistance. Similarly, four chloramphenicol-resistant isolates had the gene *mdf(A)* which is responsible for chloramphenicol resistance. However, one

isolate that was phenotypically resistant to colistin had none of the known colistin-resistance genes.

Ten *E. coli* isolates without phenotypic resistance had the sulfonamide resistance gene *sul2*, twenty-one isolates had the beta-lactam resistance gene *blaEC*, and ten isolates had *aph(6)-Id* gene, a streptomycin-resistance gene. In addition, the gene *mdf(A)* conferring resistance to broad-spectrum drugs via an efflux pump, was found in 19 isolates.

4. Discussion

4.1. Bacteria Isolation

The aim of this study was to describe the vaginal bacterial flora of mares in Sweden and to determine if antimicrobial resistance was present. A further aim was to determine suitable sampling times for a larger study in which the effects of transient exposure to antimicrobial substances in semen extenders on AMR would be investigated. A total of 530 bacteria were isolated from the vaginal swabs, with *E. coli* being isolated the most frequently (40.0%). This result is in agreement with earlier studies on vaginal bacteria in mares, where *E. coli* were the most commonly isolated bacteria in Korea (19.8%; [11]) and India (21.7%; [12]). There are, however, few previous studies on vaginal bacteria in mares; most studies focused on uterine bacteria [4]. The frequent occurrence of *E. coli* is not surprising since it is one of the most common intestinal microbes and one of the species with the shortest generation time.

Differences in bacterial species isolated among studies might be due to different environments and housing conditions for the horses and differences in culture conditions, such as types of agar plates, incubation atmosphere, and bacterial identification methods. In this study, the location of the mares tended to influence the vaginal flora, with a distinct bacterial flora being found in specific groups of mares. Of the 41 different bacterial species, only 5 were isolated from mares in both sites in Sweden: *E. coli*, *S. capitis*, *S. equisimilis*, *S. thoraltensis*, and S. *zooepidemicus*. The flora differed according to the location of the mare i.e., differed among stud farms.

4.2. Antimicrobial Susceptibility

Bacteria isolated from the equine vagina in this study were susceptible to most of the tested antibiotics with a range between 0% and 100%. This result is in contrast to most other published reports of bacteria from the equine reproductive tract, which cover uterine bacteria of mares with fertility issues, meaning that they might have received antibiotic treatment prior to sampling. Thus, the *Enterococcus faecalis* of non-inseminated mares in our study was susceptible to all 12 tested antibiotics, in contrast to previous studies in other countries where it was reported to be resistant to some antibiotics [12–14].

Some *E. coli* isolates of inseminated mares showed resistance to trimethoprim, chloramphenicol, and colistin, whereas there was no resistance to these antibiotics in *E. coli* isolated from non-inseminated mares. Age, housing condition, and whether or not the mare has been inseminated before might affect the susceptibility results since non-inseminated mares were mostly maiden mares, possibly with less chance of being in contact with antimicrobial agents than barren mares at the University. In this study, the resistance of *E. coli* isolates to trimethoprim and chloramphenicol is similar to previous reports [13,15–17]. However, colistin-resistance of *E. coli* isolates from the vagina and uterus of mares was not reported in previous studies.

Staphylococcus spp. isolates from inseminated mares were resistant to penicillin, oxacillin, erythromycin, and gentamicin in agreement with other studies [13,18,19].

Streptococcus spp. isolates of inseminated mares showed resistance to erythromycin, tetracycline, clindamycin, and trimethoprim-sulfamethoxazole. *Streptococcus equisimilis* isolates were resistant to tetracycline in both non-inseminated and inseminated mares. However, the small number of mares might confound the interpretation of this result. Moreover, differences in location, age, and previous therapeutic treatment might be involved. *Streptococcus equi* subsp. *zooepidemicus* isolates showed resistance to tetracycline,

erythromycin, and clindamycin in both inseminated and non-inseminated mares. Trimethoprim/sulfamethoxazole resistance was observed in a non-inseminated mare. Different living conditions, e.g., grouping, age, previous treatment, personnel, or location, might contribute to the different resistance results observed. The resistance of *Streptococcus* spp. isolates were similar to previous reports [12,13,15,16,18–20].

Natural or acquired AMR arises from resistance genes. Many resistance genes occur in environmental bacteria that might potentially be transferred to pathogenic and non-pathogenic bacteria in the mare [21], or they might arise following exposure of the mares' reproductive system to antibiotics. Low-level exposure to antimicrobial agents is considered to be one of the origins of AMR [22]. Such low-level exposure could arise because of exposure to antibiotics in semen extenders.

In this study, inseminated mares were exposed to penicillin and gentamicin in the semen extender. Some antibiotic resistance was observed in non-inseminated mares, i.e., although they had not been exposed to antibiotics in semen extenders, they might have come into contact with other antibiotics through unknown therapeutic use, or they might have acquired resistance genes via gene transmission between vaginal and environmental bacteria. Thus, the variation in AMR apparently occurs among mares in groups with different backgrounds.

From our results, a higher number of isolates was found on D3 than at the other time points in the study. Therefore, this time point was chosen for a future larger study investigating changes in antibiotic resistance following exposure to antibiotics in semen extenders in future studies.

4.3. Whole-Genome Sequencing

Following antimicrobial resistance testing results, sequenced *E. coli* isolates from the inseminated mares could be differentiated into three MST clusters. Cluster 2 comprised a chloramphenicol-resistant isolate and a colistin-resistant isolate sampled at D5 and D7, respectively, in addition to three sensitive isolates (D3/D5), indicating the possible acquisition of AMR. A study revealed that low-level exposure to antibiotics could affect antimicrobial resistance [22]. In the case of chloramphenicol, the *mdf(A)* gene could explain this phenotypic resistance. However, since single isolates were selected for WGS based on microdilution results, it is not possible to be certain that the same bacterial strain was chosen on all sampling occasions. We cannot exclude that *E. coli* was present on D0; they could have been present in the mixed culture but were obscured by more dominant bacterial species.

One colistin phenotypically resistant isolate had none of the known responsible AMR genes, emphasizing that phenotypic resistance to these antibiotics may not be correlated to the presence of resistance genes. However, there is a measure of uncertainty in testing antimicrobial resistance by microdilution, as in most other analyses. In microdilution, one titer is considered within the margin of error of the analysis, and the *E. coli* isolate assessed as resistant in this study was only one titer away from being assessed as susceptible. An alternative explanation could be that the genes or the mechanism conferring resistance have not been detected yet or there may be a discrepancy between genotypic and phenotypic resistance.

5. Conclusions

Five bacterial species were isolated from all mares regardless of their reproductive status, *E. coli*, *S. capitis*, *S. equisimilis*, *S. thoraltensis*, and *S. zooepidemicus*. However, the vaginal bacterial flora differed according to the geographical location of the mares, although the most commonly isolated bacterium was *E. coli*, regardless of the location of the stud farm.

Overall, vaginal bacteria isolated from inseminated mares showed higher antibiotic resistance than non-inseminated mares, suggesting a possible link between the exposure to antibiotics in semen extender and the appearance of antimicrobial resistance. However, it

is possible that other factors, such as increasing age, might increase the chance of exposure to antibiotics or increase the chance of non-genetic resistance conditions.

Whole-genome sequencing revealed some resistance of *E. coli* isolates from inseminated mares which are not characteristic of induced AMR. An example is that resistance to colistin was detected, although no AMR genes or mechanism responsible for colistin resistance was found, leading to the possibility of non-genetic resistance. Genetic resistance and persistence might play an important role in the resistance to trimethoprim and chloramphenicol. However, the number of isolates of each bacterial species from individual mares was limited, which might obscure some resistance. Further studies with a larger number of inseminated mares might reveal different results. Sampling 3 days after insemination appeared to be the most appropriate timing for detecting changes in resistance due to exposure to antibiotics in the semen extender.

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Data Availability Statement: All data are supplied in the manuscript.

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III

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Article

Antimicrobial Resistance in Vaginal Bacteria in Inseminated Mares

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Abstract: Antimicrobials are added to semen extenders to inhibit the growth of bacteria that are transferred to the semen during collection. However, this non-therapeutic use of antimicrobials could contribute to the development of antimicrobial resistance. The objective of this study was to determine changes in the antibiotic susceptibility of vaginal microbiota after artificial insemination. Swabs were taken from the vagina of 26 mares immediately before artificial insemination and again 3 days later. Bacteria isolated from the vagina at both time points were subjected to antibiotic susceptibility testing and whole-genome sequencing. In total, 32 bacterial species were identified. There were increases in the resistance of *Escherichia coli* to trimethoprim ($p = 0.0006$), chloramphenicol and ($p = 0.012$) tetracycline ($p = 0.03$) between day 0 and day 3. However, there was no significant effect of exposure to antibiotics in semen extenders with respect to the resistance of *Staphylococcus simulans* and *Streptococcus equisimilis* ($p > 0.05$). Whole-genome sequencing indicated that most phenotypic resistance was associated with genes for resistance. These results indicate that the resistance patterns of vaginal bacteria may be affected by exposure to antibiotics; therefore, it would be prudent to minimize, or preferably, avoid using antibiotics in semen extenders.

Keywords: AMR; vaginal flora; horse breeding; semen extenders; resistance genes

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1. Introduction

Antimicrobial resistance (AMR) has become a serious global concern that is accelerated by the misuse and overuse of antibiotics, as well as by inadequate infection prevention and control [1]. Although efforts are being made to reduce non-therapeutic uses of antimicrobials, one major use of antibiotics is still in animal breeding. The reproductive biotechnology artificial insemination (AI) was developed originally to prevent disease transmission when animals met for breeding, but it also facilitated faster genetic gain than could be achieved with natural mating. It is now a global phenomenon with millions of semen doses being traded around the world each year. For this international trade in semen, most countries have strict requirements regarding the health of semen donors, and they also stipulate the antibiotics that will be included in semen extenders. These antimicrobials are aimed at preventing the spread of bacterial diseases to inseminated females and the deterioration of sperm quality during storage [2].

There are several reports (reviewed recently [3]) concerning the AMR of bacteria isolated from uterine swabs, uterine lavage, vaginal swabs and clitoral swabs from the equine reproductive tract, mostly in cases of clinical disease or fertility problems. Briefly, resistance among isolates from the reproductive tract of mares has been reported in several countries including France [4], Sweden [5], India [6,7], Italy [8–10], Germany [11], the US [12,13], Slovakia [14] and Turkey [15]. Few detailed studies have been conducted on AMR in the reproductive microflora in healthy horses, and no studies have been conducted to investigate the effect of antimicrobial substances in semen extenders on vaginal flora. The

purpose of this study, therefore, was to investigate whether the inclusion of antimicrobial substances in semen extenders affects the AMR patterns of the equine vaginal microbiota by conducting analyses of bacteria sampled before and after insemination.

2. Materials and Methods

2.1. Study Period

The study was conducted in Sweden during May–August 2020.

2.2. Mares

The mares (n = 26) included in the study were of different breeds and housed at the same stud farm in Boden, northern Sweden. Breeds included Swedish standardbred trotters, North Swedish Trotters, Arabian thoroughbred, Holstein and pony breeds. Their age varied from 5 to 20 years (average age 9 years); 22 mares had previously had foals (between 1 and 12 foals), and the remaining 4 mares were maiden mares. The inclusion criterion for the mares in this study was that they should not have previously been exposed to semen extenders via insemination during the breeding season of the year 2020.

Ethical approval for swabbing was available prior to the study (number 5.8.18-15533/2018).

2.3. Sampling Technique

Swabbing was conducted twice, once on day 0 (D0) immediately prior to insemination and the second time on day 3 (D3). Thus, each mare served as its own control. This sampling regimen was chosen as the most optimal to observe changes based on a pilot study [16]. For mares with a foal at foot, the D0 sample was taken in association with insemination, which took place at foal heat, approximately 7–10 days postpartum. In the case of a repeated insemination in a subsequent cycle, the swabs used were from D0 in the first insemination cycle and from D3 in the last insemination cycle.

The sampling was performed with the mare in an examination stock; the tail was wrapped before the mare's perineal area and vulva were cleaned to remove all visible dirt, with at least three washes of soap and clean lukewarm water. The vulva and surrounding skin were then dried with bleached paper, which was inspected to determine the cleanliness of the prepared area. If necessary, further washing was carried out to achieve full cleanliness. The mares were sampled under a high hygienic standard, and a sterile glove over a rectal glove was used, avoiding touching the labia and using sterile liquid paraffin and a double-guarded occluded swab. The samples were taken from an area of the cranial vagina, approximately three centimeters distal to the fornix vagina; the precise anatomical area had been chosen after a previous inspection of organs from the slaughterhouse. The sampling site was identified by palpating the cervix uteri with a finger and then withdrawing by approximately one finger joint length. The sterile swab was in contact with the ventral vaginal wall for about 15 s while being gently rotated to collect an adequate sample.

The sampling swabs were then transferred directly into Amie's transport medium, with charcoal (Copan Diagnostics, Inc. Murrieta, CA, USA) and stored at refrigerator temperature to prevent bacterial overgrowth. Samples were sent to the laboratory at the Swedish University of Agricultural Sciences (SLU) from Monday to Wednesday to reduce the risk of sitting at the post-terminal over the weekend.

2.4. Bacteriological Analyses

According to a previous study [16], various culture media were selected to increase the chances of bacterial growth based on bacteria detected in the vagina of other animal species. The sampling swab was cultured directly on cattle or horse blood agar plates (SVA, Uppsala, Sweden) for aerobic and anaerobic conditions; a direct culture was also performed on lactose purple agar (SVA, Uppsala, Sweden), MacConkey agar (SVA, Uppsala, Sweden) and Baird-Parker agar (Oxoid, Basingstoke, UK), which were incubated aerobically at 37 ± 1 °C. All plates incubated at 37 ± 1 °C were examined for bacterial growth after 24 and 48 h. For analyses of presumptive *Lactobacillus* spp., the swabs were cultured in De

Man, Rosa and Sharpe agars (MRS-agar) (Oxoid, Basingstoke, UK), which were incubated anaerobically at 25 ± 1 °C for 5 days. Bacterial colonies of different macromorphologies from the initial culture were re-cultured on two blood agar plates (for aerobic and anaerobic culture conditions) and incubated for 24 to 48 h at 37 ± 1 °C to obtain a pure culture. The colonies from the pure cultures were stored in cryotubes with brain heart infusion (BHI) broth (CM1135; Oxoid, Basingstoke, UK) with 15% glycerol at −70 °C for subsequent antimicrobial-resistance testing and whole-genome sequencing.

The bacteria were identified at the species level by the use of 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). Score values between 2.0 and 3.0 were considered accurate at both genus and species levels, whereas score values between 1.7 and 2.0 were considered reliable only at the genus level.

2.5. Antimicrobial Susceptibility Testing

The resistance to selected antimicrobial substances was determined for most commonly isolated bacteria present in samples both before (D0) and after insemination (D3) in the same mare. The isolates were cultured on horse blood agar directly from the cryotubes and incubated for 24 h at 37 °C. They were re-cultured once to ensure a pure culture.

The resistance panel used for *Staphylococcus* spp. and *Streptococcus* spp. was *Thermo Scientific™ Sensititre ™ STAFSTR*; for *Enterococcus faecalis*, it was Thermo Scientific™ Sensititre™ EUVENC; for *E. coli*, it was Thermo Scientific™ Sensititre™ EUVENSEC (Thermo Fisher Scientific, Waltham, Massachusetts, USA). Susceptibility to selected antimicrobial substances was assessed with VetMIC™ panel analysis systems: Camp EU, version 2013-10 (SVA, Uppsala, Sweden), determining the antimicrobial minimum inhibitory concentration (MIC) by broth microdilution following the standards of the Clinical and Laboratory Standards Institute [17].

A purity check and testing of concentration were performed on the culture of the final inoculum after placing the inoculum in the resistance panel on the horse blood agar, which was incubated at 37 °C for 24 h. Epidemiological cut-off (ECOFF) values for determining susceptibility were obtained from the European Committee on Antimicrobial Susceptibility Testing (EUCAST, www.eucast.org/mic_distributions_and_ecoffs, accessed on 20 February 2023). The ECOFF values classify isolates with acquired reduced susceptibility as "non-wild type" In this paper, non-wild-type isolates are called "resistant", in agreement with the Swedish Veterinary Antibiotic Resistance Monitoring report [18].

2.6. Whole-Genome Sequencing

Whole-genome sequencing (WGS) was performed to determine whether the bacterial isolates of different antimicrobial susceptibilities from the same mares sampled at both time points were likely to be of the same strain or not and if phenotypic resistance was associated with genes for resistance. Resistant bacteria were subjected to WGS, including *E. coli, Streptococcus equisimilis* and *Staphylococcus simulans*. All isolates from the individual mares with at least one isolate phenotypically resistant to any antibiotics at both points were selected for WGS. WGS was performed on 127 *E. coli,* 26 *Streptococcus equisimilis* and 4 *Staphylococcus simulans* isolates, which showed increased or decreased resistance to antibiotics according to antimicrobial susceptibility testing. "Increased resistance" means that the proportion of bacteria from an individual mare showing resistance was higher later in the sampling sequence, whereas "decreased resistance "means that the proportion of bacteria showing resistance was lower later in the sampling sequence.

Isolates were subcultured twice on horse blood agar plates and from a single colony prior to DNA extraction to ensure pure culture. DNA was extracted with the EZ1 DNA Tissue Kit (Qiagen, Hilden, Germany) according to the protocols for Gram-negative and Gram-positive bacteria, respectively, with the exception that 75U of mutanolysin (Sigma–Aldrich, St. Louis, MO, USA) was added, and the lysis time extended to 4 h for *S. equisimilis.* The extraction was

performed on Qiagen EZ1 Advanced XL utilizing the bacterial protocol. The elution volume used was 100 µL. The Qubit ds DNA HS kit (Invitrogen, Carlsbad, CA, USA) was used to measure DNA concentrations.

Sequencing libraries were constructed with Nextera XT DNA Library Preparation Kit (Illumina, San Diego, CA, USA), and the quality was assessed by the HS DNA ScreenTape Analysis D1000 (Agilent Technologies, Inc., Santa Clara, CA, USA) on a 4150 TapeStation (Agilent Technologies, Inc.). Libraries were quantified by Qubit ds DNA HS kit (Invitrogen). WGS of the prepared libraries was conducted on the Illumina NextSeq 500 system (Illumina Inc., San Diego, CA, USA) using the Mid Output kit V2.5 with 2 × 150-bp paired-end reads (Illumina Inc).

Generated sequence reads were analyzed with SeqSphere + v7.0.5 software (Ridom GmbH, Münster, Germany). Genome assembly was done *de novo* by SKESA [19] via scripts in SeqSphere+ (Ridom GmbH). Multilocus sequencing typing (MLST) profiles were allocated for *E. coli* and *S. dysgalactiae*, respectively, using available schemes at http://enterobase.warwick.ac.uk/ [20] and https://pubmlst.org/ [21] via the MLST task templates in SeqSphere+ (Ridom, GmbH). The core genome MLST (cgMLST) for *E. coli* was performed with a task template containing 2513 loci in SeqSphere+ (Ridom, GmbH). For *S. dysgalactiae* and *S. simulans*, cgMLST schemes were created in SeqSphere+ (Ridom, GmbH). For *S. dysgalactiae*, NZ_LR594046.1 was used as the seed genome, and the following genomes were used as penetration query genomes: NZ_AP018726.1, NZ_CP044102.1, NZ_CP066073.1, NZ_CP066069.1, NZ_CP068057.1, NZ_CP033391.1, NZ_CP068478.1, JAAACI000000000.1, JAAACJ000000000.1, JAAACK000000000.1 and JAAACO000000000.1. The developed cgMLST scheme for *S. dysgalactiae* contained 1133 targets in total. For *S. simulans*, NZ_LS483313.1 was used as the seed genome, and NZ_CP014016.2, NZ_CP023497.1, NZ_CP017428.1, NZ_CP017430.1, NZ_CP016157.1, NZ_CP015642.1, NZ_LR134264.1, NZ_LT963435.1, AGZX00000000.1, LRQJ00000000.1, and PPRU00000000.1 were used as penetration query genomes. The final cgMLST scheme for *S. simulans* consisted of 1979 targets. Minimum spanning trees (MST: s) based on cgMLST data were created in SeqSphere+ (Ridom, GmbH) to investigate genetic relationship between isolates, and missing alleles were ignored in pairwise comparisons. For *E. coli*, a cluster distance threshold of 10 cgMLST targets was used.

Assembled genomes were screened for genes and point mutations associated with antimicrobial resistance by AMRFinderPlus [22] via SeqSphere+ (Ridom, GmbH), and ResFinder 4.1 [23–25].

2.7. Statistical Analysis

The difference between antimicrobial resistance results from vaginal isolates before and after contact with semen extenders was analyzed using the chi-squared test. If the criteria for the chi-squared test were not fulfilled, a Fisher's exact test was used instead. A probability level of $p < 0.05$ was considered statistically significant.

3. Results

3.1. Bacterial Analyses

In total, 985 isolates of 32 different bacterial species were identified from the 26 mares. *E. coli* was the most common bacterial species isolated from 23 of 26 mares. The second and third most-isolated bacterial genera were *Streptococcus* spp. and *Acinetobacter* spp., which were identified in 24 of 26 mares and 17 of 26 mares, respectively (Table 1).

3.2. Antimicrobial Susceptibility

The resistance of *Escherichia coli* against trimethoprim ($p = 0.0006$), chloramphenicol ($p = 0.012$) and tetracycline ($p = 0.03$) increased from D0 to D3. However, *E. coli* resistance to other antibiotics was not different after exposure to semen extenders. Furthermore, there was no association between exposure to semen extenders and the resistance of *Staphylococcus simulans* and *Streptococcus equisimilis* ($p > 0.05$).

Resistance to at least 1 of 14 antibiotics was found in 284 of 520 *E. coli* isolates. Twenty-nine isolates were multidrug-resistant (resistant to at least three classes of antibiotics). There was a significant increase in resistance to trimethoprim, chloramphenicol and tetracycline after exposure to semen extenders with antibiotics. However, resistance was also found against sulfamethoxazole, ampicillin, azithromycin and tigecycline, but differences between time points were not significant. All *E. coli* isolates were susceptible to cefotaxime, ceftazidime, ciprofloxacin, colistin, gentamicin, meropenem and nalidixic acid (Table 2).

Table 1. Bacteria isolated from the cranial vagina before (D0) and after insemination (D3) from 26 Swedish mares. The percentage (%) is the percentage of all identified bacteria.

Bacteria	D0	D3	Total Number	(%)	No. of Mares
Gram-Negative					
Acinetobacter bohemicus	–	1	1	0.1	1
Acinetobacter kookii	–	2	2	0.2	1
Acinetobacter lwoffii	21	30	51	5.2	12
Acinetobacter schindleri	46	26	72	7.3	13
Acinetobacter spp.	2	–	2	0.2	2
Escherichia coli	179	341	520	52.8	23
Klebsiella oxytoca	–	21	21	2.1	1
Pantoea agglomerans	–	3	3	0.3	1
Rahnella aquatilis	1	1	2	0.2	2
Serratia marcescens	11	–	11	1.1	1
Gram-Positive					
Aerococcus viridans	6	–	6	0.6	1
Arthrobacter gandavensis	3	–	3	0.3	1
Corynebacterium callunae	–	5	5	0.5	2
Corynebacterium casei	3	–	3	0.3	1
Enterococcus casseliflarus	–	15	15	1.5	4
Enterococcus faecalis	6	40	46	4.7	4
Enterococcus mundtii	–	1	1	0.1	1
Lactococcus raffnolactis	–	2	2	0.2	1
Paenibacillus amylolyticus	–	2	2	0.2	2
Staphylococcus aureus	1	–	1	0.1	1
Staphylococcus capitis	4	5	9	0.9	3
Staphylococcus haemolyticus	–	2	2	0.2	2
Staphylococcus schleiferi	–	10	10	1.0	3
Staphylococcus simulans	3	1	4	0.4	1
Staphylococcus vitulinus	4	2	6	0.6	2
Streptococcus canis	–	2	2	0.2	2
Streptococcus equisimilis	48	90	138	14.0	18
Streptococcus equinus	1	3	4	0.4	2
Streptococcus gallolyticus	–	4	4	0.4	1
Streptococcus hyovaginalis	1	–	1	0.1	1
Streptococcus thoraltensis	1	–	1	0.1	1
Streptococcus zooepidemicus	8	27	35	3.6	9
Total	349	636	985	100.0	

No correlation was found between the resistance of *Streptococcus dysgalactiae* subsp. *equisimilis* before and after exposure to semen extenders with antibiotics. Various *Streptococcus dysgalactiae* subsp. *equisimilis* isolates were resistant to three of eight antibiotics, including tetracycline, erythromycin and nitrofurantoin. All *Streptococcus dysgalactiae* subsp. *equisimilis* isolates were susceptible to cefalothin, clindamycin, oxacillin, penicillin and trimethoprim/sulfamethoxazole. For some antibiotics, cefoxitin, enrofloxacin, fusidic acid and gentamicin, sensitivity or resistance could not be assessed since no ECOFFs were mentioned in EUCAST (Table 3).

All *Enterococcus faecalis* isolates were susceptible to the six tested antibiotics (ampicillin, ciprofloxacin, gentamicin, linezolid, teicoplanin and tigecycline). For some antibiotics

(chloramphenicol, daptomycin, erythromycin, quinupristin/dalfopristin, tetracycline and vancomycin), sensitivity or resistance could not be assessed since no ECOFFs were given in the EUCAST (Table 4). All tested *Streptococcus equi* subsp. *zooepidemicus* isolates were susceptible to clindamycin, erythromycin, nitrofurantoin, oxacillin, penicillin, tetracycline and trimethoprim/sulfamethoxazole. For some antibiotics, cefalotin, cefoxitin, enrofloxacin, fusidic acid and gentamicin sensitivity or resistance could not be assessed since no ECOFFs were given in the EUCAST (Table 5).

Resistance to at least one of seven antibiotics was detected in different *Staphylococcus simulans* isolates, including oxacillin (25%), penicillin (50%), fusidic acid (50%) and trimethoprim/sulfamethoxazole (50%). One isolate was multidrug resistant. There was no association between exposure to antibiotics via artificial insemination and change in resistance. All *Staphylococcus simulans* isolates were susceptible to erythromycin, gentamicin and tetracycline. For some antibiotics, cefalotin, cefoxitin, clindamycin, enrofloxacin and nitrofurantoin, sensitivity or resistance could not be assessed since no ECOFFs were given in the EUCAST (Table 6).

3.3. Whole-Genome Sequencing

Whole-Genome Sequencing was used to decide whether *E. coli*, *Streptococcus equisimilis* and *Staphylococcus simulans* isolates of different antimicrobial susceptibility from the same mares sampled at both time points were likely to be of the same strain or not and if phenotypic resistance was associated with genes for resistance.

One hundred twenty-seven *E. coli* isolates from inseminated mares before (D0) and after (D3) insemination, were allocated into fourteen clusters by cgMLST analysis (Figure 1). There was, at most, a one-allele difference between isolates within each cluster, except for Cluster 2 and 13 where there was a three-allele difference. Therefore, isolates within each cluster were highly genetically related, i.e. the same strain. Nine clusters consisted of isolates from one mare (Cluster 3, 6, 7, 8, 9, 10, 11 and 14), whereas five clusters consisted of isolates from 2 to 3 mares (Cluster 1, 2, 4 5 and 13 (Figure 1)). The finding of isolates from different mares in the same clusters suggests a possible spread within the herd.

Twenty-six *Streptococcus equisimilis* isolates from inseminated mares before (D0) and after (D3) insemination, were subjected to cgMLST analysis, and a minimum spanning tree (MST) was generated (Figure 2a). There were 18 allelic differences between 12 isolates from Horse H and 3 isolates from Horse J, but since the developed cgMLST scheme lacked a validated clustering distance threshold, it was not possible to determine whether the isolates belonged to 2 different clusters or 1 and the same (Figure 2a). Regardless, there were no allelic differences between isolates from each horse in this case; hence, they were of the same strain. Eleven isolates from Horse I and J clustered together with a maximum of two allelic differences, and even if the developed cgMLST scheme lacked a validated clustering distance threshold, it is likely that those isolates were genetically closely related; i.e. they were the same strain since the differences were very few (Figure 2a). The finding of genetically related isolates in two mares (Horse I and J) indicates a possible spread within the herd, as seen in *E. coli* isolates.

Four *Staphylococcus simulans* isolates from an inseminated mare (Horse K) before (D0) and after (D3) insemination, were subjected to cgMLST analysis, and a minimum spanning tree (MST) was generated (Figure 2b). There were no allelic differences between isolates P868 and PM194 and P869 and PM193, respectively; hence, isolates within each pair were genetically closely related, i.e. the same strain. Although no defined cluster distance threshold was available for this cgMLST scheme, the allelic distance between the two pairs was very large, and it is therefore unlikely that they are genetically closely related (Figure 2b).

Twenty-nine of the *E. coli* isolates were multiple drug resistant (MDR); twenty of these isolates belonged to Cluster 2 (Horse C and G), with one isolate each belonging to Cluster 4 (Horse B), Cluster 6 (Horse E), Cluster 7 (Horse D) and Cluster 14 (Horse B).

Table 2. Distribution of MICs (mg/L) and resistance of the 301 isolates of *Escherichia coli* from the cranial vagina of 14 mares sampled before (D0) and after (D3) insemination. The results are shown as the percentage of isolates at different MIC values.

		Res (%)	<0.12	0.25	0.5	1	2	4	8	16	32	64	>64
Ampicillin	D0 (*n* = 147)	1				4	33	59	3	1			
	D3 (*n* = 154)	4					21	72	3			4	
Azithromycin	D0 (*n* = 147)	1					13	51	33	2	1		
	D3 (*n* = 154)	0						38	58	4			
Cefotaxime	D0 (*n* = 147)	0		100									
	D3 (*n* = 154)	0		100									
Ceftazedime	D0 (*n* = 147)	0			99	1							
	D3 (*n* = 154)	0			99	1							
Ciprofloxacin	D0 (*n* = 147)	0	99	1									
	D3 (*n* = 154)	0	100										
Chloramphenicol *	D0 (*n* = 147)	6							94	5		1	
	D3 (*n* = 154)	15							85	14	1		
Colistin	D0 (*n* = 147)	0				99	1						
	D3 (*n* = 154)	0				100							
Gentamycin	D0 (*n* = 147)	0			73	26	1						
	D3 (*n* = 154	0			81	16	3						
Meropenem	D0 (*n* = 147)	0	100										
	D3 (*n* = 154)	0	100										
Nalidixic acid	D0 (*n* = 147)	0						99	1				
	D3 (*n* = 154)	0						99	1				
Sulfamethoxazole	D0 (*n* = 147)	90							1	1	3	5	90
	D3 (*n* = 154)	90								2	1	7	90
Tetracycline *	D0 (*n* = 147)	0					87	12	1				
	D3 (*n* = 154)	4					85	9	2	1	3		
Tigecycline	D0 (*n* = 147)	1		98	1			1					
	D3 (*n* = 154)	0		96	4								
Trimethoprim *	D0 (*n* = 147)	4		50	42	4					4		
	D3 (*n* = 154)	17		21	49	12	1				17		

White fields denote the range of dilutions tested for each antibiotic and vertical bold lines indicate cut-off values used to define resistance. Grey fields denote the range outside of dilutions tested. MICs equal to or lower than the lowest concentration tested are given as the lowest tested concentration. * Significant association between antibiotics exposure and resistance results ($p < 0.05$).

Table 3. Distribution of MICs (mg/L) and resistance of the 90 isolates of *Streptococcus dysgalactiae* subsp. *Equisimilis* from the cranial vagina of 10 mares sampled before (D0) and after (D3) insemination. The results are shown as the percentage of isolates at different MIC values.

		Res (%)	<0.03	0.06	0.12	0.25	0.5	1	2	4	8	16	32	64	> 64
Cefalotin	D0 (*n* = 48)	0						100							
	D3 (*n* = 42)	0						100							
Cefoxitin	D0 (*n* = 48)	–					17	83							
	D3 (*n* = 42)	–				2	10	88							
Clindamycin	D0 (*n* = 48)	0					100								
	D3 (*n* = 42)	0					100								
Enrofloxacin	D0 (*n* = 48)	–					15	85							
	D3 (*n* = 42)	–					21	79							
Erythromycin	D0 (*n* = 48)	2					98	2							
	D3 (*n* = 42)	0					100								
Fusidic acid	D0 (*n* = 48)	–							100						
	D3 (*n* = 42)	–							100						
Gentamicin	D0 (*n* = 48)	–							8	92					
	D3 (*n* = 42)	–							2	98					
Nitrofurantoin	D0 (*n* = 48)	0										81	19		
	D3 (*n* = 42)	2										84	14		2
Oxacillin	D0 (*n* = 48)	0				100									
	D3 (*n* = 42)	0				100									
Penicillin	D0 (*n* = 48)	0	100												
	D3 (*n* = 42)	0	100												
Tetracycline	D0 (*n* = 48)	87							13	87					
	D3 (*n* = 42)	95							5	95					
Trimethoprim/	D0 (*n* = 48)	0				100									
Sulfamethoxazole	D3 (*n* = 42)	0				98	2								

White fields denote the range of dilutions tested for each antibiotic, and vertical bold lines indicate cut-off values used to define resistance. Grey fields denote the range outside of dilutions tested. MICs equal to or lower than the lowest concentration tested are given as the lowest tested concentration.

Table 4. Distribution of MICs (mg/L) and resistance of the 20 isolates of *Enterococcus faecalis* from the cranial vagina of 2 mares sampled before (D0) and after (D3) insemination. The results are shown as the percentage of isolates at different MIC values.

		Res (%)	<0.03	0.06	0.12	0.25	0.5	1	2	4	8	16	32	64	128	256	512	1024
Ampicillin	D0 (*n* = 6)	0						83	17									
	D3 (*n* = 14)	0						100										
Chloramphenicol	D0 (*n* = 6)	–									100							
	D3 (*n* = 14)	–									100							
Ciprofloxacin	D0 (*n* = 6)	0							100									
	D3 (*n* = 14)	0						21	79									
Daptomycin	D0 (*n* = 6)	–					33	17		50								
	D3 (*n* = 14)	–					7	72		14	7							
Erythromycin	D0 (*n* = 6)	–						50	50									
	D3 (*n* = 14)	–						36	64									
Gentamicin	D0 (*n* = 6)	0									83	17						
	D3 (*n* = 14)	0									93	7						
Linezolid	D0 (*n* = 6)	0							100									
	D3 (*n* = 14)	0							100									
Quinupristin/	D0 (*n* = 6)	–									50	50						
Dalfopristin	D3 (*n* = 14)	–									36	57	7					
Teicoplanin	D0 (*n* = 6)	0					100											
	D3 (*n* = 14)	0					100											
Tetracycline	D0 (*n* = 6)	–						100										
	D3 (*n* = 14)	–						100										
Tigecycline	D0 (*n* = 6)	0		100														
	D3 (*n* = 14)	0		86	14													
Vancomycin	D0 (*n* = 6)	–						83	17									
	D3 (*n* = 14)	–						64	29	7								

White fields denote the range of dilutions tested for each antibiotic, and vertical bold lines indicate cut-off values used to define resistance. Grey fields denote the range outside of dilutions tested. MICs equal to or lower than the lowest concentration tested are given as the lowest tested concentration.

Table 5. Distribution of MICs (mg/L) and resistance of the eight isolates of *Streptococcus equi* subsp. *zooepidemicus* from the cranial vagina of two mares sampled before (D0) and after (D3) insemination. The results are shown as the percentage of isolates at different MIC values.

		Res (%)	<0.03	0.06	0.12	0.25	0.5	1	2	4	8	16	32	64	> 64
Cefalotin	D0 (*n* = 5)	–						100							
	D3 (*n* = 3)	–						100							
Cefoxitin	D0 (*n* = 5)	–					60	40							
	D3 (*n* = 3)	–					67	33							
Clindamycin	D0 (*n* = 5)	0					100								
	D3 (*n* = 3)	0					100								
Enrofloxacin	D0 (*n* = 5)	–					80	20							
	D3 (*n* = 3)	–					100								
Erythromycin	D0 (*n* = 5)	0					100								
	D3 (*n* = 3)	0					100								
Fusidic acid	D0 (*n* = 5)	–							100						
	D3 (*n* = 3)	–							100						
Gentamicin	D0 (*n* = 5)	–						100							
	D3 (*n* = 3)	–						100							
Nitrofurantoin	D0 (*n* = 5)	0										100			
	D3 (*n* = 3)	0										100			
Oxacillin	D0 (*n* = 5)	0				100									
	D3 (*n* = 3)	0				100									
Penicillin	D0 (*n* = 5)	0	100												
	D3 (*n* = 3)	0	100												
Tetracycline	D0 (*n* = 5)	0							100						
	D3 (*n* = 3)	0							100						
Trimethoprim/	D0 (*n* = 5)	0				100									
Sulfamethoxazole	D3 (*n* = 3)	0				100									

White fields denote the range of dilutions tested for each antibiotic, and vertical bold lines indicate cut-off values used to define resistance. Grey fields denote the range outside of dilutions tested. MICs equal to or lower than the lowest concentration tested are given as the lowest tested concentration.

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Table 6. Distribution of MICs (mg/L) and resistance of the four isolates of *Staphylococcus simulans* from the cranial vagina of one mare sampled before (D0) and after (D3) insemination. The results are shown as the percentage of isolates at different MIC values.

Table 6. Distribution of MICs (mg/L) and resistance of the four isolates of *Staphylococcus simulans* from the cranial vagina of one mare sampled before (D0) and after (D3) insemination. The results are shown as the percentage of isolates at different MIC values.

		Res (%)	<0.03	0.06	0.12	0.25	0.5	1	2	4	8	16	32	64
Cefalotin	D0 (*n* = 3)	–						100						
	D3 (*n* = 1)	–						100						
Cefoxitin	D0 (*n* = 3)	–							67	33				
	D3 (*n* = 1)	–							100					
Clindamycin	D0 (*n* = 3)	–					100							
	D3 (*n* = 1)	–					100							
Enrofloxacin	D0 (*n* = 3)	–				100								
	D3 (*n* = 1)	–				100								
Erythromycin	D0 (*n* = 3)	0					100							
	D3 (*n* = 1)	0					100							
Fusidic acid	D0 (*n* = 3)	33						67	33					
	D3 (*n* = 1)	100							100					
Gentamicin	D0 (*n* = 3)	0						100						
	D3 (*n* = 1)	0						100						
Nitrofurantoin	D0 (*n* = 3)	–										100		
	D3 (*n* = 1)	–										100		
Oxacillin	D0 (*n* = 3)	33				67	33							
	D3 (*n* = 1)	0				100								
Penicillin	D0 (*n* = 3)	67	33					67						
	D3 (*n* = 1)	0	100											
Tetracycline	D0 (*n* = 3)	0						67	33					
	D3 (*n* = 1)	0						100						
Trimethoprim/	D0 (*n* = 3)	67					33			67				
Sulfamethoxazole	D3 (*n* = 1)	0				100								

White fields denote the range of dilutions tested for each antibiotic, and vertical bold lines indicate cut-off values used to define resistance. Grey fields denote the range outside of dilutions tested. MICs equal to or lower than the lowest concentration tested are given as the lowest tested concentration.

1). There was, at most, a one-allele difference between isolates within each cluster, except for Cluster 2 and 13 where there was a three-allele difference. Therefore, isolates within each cluster were highly genetically related, i.e. the same strain. Nine clusters consisted of isolates from one mare (Cluster 3, 6, 7, 8, 9, 10, 11 and 14), whereas five clusters consisted of isolates from 2 to 3 mares (Cluster 1, 2, 4 5 and 13 (Figure 1)). The finding of isolates from different mares in the same clusters suggests a possible spread within the herd.

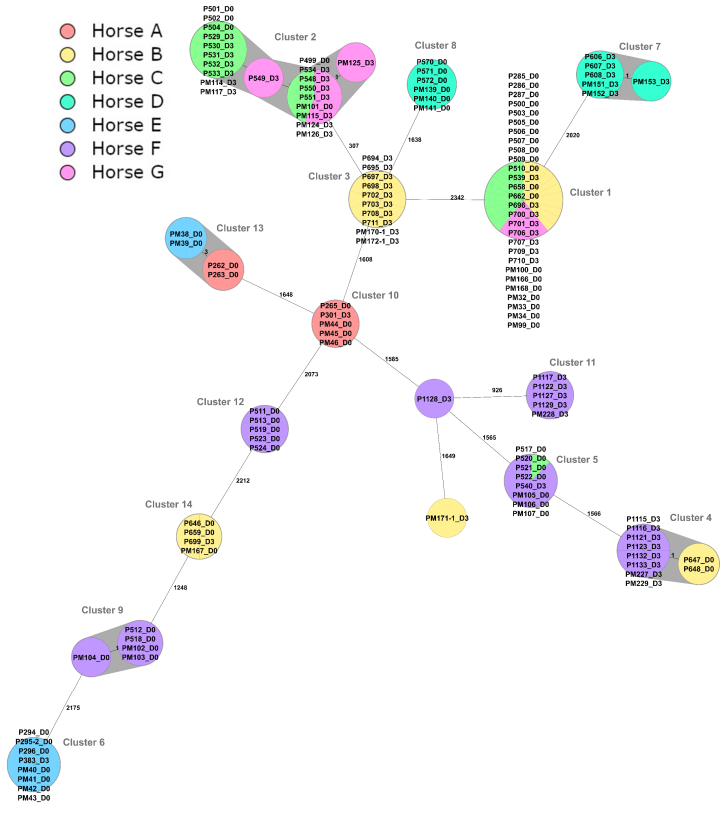

Figure 1. Minimum spanning tree (MST) created for 127 *Escherichia coli* from mares in Boden in northern Sweden visualizing core genome multi-locus sequence typing (cgMLST) results. Nodes corresponding to sequenced isolates are colored according to individual mares. D0 and D3 refer to isolates before and after insemination, respectively. The numbers between nodes represent allelic differences. Line lengths are not proportional to the number of differences. Identified clusters have been highlighted in grey.

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Eighteen of the *Streptococcus equisimilis* isolates were phenotypically resistant to tetracycline; eight clustered together from Horse H; ten clustered with isolates from Horse I and J. One isolate from Horse H was phenotypically resistant to erythromycin. One isolate was phenotypically resistant to nitrofurantoin, and it clustered together with isolates from Horse I and J.

Two isolates (P868 and PM194), which clustered together, were phenotypically resistant to fusidic acid. Two other isolates that clustered together (P869 and PM193) were phenotypically resistant to penicillin and trimethoprim/sulfamethoxazole. One isolate (P869) was phenotypically resistant to oxacillin.

The phenotypic resistance was compared between isolates from individual mares before and after the exposure to antibiotics within the same cluster, i.e. isolates of the same strain (Tables 7 and 8). For *E. coli,* increased resistance to sulfamethoxazole (Cluster 1), trimethoprim (Cluster 2 and 10), chloramphenicol (Cluster 2, 6 and 14) and tetracycline (Cluster 6) was found.

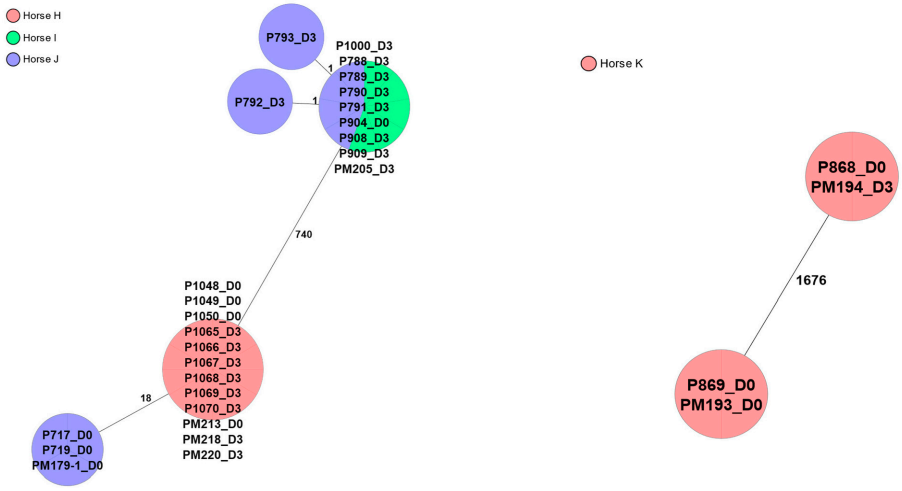

Figure 2. (**a**) Minimum spanning tree (MST) created for 26 *Streptococcus equisimilis* from mares in Boden in northern Sweden visualizing core genome multi-locus sequence typing (cgMLST) results. (**b**) Minimum spanning tree (MST) created for four *Staphylococcus simulans* from mares visualizing core genome multi-locus sequence typing (cgMLST) results. Nodes corresponding to sequenced isolates are colored according to individual mares. D0 and D3 are referring to isolates before and after insemination, respectively. The numbers between nodes represent allelic differences. Line lengths are not proportional to the number of differences.

Table 7. Distribution of resistant *E. coli* isolates and corresponding cluster and horse from which samples were originating.

Antibiotics (Total No. of Resistant Isolates).	No. of Resistant Isolates/Cluster/Horse
Trimethoprim (*n* = 32)	20/2/C; 2/4/B; 6/7/D; 4/10/A
Tetracycline (*n* = 10)	1/10/G; 2/2/G; 1/6/E; 6/7/D
Azithromycin (*n* = 1)	1/14/B
Chloramphenicol (*n* = 31)	20/2/C+G; 1/4/F; 1/5/F; 1/6/E; 1/8/D; 4/11/F; 3/14/B
Tigecycline (*n* = 1)	1/14/B
Ampicillin (*n* = 7)	1/4/B; 6/7/D

AMR Genes

All sequenced *Escherichia coli* isolates were resistant to sulfamethoxazole both phenotypically and genotypically; 6 isolates had the *sul1* gene, and 26 isolates had the *sul2* gene, which are responsible for sulfamethoxazole resistance. However, no resistance genes were found in 95 of the isolates. Thirty-two *E. coli* isolates were phenotypically resistant to trimethoprim; twenty-six of these isolates had *dfrA1*; seventeen isolates had *dfrA14;* no resistance genes were found in two isolates. Ten *E. coli* isolates were phenotypically resistant to tetracycline; six of these isolates had *tet(A)*; one isolate had *marR_S3N*; no resistance genes were found in the other three isolates. All 32 *E. coli* isolates that were phenotypically resistant to chloramphenicol had *mdf(A)*. One *E. coli* isolate phenotypically

resistant to tigecycline had *mdf(A)*. Of the seven *E. coli* isolates phenotypically resistant to ampicillin, six isolates had *blaEC-5/blaTEM-1* and *blaTEM-1B*; one isolate had *blaEC*.

Table 8. Phenotypic antimicrobial resistance of bacteria isolates from the vagina of mares before (D0) and after (D3) insemination.

Bacteria.	Cluster/ Horse	Day 0		Day 3	
		No. of Isolates	Resistance	No. of Isolates	Resistance
E. coli	1/C	10	Sul	1	Sul
	1/B	3	Sul	7	Sul
		1	–		
	2/C	4	Sul, Tri, Chl	9	Sul, Tri, Chl
		1	Sul		
	6/E	7	Sul	1	Sul, Tet, Chl
	10/A	1	Sul	4	Sul, Tri
	14/B	1	Sul, Azi, Chl, Tig		
		1	Sul, Chl	1	Sul, Chl
		1	Sul		
S. equisimilis	NA/I	1	–	4	Tet
	NA/H	2	Tet	8	Tet
		1	Ery		–
		1	–		
S. simulans	NA/K	1	Fus	1	Fus

Sul—sulfamethoxazole; Tet—tetracycline; Tri—trimethoprim; Chl—chloramphenicol; Azi—azithromycin; Tig—tigecycline; Ery—erythromycin; Fus—fusidic acid, NA—clustering is not available.

The gene *marR_S3N* was found in twenty-seven *E. coli* isolates susceptible to ciprofloxacin, chloramphenicol and tigecycline and twenty-six isolates susceptible to tetracycline. The gene *blaEC* was found in 119 isolates susceptible to meropenem, cefotaxime and ceftazidime and 118 isolates susceptible to ampicillin. The gene *blaEC-5/blaTEM-1* was found in six isolates susceptible to meropenem, cefotaxime and ceftazidime.

The gene *catB3* was found in six *E. coli* isolates susceptible to chloramphenicol. The gene *tet(A)* was found in six isolates susceptible to tigecycline. The gene *aadA5* was found in six isolates susceptible to gentamicin. The gene *aph(6)-ld* was found in 26 isolates susceptible to gentamicin. In addition, the gene *mdf(A)*, conferring resistance to broad-spectrum drugs via an efflux pump, was found in 92 isolates.

Five *Streptococcus equisimilis* isolates had *lsa*C as a resistance gene; one of these isolates had phenotypical resistance to erythromycin. Eighteen isolates phenotypically resistant to tetracycline were not shown to have resistance genes. Furthermore, one isolate, which was phenotypically resistant to nitrofurantoin, did not have resistance genes.

Two *Staphylococcus simulans* isolates had the *blaZ* resistance gene responsible for phenotypical resistance to penicillin and oxacillin. However, two isolates phenotypically resistant to fusidic acid and two isolates phenotypically resistant to trimethoprim/sulfamethoxazole did not have any resistance genes.

4. Discussion

The purpose of this study was to determine if the vaginal microbiota of mares was affected by exposure to antibiotics in semen extenders and also whether this exposure affected the AMR of the bacteria isolated from the vagina.

4.1. Bacterial Analyses

In the present study, 300 Gram-positive and 685 Gram-negative bacterial isolates were identified from the vagina. This result is in agreement with other studies where Gram-negative

bacteria dominated the microbiota in the vagina [7,26]. The isolates most frequently found in our study included *E. coli* (52.8%), *S. equisimilis* (14.0%) and *S. zooepidemicus* (3.6%). A study in Korea revealed that the most frequently isolated species of bacteria were *Escherichia coli* (19.8%), *Staphylococcus aureus* (14.9%) and *Proteus mirabillis* (14.9%); the samples were collected from vaginal mucosa and clitoral fossa [26]. In contrast, in an Indian report, the dominating bacteria in the vagina were *E. coli* (21.7%), *Enterobacter agglomerans* (16.7%), *Enterococcus faecalis* (15.6%) and *Enterococcus faecium* (15.6%) [7]. However, bacteria in these other studies were isolated using various types of agar plates and cultured under different conditions compared to our study, and they were identified at the species level using different methods compared to ours. Although other methods of analysis were used in different studies, *E. coli* seems to be the most isolated bacterial species, most likely because it is one of the most common bacteria in the reproductive tract and the surroundings.

Lactobacillus spp. were not detected in our study, although the selective culture medium, MRS agar, was used. This observation is in contrast to a study performed in Uruguay, in which *Lactobacillus* spp. were isolated from all vaginal samples [27]. Time and temperature might affect the viability of *Lactobacilli*; in the study by Fraga et al. [27], the swabs were kept at 4 °C and transported to the laboratory within 3 h. In our study, the stud was at a considerable distance (> 860 km) from the laboratory, necessitating overnight transport of the swabs, which took place at ambient temperatures.

4.2. Antimicrobial Susceptibility

The vaginal microbiota of mares in our study was susceptible to most of the tested antibiotics. In fact, all *Enterococcus faecalis* isolates were susceptible to all 12 tested antibiotics in our study. In other studies, most isolates were obtained from infertile mares, which may have been treated with antibiotics. In other studies, *Enterococcus* spp. isolates have been described as resistant to all antibiotics tested [7] and resistant to some antibiotics except penicillin, cefquinome, florfenicol, amoxicillin/clavulanic acid, amoxicillin, gentamicin and colistin [15] or resistant to some antibiotics except for gentamicin, vancomycin, linezolid, penicillin and tetracycline [9].

Interestingly, all *Streptococcus equi* subsp. *zooepidemicus* isolates of the exposed mares were susceptible to all tested antibiotics. This result is in line with monitoring antibiotic resistance in veterinary medicine in Sweden, where *Streptococcus zooepidemicus* isolated from horses have remained uniformly susceptible over the years [18]. In contrast, studies of this bacterium from other countries reported resistance to most antibiotics [10,12,28].

Resistance to seven different antibiotics was found in different *E. coli* isolates in our study, including sulfamethoxazole (90%), trimethoprim (10.7%), chloramphenicol (10.6%), and tetracycline (2%) ampicillin (2.5%), azithromycin (0.5%) and tigecycline (0.5%). Trimethoprim, chloramphenicol and tetracycline showed increased resistance three days after AI. However, these antibiotics were not included in the semen extender. Genes conferring resistance to antibiotics may not be specific for these antibiotics but 'merely' allow survival in a hostile environment, with resistance to antibiotics being coincidental. Therefore, bacteria do not necessarily need to be exposed to an antibiotic to develop resistance to it. Alternatively, exposure to another antibiotic could cause a transfer of resistance between bacteria, e.g. via plasmids [29]. Other factors might influence the association, such as previous treatment or exposure to environmental bacteria on personnel. The resistance of *E. coli* isolates to some tested antibiotics in our study was similar to other studies, where trimethoprim, chloramphenicol, tetracycline, ampicillin and azithromycin resistance was found [11,13,15]. To our knowledge, there have not been any previous reports of resistance to tigecycline in *E. coli* isolates from the vagina of mares. Resistance to 4 of 14 antibiotics could be detected in different *Staphylococcus simulans* isolates in our study, including oxacillin (25%), penicillin (50%), fusidic acid (50%) and trimethoprim/sulfamethoxazole (50%). Other studies showed the resistance of *Staphylococcus* spp. to most antibiotics tested, including amoxicillin/clavulanic acid, cefotaxime, cefquinome, colistin, enrofloxacin, erythromycin, gentamicin, kanamycin, marbofloxacin, penicillin, ri-

fampicin, sulfisoxazole, tetracycline and trimethoprim/sulfamethoxazole [8,14,15]. As in our study, penicillin and trimethoprim/sulfamethoxazole resistance were found in these studies.

Many isolates (91.1%) of *Streptococcus equisimilis* were resistant to tetracycline but showed low resistance to erythromycin (1.1%) and nitrofurantoin (1.1%). Furthermore, all *Streptococcus equisimilis* isolates in this study were susceptible to cefalotin, clindamycin, oxacillin, penicillin and trimethoprim/sulfamethoxazole, which differed significantly from some other studies where resistance to all tested antibiotics was reported [7,8].

4.3. Whole-Genome Sequencing

Following antimicrobial resistance testing results, sequenced *E. coli* isolates from inseminated mares at both time points were separated into 14 MST clusters; 9 clusters consisted of isolates from 1 mare, whereas 5 clusters consisted of isolates from 2 to 3 mares. The finding of genetically related isolates in more than one mare indicates a possible spread within the herd. Moreover, for *Streptococcus equisimilis*, there was an indication of spread within the herd, since the same isolate was potentially detected in isolates from two horses (Horse I and J). Twenty-nine of the sequenced *E. coli* isolates (22.8%) were MDR, and they were found in five clusters; one cluster consisted of isolates from two mares. The possibility of spreading within the herd might occur also for MDR isolates.

Increased or decreased resistance was observed from isolates from the same horse within the same cluster. Following antimicrobial resistance testing results, the proportions of resistant isolates before and after insemination were compared. Increased resistance to sulfamethoxazole, trimethoprim, chloramphenicol and tetracycline and decreased resistance to azithromycin and tigecycline were found in *E. coli* isolates. *Streptococcus equisimilis* showed increased resistance to tetracycline and decreased resistance to erythromycin. An increase in phenotypic resistance might be caused by different mechanisms after experiencing an unfavorable environment; for example, it was reported previously that low-level exposure to antibiotics could lead to antimicrobial resistance [30]. The vaginal bacteria of the mares in this study were exposed to the antibiotics in the semen extender used to prepare the semen doses. However, since single isolates were selected for WGS based on microdilution results in our study, it is not possible to be certain that the same bacterial strain was chosen on all sampling occasions.

Two *E. coli* isolates and one *Streptococcus equisimilis* isolate, which had phenotypic resistance, did not contain resistance genes. Furthermore, there were no resistance genes in two *Staphylococcus simulans* isolates showing phenotypic resistance to fusidic acid and trimethoprim/sulfamethoxazole. Natural or acquired resistance usually results from resistance genes. A study showed that many resistance genes occurring in environmental bacteria might potentially be transferred to bacteria in the mares' reproductive system [31]. Gene transmission in nature occurs via three mechanisms: conjugation, transduction and transformation [32]. Even dead bacteria can pass the resistance gene to other bacteria by transformation [33]. In the present study, possible explanations for the difference between phenotypic and genotypic resistance could be that the genes or the mechanisms conferring resistance have not been detected yet, or there may be a discrepancy between genotypic and phenotypic resistance.

In conclusion, most isolates from the cranial vagina of mares were Gram-negative bacteria. The exposure of vaginal bacteria to antibiotics in the semen extender was associated with a change in the resistance of *Escherichia coli* against trimethoprim, chloramphenicol and tetracycline. The antibiotic resistance pattern of the genetically related isolates in the same horse increased or decreased in some *Escherichia coli* and *Streptococcus equisimilis* isolates after exposure to the semen extender. These results are interesting and warrant further study.

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Doctoral Thesis No. 2023:77

Bacteria in the reproductive tract of horses were studied. Vaginal swabs from mares before and after sham insemination, revealed a predominance of *E. coli*. Variations in bacteria among mares were linked to environmental factors, breeding status and insemination. The isolates exhibited diverse antibiotic susceptibility, influenced by breeding status and environment, and history. Stallions from different countries showed distinctive seminal microbiomes, necessitating further study for customized semen preservation strategies. In addition, separating spermatozoa from seminal plasma using a low density colloid lowered bacterial counts without harming sperm quality, indicating a potential alternative to antibiotics in semen extenders.

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