Characterization, quantification and removal of potential pathogens from stallion semen

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Abstract

In Sweden, equine artificial insemination is most frequently carried out with liquid semen rather than frozen semen. Many factors affect sperm quality during semen storage, including the presence of bacteria and addition of antibiotics. Use of Single Layer Centrifugation (SLC) or Modified SLC (MSLC) has been shown to improve sperm quality, resulting in an increase in the time for which the sperm sample retains its function during storage. These techniques can also separate spermatozoa from bacteria. The purpose of this thesis was to investigate the role of MSLC, antibiotics and bacteria on sperm quality and storage time. This thesis comprised 4 studies: **Study I** was a retrospective study of potential pathogenic bacteria isolated from Swedish stallion semen during the period 2007 to 2017. **Study II** was to identify bacteria in semen by conventional laboratory culture methods. **Study III** was to isolate pathogenic and non-pathogenic bacteria in stallion semen using metagenomic analysis. **Study IV** was to investigate the effect of MSLC and the effect of presence or absence of antibiotics in the extender on sperm quality. Our results showed that potentially pathogenic bacteria such as *Taylorella equigenitalis, Klebsiella pneumoniae*, beta haemolytic streptococci and *Pseudomonas aeruginosa* appeared infrequently in Sweden. However, many non-pathogenic bacteria were found. Metagenomic analysis enabled more bacteria to be identified than other methods. The bacterial genera identified were different between studies, animals and ejaculates, even from animals kept on the same stud. *Corynebacterium* spp. were the most frequently found non-pathogenic bacteria identified in all our studies. The bacterial population was decreased using MSLC, sperm quality was improved and the shelf-life of the sperm samples was increased. The presence of antibiotics in the extender did not affect sperm viability. The bacterial population was greater in samples without antibiotics than in samples with antibiotics; bacteria appeared in all samples, even those with extender containing antibiotics. There was no evidence that bacteria isolated from a stallion on one stud should also be isolated from other individuals on the same premises. More work is needed to investigate the effects of particular bacterial genera on sperm quality. In addition, it would be interesting to investigate the shape and size of bacteria in relation to spermatozoa and the proportions of different bacteria removed using MSLC. Further modifications to the SLC technique might enable the removal of more bacteria.

Keywords: Modified Single Layer Centrifugation, male fertility, microorganism, liquid semen, sperm quality, 16S sequencing, pony stallions, semen evaluation

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Karaktärisering, kvantifiering och borttagning av potentiella patogener i hingstsperma

Abstrakt

Sperma från hingstar för artificiell insemination sker i Sverige vanligen med kyld sperma istället för djupfryst. Många faktorer påverkar spermiekvaliteten under samling och lagring innefattande också bakterieförekomst och tillsats av antibiotika till spädningsvätskorna. Användning av Single Layer Centrifugation (SLC) eller Modified SLC (MSLC) har visat sig öka spermiekvaliteten, vilket resulterat i en ökning av spermiernas hållbarhet. Dessa tekniker kan också separera spermier från bakterier.

Målet med avhandlingen var att studera och undersöka betydelsen av MSLC, antibiotika och bakterier på spermiekvalitet under lagring samt spermiernas överlevnadstid. Avhandlingen består av 4 studier. Den första studien (nr I) är en retrospektiv undersökning av potentiellt patogena bakterier isolerade från hingstar i Sverige under perioden 2007 - 2017. I studie nummer två (nr II) identifierades bakterier i sperma med konventionella laboratoriemetoder. I tredje studien (nr III) isolerades patogena och icke-patogena bakterier i hingstsperma med hjälp av metagenomisk analys. I den sista studien, (nr IV) undersöktes effekterna av spermiekvaliteten med eller utan MSLC samt med eller utan antibiotika i spädningsvätskan. Våra resultat visar att potentiellt patogena bakterier som *Taylorella equigenitalis*, *Klebsiella pneumoniae*, beta-haemolytiska streptokocker och *Pseudomonas aeruginosa* sällan förekom i Sverige, men många icke-patogena bakterier hittades. Metagenomiska analyser möjliggjorde att fler bakterier identifierades än med hjälp av andra metoder. De olika bakteriearter som isolerats skilde sig mellan olika studier, hingstar och ejakulat, även ibland hingstar som stått på samma ställe. *Corynebacterium* spp. var den vanligaste icke-patogena bakterier som identifierades i alla studierna. Bakteriepopulationen sjönk när MSLC användes, spermiekvaliteten förbättrades och spermiernas överlevnadstid ökade. Bakteriepopulationerna var större i prover utan antibiotika än i prover med antibiotika men varken bakterier eller antibiotika i spädningsvätskan påverkade spermiernas livsduglighet. Det fanns inga bevis att bakterier isolerade från en hingst i ett stall också skulle isoleras från andra hingstar på samma plats. Fler studier behövs för att undersöka effekten av vissa specifika bakterie på spermiekvalitet. Det skulle vara intressant att undersöka formen och storleken av bakterier i relation till spermierna och vilka bakterier kan användas för att ta bort bakterier via MSLC. Modifiering av SLC tekniken kanske kan användas för att ta bort ytterligare några eller alla.

Nyckelord: Modified Single Layer Centrifugation, Hanlig fertilitet, Mikroorganism, Kyld sperma, Spermiekvalité, 16S sekvensering, Ponny hingstar, Sperma utvärdering

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Dedication

To my country Iraq, my family (RASHA, YOUSIF and MATTI), my father and mother, my teachers, and 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 Al-Kass, Z., Eriksson, E., Bagge, E., Margareta, W. & Morrell, JM.*. Bacteria detected in semen and pre-ejaculatory fluid of Swedish stallions from 2007 to 2017. (Submitted).

II Al-Kass, Z., Eriksson, E., Bagge, E., Margareta, W. & Morrell, JM.* Conventional diagnostics to identify bacteria in semen from stallions in Sweden. (Manuscript).

III Al-Kass, Z., Guo, YZ., Pettersson, OV., Niazi, A. & Morrell, JM.*. Metagenomic analysis of bacteria in semen from Swedish stallions. (Manuscript).

IV Al-Kass, Z., Spergser, J., Aurich, C., Kuhl, J., Schmidt, K., Johannisson, A. & Morrell, JM.* (2018). Sperm Quality during Storage Is Not Affected by the Presence of Antibiotics in EquiPlus Semen Extender but Is Improved by Single Layer Centrifugation. Antibiotics, 7, 1-13.

V Al-Kass, Z., Spergser, J., Aurich, C., Kuhl, J., Schmidt, K. & Morrell, JM* (2019). Effect of presence or absence of antibiotics and use of modified single layer centrifugation on bacteria present in pony stallion semen. Reproduction in Domestic Animals, 54, pp. 342–349. https://doi.org/10.1111/rda.13366.

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Papers IV and V are reproduced with permission of the publishers.

The contribution of Ziyad Al-Kass to the papers included in this thesis was as follows:

I. Contributed to the planning and design of the experiment, data analysis and statistical analysis; drafted the paper with regular input from the co-authors.

II. Contributed to the planning and design of the experiment, culture and identification of bacteria, data analysis and statistical analysis; drafted the paper with regular input from the co-authors.

III. Contributed to the planning and design of the experiment, extraction of DNA, data analysis and statistical analysis; drafted the paper with regular input from the co-authors.

IV. Designed the experiment, collected semen, performed most of the laboratory work, and analyzed the data; drafted the paper with regular input from the co-authors and revised the final version of the article.

V. Designed the experiment, collected semen, contributed to the laboratory work and data analysis; drafted the paper with regular input from the co-authors and revised the final version of the article.

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Abbreviations

%DFI	DNA fragmentation index
AI	Artificial insemination
ALH	Lateral head displacement
AV	Artificial vagina
BCF	Beat cross frequency
CA	Control extender with antibiotics
CASA	Computer-assisted sperm analysis
CEM	Contagious equine metritis
CEMO	Contagious equine metritis organism
cfu/mL	Colony forming units/mL
COBA	Colistine oxolinic blood agar
CW	Control extender without antibiotics
DGC	Density gradient centrifugation
FAA	Fastidious anaerobic agar
FC	Flow cytometer
JC-1	5,59,6,69-tetrachloro-1,19,3,39-tetraethylbenzimidazolylcarbocyanine iodide
K. pneumoniae	*Klebsiella pneumoniae*
LIN	Linearity
M. subdolum	*Mycoplasma subdolum*
MALDI-TOF MS	Matrix-assisted laser desorption ionization time of flight mass spectrometry
MAST	Mannitol salt agar
MI	Membrane Integrity
MMP	Mitochondrial Membrane Potential
MSLC	Modified Single Layer Centrifugation
MSP	Main spectra projections
P. aeruginosa	*Pseudomonas aeruginosa*

PI	Propidium iodide
PIA	*Pseudomonas* isolation agar
PM	Progressive motility
PPLO	Pleuropneumonia-Like-Organism
ROS	Reactive oxygen species
SA	Modified Single Layer Centrifugation, extender with antibiotics
SCSA	Sperm Chromatin Structure Assay
SLC	Single Layer Centrifugation
SLU	Swedish University of Agricultural Sciences
sp. and spp.	Species
STR	Straightness
SVA	National Veterinary Institute
SW	Modified Single Layer Centrifugation, extender without antibiotics
T. equigenitalis	*Taylorella equigenitalis*
TM	Total motility
VAP	Velocity of the average path
VCL	Curvilinear velocity
VSL	Straight line velocity
WOB	Wobble

1 Introduction

1.1 Stallion artificial insemination (AI)

The systematic development of AI in horses started in Russia from 1899 (Ivanoff, 1922). At first, semen was collected using a rubber bag in the vagina of an estrus mare, then in the 1930s and 1940s, various artificial vaginas (AV) were developed (Perry, 1968). Research continues currently to improve methods for semen collection, and to improve sperm quality during storage. Short term storage of equine sperm at 5ºC for up to 72 h in extenders based on milk and/or egg yolk is claimed to give acceptable pregnancy rates, fertility and viability (Aitken *et al.*, 2012; Jasko *et al.*, 1993), but generally equine AI with liquid extended semen is done within 48h of semen collection (Foote, 2002).

According to reported statistics, there were 355500 horses in Sweden in 2016 (Jordbruksverket, 2017). In 2017, according to the relevant breed associations, approximately 68% warmblood Swedish mares and 46% warmblood trotters were inseminated using liquid semen (A-M Dalin, personal communication). There are many AI centers in Europe; for example, in 2012 there were 26 in Sweden and 119 in Germany (Aurich, 2012). These numbers are increasing yearly. Liquid stallion semen is used widely in equine reproduction, and the main challenges are how to extend storage time, increase sperm quality, control bacterial contamination, and reduce antibiotic usage. During the last few decades, technical advances in equine AI include improved evaluation and storage (Colenbrander *et al.*, 2003), using sperm selection (Morrell & Rodriguez-Martinez, 2009), sperm sexing (Buchanan *et al.*, 2000) and intracytoplasmic sperm injection (Dell Aquila *et al.*, 1997).

Semen can be collected by different methods: 1) use of a condom; 2) pharmacologically; 3) manual manipulation of the penis; 4) electroejaculation; and 5) AV. However, the most commonly used method for commercial purposes

and research is by AV. Several models are available: Missouri (commonly used in the United States of America), Colorado, Nishikawa, Polish models and Hanover. All of these models work on the same principle (Hurtgen, 2009), namely to provide sufficient stimulation to the penis to promote ejaculation.

1.2 Sperm quality

Samples that are suitable for insemination or for storage as liquid or frozen samples, must be of good quality. Sperm quality is the ability of spermatozoa to survive during storage and to reach the oocyte and accomplish fertilization.

Several methods are available to evaluate sperm quality before using it for insemination or for research purposes. In commercial AI stations, sperm concentration, total and progressive motility (PM), and sometimes morphology are used (Rodriguez-Martinez, 2013). Based on these evaluations, a decision is made on whether the sperm quality is acceptable or not, and for calculating the number of AI doses that can be prepared (Malmgren, 1997). A study showed that there was a relationship between sperm concentration and sperm characteristics after thawing in donkey semen, with the best results obtained at sperm concentrations of 100 and 250×10^6 spermatozoa/mL (Contri *et al.*, 2012). Regarding motility and morphology, defective spermatozoa might not reach the oocyte or might be unable to fertilize it (Foxcroft *et al.*, 2008).

More advanced methods are employed to evaluate sperm quality for research, including sperm mitochondrial status, Sperm Chromatin Structure Assay (SCSA), acrosome integrity, detection of oxidative stress and lipid peroxidation (Hossain *et al.*, 2011). Mitochondria are present in the sperm mid-piece and are important in producing energy. Evaluating adenosine triphosphate and measurement of mitochondrial statues provide an indication of sperm quality (Gravance *et al.*, 2000). The main source of adenosine triphosphate in stallion spermatozoa is from mitochondrial oxidative phosphorylation (Gibb *et al.*, 2014). The SCSA measures the denaturability of sperm chromatin after challenging with acid treatment. Denaturation is linked with DNA strand breaks (Evenson *et al.*, 1995). Studies showed that there was a significant association between oxidative stress parameters and some sperm motility parameters, with the most fertile semen samples producing more reactive oxygen species (ROS) (Luo *et al.*, 2013; Gibb *et al.*, 2014). Membrane integrity (MI) is evaluated as an indicator of fluid transportation across the membrane, which is important in the fertilization process (Rodriguez-Martinez, 2003).

1.3 Bacterial contamination and antibiotics

Bacterial contamination in semen samples is one of the most important points influencing sperm quality during storage. Bacteria come from the surface of the penis, the prepuce, and the skin of the animal. Most of them are not pathogenic, but there are a few bacteria that are pathogenic and some that can become pathogenic under certain circumstances, leading to endometritis and subfertility in mares. Thus, the stallion represents an important way to transmit bacteria to mares and, potentially, cause disease (Aurich *et al.,* 2003). Even semen processing can cause bacterial contamination (Althouse, 2008). Stallions used for natural mating have a higher total bacterial flora on the genital mucosa during the breeding season than in the non-breeding season (Klug & Sieme, 1992). The most important pathogens transmitted during coitus or AI are those responsible for venereal disease, for example equine herpesvirus 3, *Taylorella equigenitalis*, *Pseudomonas aeruginosa*, and *Klebsiella pneumoniae* (Blanchard *et al.,* 1992). Other studies showed an important role for *Mycoplasma equigenitalium*, *M. subdolum* and *Acholeplasma* species (spp.) in infertility, endometritis and abortions in mares (Kirchhoff *et al.,* 1980; Heitmann *et al.,* 1979). On the other hand, many bacteria identified on the exterior of the stallion´s penis are not usually pathogenic e.g. *Escherichia coli*, *Streptococcus equisimilus*, *Streptococcus zooepidemicus*, nonpathogenic strains of *P. aeruginosa*, *K. pneumoniae* and *Bacillus* spp. (Tibary *et al.,* 2009; Samper & Tibary, 2006), Many other genera of nonpathogenic bacteria were identified in different studies on stallion semen (e.g. Althouse *et al.,* 2010; Ortega-Ferrusola *et al.,* 2009; Corona & Cherchi, 2009; Lu & Morresey, 2007; Pasing *et al.,* 2013) and these bacteria can have a negative effect on the quality of liquid stallion semen (Aurich & Spergser, 2007). They are associated with decreased sperm viability and motility, and also an increased proportion of defective acrosomes (Althouse *et al.,* 2000; Ortega-Ferrusola et *al.,* 2009; Kuster and Althouse, 2016). In addition, bacteria in frozen semen may cause early embryonic death and/or endometritis in females inseminated with contaminated semen (Maes *et al.,* 2008). Therefore, antibiotics are added to semen extenders to control bacterial growth (Pickett *et al.,* 1999).

According to Appendix C of the European Union guideline 92/65, it is mandatory to add antibiotics to extenders for stallion semen sold for commercial purposes (European Commission, 1992). Antibiotics commonly used in stallion semen extender are amikacin, gentamicin, streptomycin, penicillin, ticarcillin and polymyxin (Althouse, 2008; Pickett *et al.,* 1999). However, some studies showed that using different antibiotics at various concentrations in semen extender affected sperm motility (Varner *et al.,* 1998; Pickett *et al.,* 1999; Jasko

et al., 1993). Furthermore, different extenders had an effect on sperm viability (Pagl *et al.*, 2006). Even in other species, antibiotics in semen extenders had a negative effect on sperm viability, for example in ram (Yaniz *et al.*, 2010), buffalo bull (Akhter *et al.*, 2008) and boar spermatozoa (Schulze *et al.*, 2014). This intensive use of antibiotics in semen extenders could lead to bacterial resistance, which occurs when bacteria survive and grow in the presence of antimicrobials (Levy & Marshall, 2004). Development of resistance occurs either intrinsically in bacteria or by acquiring the genetic material responsible for antibiotic resistance from other bacteria by conjugation, transduction or transformation (MacGowan & Macnaughton, 2017).

A report from the European Centre for Disease Prevention and Control (European Centre for Disease Prevention and Control, 2015), stated that around 25000 people in Europe die every year because patients become infected with resistant bacteria in hospital, at a cost of around €1.5 billion. Therefore bacteria which are resistant to antibiotics are considered as an important challenge, causing high morbidity and mortality (Frieri *et al.*, 2017). We need to be concerned about bacterial resistance (European Centre for Disease Prevention and Control, 2015) and make every effort to use antibiotics in a sustainable manner.

1.4 Bacterial identification

To be able to identify bacteria, methods are needed that are fast, inexpensive, easy to use and accurate. Matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS), covering all these specifications, was developed for identification of bacteria (Croxatto *et al.*, 2011). It was used for the first time in 1975 by Anhalt & Fenselau (1975). Now, most laboratories use MALDI-TOF MS for routine diagnostic work, but there are some disadvantages, because it is still necessary to culture bacteria on agar and select suitable colonies for identification. Therefore, it is only suitable to identify bacteria that are able to grow, and only those which are already contained in the database can be identified. Most databases contain data on bacteria that are relevant for human medicine (Croxatto *et al.*, 2011), which may be of limited value for veterinary medicine or general bacteriology. In contrast, 16S sequencing to identify bacterial DNA, is capable of identifying both live and dead bacteria even at low levels, no culture is needed, and it is able to provide a higher specific identification (Biswas & Rolain, 2013).

1.5 Sperm selection methods

Removal of seminal plasma and selection of spermatozoa can be done either for AI or for research. Sperm selection is used to separate out motile spermatozoa with normal morphology, good chromatin integrity and an intact acrosome from the rest of the sample, all of which are needed for successful fertilization of the oocyte and continued development of the zygote (Morrell *et al.*, 2009a). The presence of seminal plasma is necessary for sperm function, but is harmful to spermatozoa during storage because it contains decapacitation factors (Bjorndahl *et al.*, 2005), motility inhibitors (Kordan *et al.*, 1998), and other detrimental factors. Therefore, researchers started to remove seminal plasma and select spermatozoa with desirable properties for research and AI.

A washing method can be used for separation of spermatozoa from seminal plasma, whereas such separation combined with selection of good spermatozoa can be done with sperm migration, filtration or colloid centrifugation (Morrell & Rodriguez-Martinez, 2009). Semen centrifugation through a colloid is reported to result in sperm samples with improved sperm motility, viability and chromatin integrity (Morrell *et al.*, 2009b), and an intact acrosome (Costa *et al.*, 2012). Single Layer Centrifugation (SLC) i.e. centrifugation through one layer of colloid, can be considered to be the most useful selection method since density gradient centrifugation (DGC), requiring two or more colloids, is more time consuming to prepare and cannot be scaled-up easily to process large volumes of ejaculate. The methods used routinely on some studs include sperm washing and SLC. There are many advantages to using SLC because this method is able to remove seminal plasma, which may contain pathogens, sources of ROS, debris, leukocytes etc. from samples, as well as to select motile spermatozoa and those with an intact acrosome. There are also disadvantages in that it is more expensive than other methods and some training is required (Morrell & Rodriguez-Martinez, 2009).

Colloid centrifugation (either DGC or SLC) has been used to prepare semen from men (Nicholson *et al.*, 2000), bulls (Ock *et al.*, 2006; Goodla *et al.*, 2014; Nongbua *et al.*, 2017), rams (Correa & Zavos, 1996; Sterbenc *et al.*, 2019), boars (Popwell & Flowers, 2004), stallions (Brum *et al.*, 2008; Al-Essawe *et al.*, 2018), turkeys (Morrell *et al.*, 2005), dogs (Morrell *et al.*, 2008a), giant pandas (Cai *et al.*, 2018), and cats (Chatdarong *et al.*, 2010), among others. It has also been used to remove bacteria from human semen (Nicholson *et al.*, 2000), boar semen (Morrell and Wallgren, 2011; Morrell *et al*., 2019) and stallion semen (Morrell *et al.*, 2014; Guimaraes *et al.*, 2015).

Morrell *et al.* (2014) modified the SLC technique by including an inner tube to avoid re-contaminating the sperm pellet after centrifugation, which had the additional advantage of decreasing processing time. This method was used to reduce the bacterial load in stallion semen (Morrell *et al.*, 2014) and in boar semen (Morrell *et al*., 2019).

2 Aims

The general aim of this study was to investigate bacterial contamination in stallion semen during collection and processing, to identify the bacteria and determine their effect, or the presence of antibiotics, on sperm quality during storage.

Study I: to determine the occurrence of potentially pathogenic bacteria over a ten - year period (2007 to 2017) from Swedish stallions, using semen samples submitted to the National Veterinary Institute (SVA).

Study II: to identify the bacteria isolated from Swedish stallions after using different aerobic and anaerobic culture methods, identifying the isolated bacteria by MALDI-TOF MS.

Study III: to examine the presence of pathogenic and nonpathogenic bacteria in Swedish stallion semen from one stud, using 16S sequencing for identification, and to study differences in bacteria isolated from the same animal.

Study IV: to evaluate sperm quality during storage in extender with and without antibiotics, to determine the effect of antibiotics on spermatozoa. The bacteria present in stallion semen and the effect of using MSLC to remove bacteria on sperm quality were also investigated.

3 Materials and methods

A general review of material and methods is described. For more information see papers I-V.

3.1 Study design

The studies in this thesis took place at the Swedish University of Agricultural Sciences (SLU), SVA, SciLifeLab-Uppsala, Sweden, and the Center for Artificial Insemination and Embryo Transfer, and the Institute of Microbiology, Department of Pathobiology, Vienna University for Veterinary Sciences, Austria.

Study I: the presence of potential pathogens in samples submitted to the SVA between 2007 and 2017 for routine testing of breeding stallions before the start of each breeding season was surveyed. In total, material from 2308 stallions was submitted for testing for *T. equigenitalis*, the causal agent of contagious equine metritis (CEM), and 730 semen samples were submitted for general bacterial screening from stallions in Sweden.

Study II: semen from five Swedish stallions was used to investigate the bacteria commonly found. Bacterial culture was performed under different conditions, followed by identification using MALDI-TOF MS.

Study III: Metagenomic analysis was used to identify bacteria in semen from Swedish stallions.

Study IV (Papers IV and V): sperm quality was studied in extenders with or without antibiotics, with or without centrifugation through a species-specific colloid formulation, to determine the effects of these factors on number of bacteria and sperm quality (Figure 1).

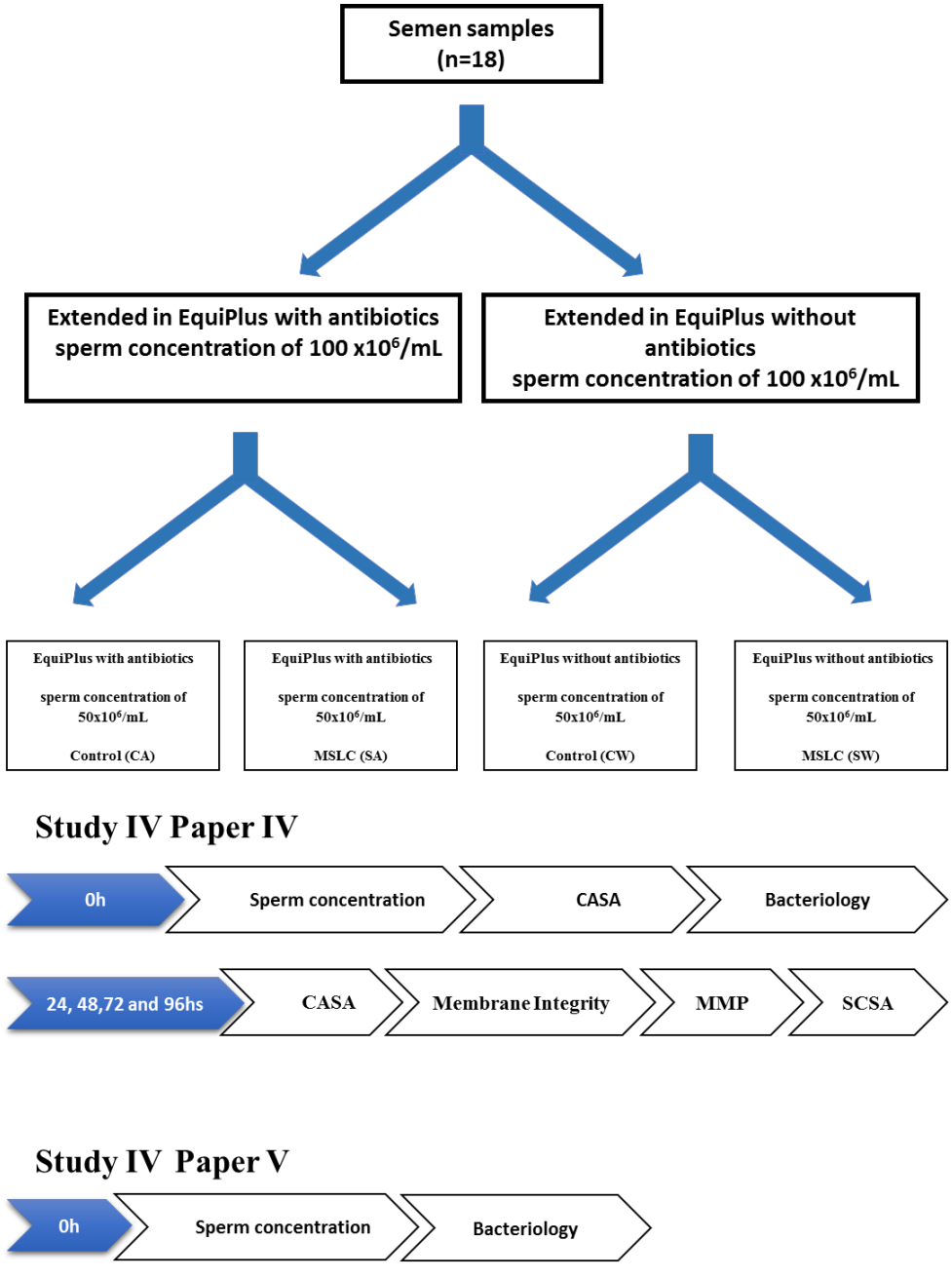

Figure 1. Study IV Design. Abbreviations, CASA: Computer-Assisted Sperm Analysis, MMP: Mitochondrial Membrane Potential & SCSA: Sperm Chromatin Structure Assay.

3.2 Ethical approval

Studies I, II and III: No ethical approval is needed in Sweden for semen collection from stallions using an AV. The stallions were housed at commercial studs in Sweden according to standard husbandry conditions for this species.

Study IV: Semen collection for research purposes was approved by the Austrian Federal Ministry for Science and Research (license number BMWFW-68.205/0150-WF/V/3b/2015). The stallions were housed according to standard husbandry methods at the Center for Artificial Insemination and Embryo Transfer, Vienna University for Veterinary Sciences, Austria.

3.3 Animals and samples

Study I: Data were available from routine testing of stallion semen samples sent to SVA between 2007 and 2017 from studs all over Sweden. The samples had been taken from semen and from different parts of the reproductive tract as follows: urethral fossa, male genital organs, penile shaft and prepuce, urethral orifice and pre-ejaculate secretion. In addition, detailed bacterial identification of semen samples from six stallions (8–18 years old) at one stud in Sweden was available from a separate experiment in 2016.

Study II: semen samples from five adult warmblood stallions (7–17 years old), were collected in May and June 2015, using a sterilized Missouri model AV fitted with an inline filter to remove gel. The semen was extended with EquiPlus (Minitüb, Tiefenbach, Germany) to a final sperm concentration of 100×10^6 /ml, and was immediately transferred to sterile tubes and placed on ice for transfer to SVA for bacterial culture.

Study III: stallion ejaculates were obtained in March 2015, from seven warmblood stallions (7–17 years old) on a commercial stud in Sweden. Semen was collected as described for Study II. The extended semen was immediately transferred to sterile tubes and placed on ice for transfer to SLU, for subsequent storage at -80 °C.

Study IV: semen samples from six adult pony stallions (5–25 years old) were collected between February and April 2017, once weekly (3 ejaculates per animal), using a sterilized Hannover AV after the stallion had mounted a phantom.

3.4 Semen evaluation

3.4.1 Sperm concentration

Studies II & IV: Sperm concentration was evaluated using a Nucleocounter-SP 100 (ChemoMetec, Allerød, Denmark) according to the manufacturer´s instructions.

3.4.2 Computer-assisted sperm analysis

Study IV (paper IV): Sperm kinematics were evaluated using a SpermVision analyzer (Minitűb GmbH, Tiefenbach, Germany), connected to an Olympus BX 51 microscope (Olympus, Tokyo, Japan) with a heated stage (38°C). Motility analysis was carried out in eight fields (at least 1000 spermatozoa in total). Total and progressive motility (TM, %; PM, %), curvilinear velocity (VCL, μm/s), velocity of the average path (VAP, μm/s), wobble (WOB) lateral head displacement (ALH, μm), straight line velocity (VSL, μm/s), linearity (LIN), straightness (STR), and beat cross frequency (BCF, Hz) were calculated, The software program for the SpermVision used settings adjusted for stallion spermatozoa. Spermatozoa were considered as immotile if VAP <20; locally motile if VAP > 20 and <30, STR <0.5, VCL <9. Assessment was performed at 0, 24, 48, 72 and 96 h after collection.

3.4.3 Membrane Integrity

Study IV (paper IV): Assessment of MI was carried out after staining with SYBR14 and propidium iodide (PI) (Live-Dead Sperm Viability Kit L-7011; Invitrogen, Eugene, OR, United States of America. In this assay, PI can only penetrate damaged sperm membranes, whereas SYBR14 can pass into all sperm cells. Aliquots of samples diluted to a sperm concentration of approximately 2 million spermatozoa/mL with CellWASH (Becton Dickinson, San José, CA, USA) were stained with 0.6 μL of 0.02 μM SYBR14 and 3 μL of 12 μM PI, followed by incubation for 10 min at 37 ºC (Johannisson *et al.*, 2009). Red and green fluorescence, as well as forward and side scatter, were measured using a FACSVerse™ flow cytometer (BD Biosciences) (FC). Samples were assessed at 24, 48, 72 and 96 h after semen collection, classifying SYBR14 positive or negative/PI positive spermatozoa as having a damaged membrane and SYBR14 positive, PI negative spermatozoa as having an intact membrane.

3.4.4 Mitochondrial Membrane Potential

Study IV (paper IV): Assessment of MMP was done using FC, according to the method reported by Morrell *et al.* (2017). Sample aliquots (1000 µL) were stained with 0.5 µL of 3 mM 5,59,6,69-tetrachloro-1,19,3,39-tetraethylbenzimidazolylcarbocyanine iodide (JC-1) and the mixture was incubated at 37 °C for 30 min. Aliquots were stained and evaluated at 24, 48, 72 and 96 h after semen collection.

3.4.5 Sperm Chromatin Structure Assay

Study IV (paper IV): The sperm concentration of the samples was adjusted to approximately 2 million spermatozoa/mL by mixing aliquots (50 µL) with buffer containing 0.01 M Tris-HCl, 0.15 M sodium chloride and 1 mM EDTA (pH 7.4; TNE buffer). After snap-freezing in liquid nitrogen, the samples were stored at -80 °C until analysis.

Aliquots were frozen at 24, 48, 72 and 96 h after collection. Evaluations were made using FC. Samples were thawed on ice and stained with acridine orange (Johannisson *et al.*, 2014). Spermatozoa with single stranded DNA fluoresce red, whereas those with normal double stranded DNA fluoresce green. The ratio of red to (green + red) fluorescence provides a measure of the proportion of spermatozoa with damaged DNA in the population, i.e. the DNA fragmentation index (%DFI). The green and red fluorescence, as well as forward and side scatter, were collected and the ratio for each of the cells was calculated using FCSExpress version 2 (DeNovo Software, Thornhill, ON, Canada).

3.5 Modified Single Layer Centrifugation

Study IV: Two holes were cut in the lid of 50 mL centrifuge tubes, one in the middle to accommodate a sterile 5 mL plastic tube (Cytology Brush-Minitube-Celadice - Slovakia), and the second near the edge of the lid through which the sample could be added. Equicoll (Morrell *et al.*, 2014), 15 mL, was poured into each tube. An aliquot of each semen sample, adjusted to a sperm concentration of 100 $x10^6$/mL, was pipetted on top of the colloid through the second small hole at the edge of the lid. After centrifugation at 300×g for 20 mins using a swing-out rotor, the sperm pellet was recovered using a Pasteur pipette passed through the tube in the middle of the lid (Morrell *et al.*, 2014) and was resuspended in extender. Four treatment groups were formed: control and MSLC in EquiPlus with antibiotics (CA and SA, respectively); control and MSLC in EquiPlus without antibiotics (CW and SW, respectively), with a final

sperm concentration of $50x10^6$/mL in all samples. These samples were stored at 6°C for 96 h, considering the day of collection as day 0.

3.6 Bacteriology

3.6.1 Bacterial culture

Study I: Swabs or samples for the isolation of *T. equigenitalis* were streaked on to three agar plates: hematin agar, hematin with streptomycin and hematin with antibiotics (all agar plates manufactured at SVA for CEMO). The plates were incubated at 37°C in CO_2 for 3 and 7 days.

To identify other potentially pathogenic bacteria, samples were cultured on cattle blood agar, pseudomonas isolation agar (PIA) and bromocresol lactose purpur agar, and the plates were incubated at 37°C for 48 h. The agar plates were checked at 24 h and 48 h. If necessary, the relevant colonies were re-cultured on horse blood agar to obtain pure colonies before biochemical identification.

For study II, semen was serially diluted 4 times; samples from each dilution were plated and cultured under aerobic and anaerobic conditions. The following agars were incubated at 37°C for 48 h under aerobic conditions: McConkey agar, cattle blood agar, PIA and mannitol salt agar (MAST), whereas streptococcal selective agar, colistine oxolinic blood agar (COBA) and chocolate agar were incubated at 37°C for 48 h under carbon dioxide conditions. For the anaerobic culture, fastidious anaerobic agar (FAA) was used, incubating at 37°C for 48 h under anaerobic conditions.

3.6.2 Bacterial identification

Study I: Several methods were used to identify bacteria in different parts of the project.

Contagious equine metritis organism (CEMO)

Colonies were identified by appearance, enzyme kits (API-zym; BioMerieux, USA) used according to the manufacturer´s recommendation, (Engvall, 1985), monotail (Mono-Tayl-agglutination test (BioNor, Norge), ALA-test, catalase test (hydrogen peroxide). Gram Stain, oxidase test, and growth on horse blood agar in oxygen and CO_2 were used for identification. From 2013, it was possible to identify *T. equigenitalis* by MALDI-TOF MS.

Gram staining

Gram staining is used to differentiate between gram-positive and gram-negative bacteria, depending on the cell wall staining (Michael, 1983).

Coagulation test

This test was used for staphylococci, incubating one loop of bacteria with 0.5 mL rabbit plasma (diluted in 1:4 with water) at 37°C for 4 h. If coagulation occurred, the sample was positive, whereas a liquid sample was negative.

Analytical Profile Index API 20 E & API NE for oxidase-positive bacteria (BioMerieux, USA)

This index was used to identify members of the family of Enterobacteriaceae, performed according to the manufacturer´s recommendation.

MALDI-TOF MS (Bruker Daltonics, Germany) (Studies I, II and IV)

This mass spectrometry technique was used to identify bacteria in study IV, by pipetting pure single bacterial colonies, 1 µl of protein extract (acetonitrile/formic acid extraction) from 2 mL of late-exponential phase broth cultures on MALDI 96-target plate, two per bacterium, then coating with 1 µl matrix of an energy-absorbent organic compound (α-cyano-4-hydroxycinnamic acid matrix solution (10 mg/mL in 50% acetonitrile and 2.5% trifluoroacetic acid) (Singhal *et al.*, 2015). In studies I & II, the same method was used without the protein extract. Unidentified colony material from pure cultures of bacteria was mixed with serum broth containing 15% glycerol and frozen at -80 °C in 1mL sterile tubes.

In Study II, a four-step procedure was followed in an attempt to identify more bacteria: i) bacteria were analysed immediately after culture using MALDI-TOF MS; ii) all samples were re-run against several updated MALDI-TOF MS databases; iii) main spectra projections (MSP) were made for creation of a dendogram, to group together similar samples; and iv) representative bacteria were identified by 16S sequencing and new constructed MSP:s from sequenced bacteria were used for identification of other bacteria. In Study IV, unidentified samples after MALDI-TOF MS analysis were classified by partial 16S rRNA gene sequencing (Lane, 1991).

DNA extraction and 16S sequencing (Study III)

DNA extraction was done in the Clinical Sciences Research Laboratory at SLU using AllPrep DNA/RNA/miRNA Universal Kit Cat No. /ID: 80224 following the manufacturer´s instructions.

The DNA quality was tested using a Nano Drop 8000 Spectrophotometer (Thermo Scientific). The quality of the sample ratio ($A_{260/280}$) was 1.7 to 1.9, and the concentration from 7.3 to 38.6 ng/μL. The DNA samples were stored at -80 °C until sequencing.

The DNA samples were transported to SciLifeLab, Uppsala, on dry ice for sequencing. The 16S hypervariable regions were amplified according to the Ion 16S™ Metagenomics Kit user guide (rev C.0). The amplicon products were purified according to the same instructions, and were quantified on Agilent Bioanalyzer using the High Sensitivity DNA kit. Library preparation was performed on The AB Library Builder System according to the AB Library Builder™ System user guide, Ion Xpress™ Plus and Ion Plus Library Preparation, pp 22–30, and 40–41, using the “Ion Plus and Ion Xpress Plus" protocol card with “No Size Selection” and “Pre-Sheared” selected when starting the run. In total, 100 ng of input material was used for each sample. Ion Xpress P1 Adapter and Barcodes were employed. The library was amplified and purified (Ion 16S™ Metagenomics Kit user guide (rev C.0), pp22–24), with 5 cycles of the PCR programme. The final libraries were assessed and quantified with the Fragment Analyzer system, using the DNF- 474 High Sensitivity NGS Fragment analysis kit. Template preparation and chip loading were performed on the Ion Chef system using the ion 530 chip; sequencing was performed with the Ion S5™ XL system (Ion 520™ & 530™ Kit–Chef user guide (rev D.0)). Raw data was uploaded to Ion Reporter in BAM format and analyzed using Metagenomics 16S w1.1 workflow under default parameters (ion torrent sequencing for all, 2018). Both Curated MicroSEQ (R) 16S Reference Library v2013.1 and Curated Greengenes v13.5 databases were chosen as references (Ion Reporter, 2016).

Study IV

Sperm samples, as well as extenders with and without antibiotics, were diluted by adding an aliquot (1mL) to 9 mL 2SP medium (0.2 mol/L sucrose in 0.02 mol/L phosphate buffer, supplemented with 10% fetal calf serum), 1 to 5 h after collection. The mixture was vortexed, and serially diluted to 1×10^{-8}. Aliquots (0.1 mL) from the diluted samples were plated on agar plates in triplicate as follows: Columbia agar with 5% sheep blood, Schaedler agar with vitamin K1 and 5% sheep blood (both BBL™, BD Diagnostics, Schwechat, Austria) and Pleuropneumonia-Like-Organism (PPLO) agar (Difco™, BD

Diagnostics, Schwechat, Austria) supplemented with 20% horse serum (Gibco™, Thermo Fisher Scientific, Vienna, Austria). Agar plates were incubated at 37 ºC under microaerobic conditions for PPLO agar, at 37 ºC in an anaerobic jar with gas packs (BD Diagnostics, Schwechat, Austria) for Schaedler agar, and Columbia agar plates were incubated in ambient air at 33ºC. Bacterial growth on plates was examined daily up to 96 h of incubation. Total bacterial concentration was calculated by counting bacterial colonies from triplicates. For identification of mycoplasma colonies, PPLO broth (Difco™, BD Diagnostics, Austria) was used, transferring mycoplasma colonies from PPLO agar and incubating at 37 ºC until the broth medium changed color.

To identify bacteria, the colonies were selected by morphological examination, both macro- and microscopically, for MALDI-TOF MS. If identification was not possible by MALDI - TOF MS, they were identified by partial 16S rRNA gene sequencing (Lane, 1991). Resultant sequences were subjected to a similarity search against the EzBioCloud database (Yoon *et al.*, 2017) (http://www.ezbiocloud.net/identify). Sequence similarity values of ≥ 98.7% and ≥ 95% were applied as indicatory cut-off values for genus and species affiliation, respectively.

3.7 Statistical analysis

Data were analysed using Statistical Analysis System software (ver. 9.4, SAS Inst. Inc., Cary, NC) and Pearson correlations; a p value <0.05 was considered to be statistically significant. Graphics were drawn using Microsoft excel 2013 software and R Studio software.

Study I and II: Data were analysed using PROC FREQ.

Study III: Data were analysed using R 3.3.1 software. Pearson correlations were made between the various bacterial genera and bacterial count.

Study IV:Paper IV: Diagnostic plots were used to test normality; data were analyzed using repeated measures, with stallions and ejaculates as random factors, and treatments and days as variables, using PROC MIXED (Wang & Goonewardene, 2004). The results are presented as Least Squares Means ± Standard Error of Mean. Pearson correlations were made between the various parameters of bacterial count and sperm quality.

Paper V: Before analysis, data were tested for normal distribution by diagnostic plots, and were log transformed if they were not normally distributed; PROC MIXED was used; random factors were ejaculates and stallions, treatments were variable factors. Data are presented as Least Squares Means ± Standard Error of Mean. Pearson correlations were made between the various bacterial species and total bacterial concentration (Olsson, 2011).

4 Results

The results for Studies I – IV are as follows:

4.1 Study I

From the 25512 samples tested for CEMO in Sweden in the period 2007 to 2017, 11 positive animals (53 positive samples) were identified (Table 1). The last positive animal was in 2015. Positive animals were re-examined after treatment; 10 of the positive animals were treated successfully, becoming negative, whereas no information is available about the remaining animal.

Table 1. *Distribution of samples for bacterial growth and CEMO according to year.*

	2007	2008	2009	2010	2011	2012	2013	2014	2015	2016	2017
bacterial growth											
Semen samples	65	145	141	127	40	70	41	36	35	21	9
Animals per year	57	72	87	85	40	66	38	35	27	20	8
Positive samples	1	8	3	2	4	3	1	2	6	4	3
Positive animals	1	5	3	2	4	2	1	2	5	4	3
CEMO											
Stallions tested/ year	511	494	485	532	478	482	452	480	469	424	423
Positive stallions	3 (0.6%)	1 (0.2%)	3 (0.6%)	2 (0.4%)	1 (0.2%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	1 (0.2%)	0 (0.0%)	0 (0.0%)
Samples	2434	2504	2518	2570	2410	2556	2381	2471	2111	1804	1753
Positive samples	20 (0.8%)	2 (0.1%)	15 (0.6%)	5 (1.2%)	7 (0.3%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	4 (0.2%)	0 (0.0%)	0 (0.0%)

The samples originated from more than 70 studs distributed all over Sweden; from 730 animals, 32 were positive for the presence of potentially pathogenic bacteria (Table 1). The most frequently detected bacteria were *K. pneumoniae* (12 animals) and beta haemolytic streptococci (10 animals), with *P. aeruginosa* (5 animals) being isolated less frequently (Table 2).

According to geographical distributed, three clusters of animals were observed, one cluster in Skåne (117 animals), the second in Stockholm, Uppsala and Örebro counties (88 animals) and the third in Western Götaland and Östergötland counties (70 animals). These studs were distributed mostly in the middle and south of Sweden.

Only potentially pathogenic bacteria were identified. Non-pathogenic bacteria were classified as “no specific infection detected”.

Table 2. *Proportion of samples positive for potential pathogens.*

Reported bacteria	Samples	animals
Klebsiella pneumoniae	15	11
beta haemolytic streptococci	10	10
Klebsiella oxytoca	2	2
Pseudomonas aeruginosa	5	4
Klebsiella pneumoniae/*Pseudomonas aeruginosa*	2	1
Total	34	28

4.2 Study II

Sixtyfour % (121) of the bacteria were identified by MALDI-TOF; twenty genera were found. *Micrococcus* spp. and *Staphylococcus* spp. were isolated from all stallions and also from the extender. *Kocuria* sp., *Mycoplasma* spp., *Neisseria* sp., *Serratia* sp., *Arthrobacter* spp., *Bacillus* spp., *Kytococcus* sp., *Psychobacter* spp. and *Bacteroides* spp. were isolated from any one of the five animals (Table 3). Regarding bacterial load, the results showed different numbers of bacterial genera in different agars, and the count varied between animals; the lowest total was 40700 colony-forming units/ml (cfu/mL) in stallion 5, whereas the highest total bacterial count was 915000 in stallion 1. Semen from stallions 2, 3 and 4 had 102000 cfu/mL, 189000 cfu/mL, 100500 cfu/mL respectively, and the semen extender contained 80 cfu/mL (Table 4).

Table 3. *Bacteria and number of positive colonies isolated per animal and from the extender.*

	Bacteria	stallion 1	stallion 2	stallion 3	stallion 4	stallion 5	extender
1	*Micrococcus* spp.	+	+	+	+	+	+
2	*Acinetobacter* spp.	+	0	+	+	+	0
3	*Kocuria* sp.	+	0	0	0	0	0
4	*Mycoplasma* spp.	0	0	+	0	0	+
5	*Staphylococcus* spp.	+	+	+	+	+	+
6	*Streptococcus* spp.	+	0	+	+	+	+
7	*Neisseria* sp.	+	0	0	0	0	0
8	*Pseudomonas* spp.	+	+	0	0	+	0
9	*Serratia* sp.	+	0	0	0	0	0
10	*Bacillus* spp.	0	0	0	0	+	+
11	*Corynebacterium* spp.	0	+	0	0	+	0
12	*Oligella* spp.	0	+	+	+	0	0
13	*Arthrobacter* spp.	0	0	+	0	0	0
14	*Brevibacterium* spp.	0	0	+	+	0	0
15	*Kytococcus* sp.	0	0	0	+	0	0
16	*Aerococcus* spp.	+	+	0	+	+	0
17	*Bacteroides* spp.	0	0	0	+	0	0
18	*Advenella* spp.	+	0	0	0	+	0
19	*Psychobacter* spp.	+	0	0	0	0	0
20	*Propionobacterium* spp.	0	+	0	+	0	0
	Total	11	7	8	10	9	5

Table 4. *Number of bacteria isolated on different agar plates (Colony-forming units/mL).*

Agar plate	Bacteria	stallion 1	stallion 2	stallion 3	stallion 4	stallion 5	extender
blood	*Aerococcus* spp.	1000	200	0	10	800	0
	Staphylococcus spp.	9000	100	0	250	35	1
	Bacillus spp.	0	0	0	0	10	1
	Acinetobacter spp.	5000	0	0	0	0	0
	Streptococcus spp.	10000	0	2500	0	0	0
	Psychobacter spp.	10000	0	0	0	0	0
	Advenella spp.	12500	0	0	0	0	0
	Neisseria sp.	100	0	0	0	0	0
	Oligella spp.	0	200	2000	0	0	0
	Micrococcus spp.	0	20	0	0	0	20
	Brevibacterium spp.	0	0	21000	100	0	0
	Arthrobacter spp.	0	0	100	0	0	0
	Corynebacterium spp.	0	0	0	200	0	2
Chocolate	*Micrococcus* spp.	1000	0	30000	200	1100	7
	Aerococcus spp.	2000	100	0	0	400	0
	Staphylococcus spp.	0	0	3000	550	100	10
	Acinetobacter spp.	3000	0	40000	0	0	0
	Kocuria sp.	200	0	0	0	0	0
	Mycoplasma spp.	0	0	20000	0	0	10
	Oligella spp.	0	0	0	4000	0	0
	Kytococcus sp.	0	0	0	100	0	0
COBA	*Staphylococcus* spp.	0	0	0	0	150	0
	Aerococcus spp.	0	10	0	0	0	0
FAA	*Corynebacterium* spp.	0	7000	0	0	0	0
	Propionobacterium spp.	0	1600	0	3000	0	0
	Bacteroides spp.	0	0	0	100	0	0
	Streptococcus spp.	0	0	200	0	0	0
	Acinetobacter spp.	2000	0	10	1	16	0
McConkey	*Advenella* spp.	2000	0	0	0	10	0
	Pseudomonas spp.	0	0	0	0	1	0
	Oligella spp.	0	0	100	0	0	0
MAST	*Staphylococcus* spp.	5000	0	0	900	0	1
PIA	*Pseudomonas* spp.	1	2	0	0	1	0
PCA		915000	102000	189000	100500	40700	80

4.3 Study III

In this study, 83 bacterial genera (Tables 5, 6 & 7) were identified using 16 S sequencing. The bacteria found most frequently were *Porphyromonas* spp., *Corynebacterium* spp., *Finegoldia* spp., *Peptoniphilus* spp., *Mobiluncus* spp., *Chondromyces* spp., *Suttonella* spp., *Treponema* spp., *Acinetobacter* spp., and *Campylobacter* spp. Some of these bacteria were isolated from all seven stallions (Table 5).

Table 5. *Bacteria identified from all seven stallions. (operational taxonomic units).*

	Bacteria	**1**	**2**	**3**	**4**	**5**	**6**	**7**
1	*Acinetobacter* spp.	5705	3413	3458	938	8488	141	769
2	*Facklamia* spp.	103	568	63	115	172	71	28
3	*Peptoniphilus* spp.	27504	25327	5047	4590	7317	1723	563
4	*Finegoldia* spp.	65365	30713	10515	33873	13820	31572	6553
5	*Porphyromonas* spp.	37147	44236	92553	6645	2777	9321	44352
6	*Corynebacterium* spp.	9865	32244	11456	17734	112738	25202	3246
7	*Tessaracoccus* spp.	105	4249	156	101	1322	653	263
8	*Propionibacterium* spp.	192	1056	194	227	1481	731	379
9	*Mobiluncus* spp.	4377	27030	2942	7280	507	2594	6309
10	*Propioniferax* spp.	10	797	134	85	415	148	40
11	*Suttonella* spp.	3355	276	208	3268	601	814	22012

Table 6. *Bacteria isolated from semen samples from stallions (operational taxonomic units).*

No	Bacteria	1	2	3	4	5	6	7
1	*Saccharofermentans* spp.	3431	9239	1333	31	812	1242	0
2	*Brooklawnia* spp.	121	810	263	0	207	135	57
3	*Oligella* spp.	28	683	174	139	26	27	0
4	*Campylobacter* spp.	15	443	16942	541	0	573	2201
5	*Pseudoclavibacter* spp.	0	17	68	140	5413	148	15
6	*Granulicatella* spp.	0	280	109	189	79	138	56
7	*Devriesea* spp.	31	150	109	0	23	104	43
8	*Desulfovibrio* spp.	160	14	1578	0	946	1644	0
9	*Brachybacterium* spp.	149	55	130	10	211	0	0
10	*Pseudomonas* spp.	0	17	91	12	13	0	21
11	*Jonesia* spp.	13	301	238	0	0	193	48
12	*Aerococcus* spp.	305	517	14	130	675	0	0
13	*Anaerococcus* spp.	0	459	0	86	174	0	158
14	*Staphylococcus* spp.	0	927	0	22	65	0	11
15	*Pyramidobacter* spp.	899	0	107	0	2764	2476	0
16	*Advenella* spp.	57	31	73	0	0	0	38
17	*Brevibacterium* spp.	0	0	0	141	5125	139	0
18	*Kocuria* spp.	14	23	0	0	328	0	0
19	*Herbiconiux* spp.	0	0	0	42	3684	62	0
20	*Treponema* spp.	0	0	0	10458	4170	9598	0
21	*Chondromyces* spp.	0	0	0	0	14269	27844	0
22	*Paracoccus* spp.	0	0	24	0	0	0	14
23	*Carnobacterium* spp.	14	0	0	0	39	0	0
24	*Streptomyces* spp.	0	27	0	0	55	0	0
25	*Sphingobacterium* spp.	20	0	0	14	0	0	0
26	*Dialister* spp.	0	0	629	12	0	0	0
27	*Jeotgalicoccus* spp.	52	0	0	0	70	0	0
28	*Dermabacter* spp.	0	0	0	0	173	10	0
29	*Citricoccus* spp.	0	11	15	0	0	0	0
30	*Synergistes* spp.	0	0	0	0	951	37	0
31	*Nocardioides* spp.	0	11	0	0	46	0	0
32	*Jonquetella* spp.	0	12	0	0	0	0	14

Table 7*: Bacteria isolated from any one of the seven stallions.*

	Bacteria	1	2	3	4	5	6	7
1	*Brachymonas* spp.	0	0	0	43	0	0	0
2	*Ralstonia* spp.	0	2822	0	0	0	0	0
3	*Rhodococcus* spp.	0	45	0	0	0	0	0
4	*Hydrogenophaga* spp.	0	0	0	181	0	0	0
5	*Macrococcus* spp.	0	0	0	0	104	0	0
6	*Phenylobacterium* spp.	0	0	0	0	10	0	0
7	*Peptostreptococcus* spp.	0	0	367	0	0	0	0
8	*Cardiobacterium* spp.	0	0	0	0	0	0	27
9	*Moryella* spp.	0	0	111	0	0	0	0
10	*Marmoricola* spp.	0	0	0	0	25	0	0
11	*Varibaculum* spp.	0	67	0	0	0	0	0
12	*Proteiniphilum* spp.	0	0	0	0	0	0	24
13	*Leuconostoc* spp.	0	246	0	0	0	0	0
14	*Petrobacter* spp.	0	1612	0	0	0	0	0
15	*Sphingomonas* spp.	0	0	0	10	0	0	0
16	*Diaphorobacter* spp.	0	59	0	0	0	0	0
17	*Microbacterium* spp.	21	0	0	0	0	0	0
18	*Bacteroides* spp.	0	30	0	0	0	0	0
19	*Hydrogenophilus* spp.	0	38	0	0	0	0	0
20	*Acidocella* spp.	0	399	0	0	0	0	0
21	*Desemzia* spp.	0	0	0	0	12	0	0
22	*Arthrobacter* spp.	0	0	11	0	0	0	0
23	*Actinomyces* spp.	0	52	0	0	0	0	0
24	*Pseudoxanthomonas* spp.	0	0	0	0	11	0	0
25	*Cellvibrio* spp.	0	0	0	0	0	11	0
26	*Tepidiphilus* spp.	0	332	0	0	0	0	0
27	*Wautersiella* spp.	0	0	34	0	0	0	0
28	*Streptococcus* spp.	0	753	0	0	0	0	0
29	*Pelomonas* spp.	0	10	0	0	0	0	0
30	*Moraxella* spp.	0	0	0	0	52	0	0
31	*Negativicoccus* spp.	0	11	0	0	0	0	0
32	*Propionimicrobium* spp.	0	11	0	0	0	0	0

continued

	Bacteria	1	2	3	4	5	6	7
33	*Prevotella* spp.	0	57	0	0	0	0	0
34	*Bergeyella* spp.	0	0	11	0	0	0	0
35	*Burkholderia* spp.	0	165	0	0	0	0	0
36	*Neisseria* spp.	0	203	0	0	0	0	0
37	*Mycoplasma* spp.	0	0	0	2626	0	0	0
38	*Veillonella* spp.	0	82	0	0	0	0	0
39	*Micrococcus* spp.	0	10	0	0	0	0	0
40	*Haemophilus* spp.	0	47	0	0	0	0	0

4.4 Study IV

This study is reported in detail in papers IV and V.

4.4.1 Paper IV:

The focus of this paper was the effect of antibiotics and preparation by MSLC on sperm quality during storage.

Sperm Motility and kinematics

Sperm total motility was improved in the MSLC samples compared to controls; the difference increased with storage time i.e. the controls deteriorated more rapidly than the MSLC samples (Figure 2).

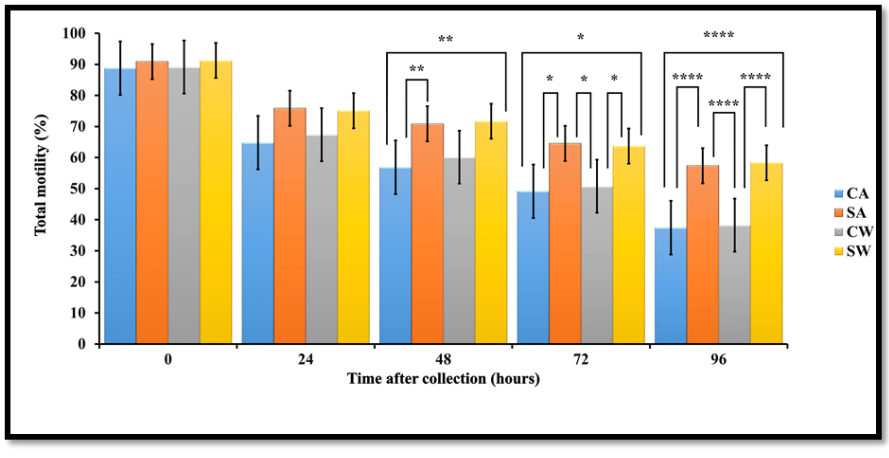

Figure 2. Total motility in control and MSLC samples, with and without antibiotics, during storage for 96 h at 6 ˚C. Values are Least Squares Means ± Standard Error of Mean (n = 18), * $P < 0.05$, ** $P < 0.01$, **** $P < 0.0001$.Abbreviations: CA, control with antibiotics; SA, modified single layer centrifugation with antibiotics; CW, control without antibiotics; SW, modified single layer centrifugation without antibiotics.

A similar pattern was seen for the kinematics (Table 8).

Table 8. *Sperm kinematics for control and treatment groups, with and without antibiotics at 0 to 96 h (Least Squares Means ± Standard Error of Mean; n = 18), Similar letters within rows indicate statistical difference between columns for the same parameter,* [a,b] *P < 0.05,* [c,d] *P < 0.01. Abbreviations: CA, control with antibiotics; SA, modified single layer centrifugation with antibiotics; CW, control without antibiotics; SW, modified single layer centrifugation without antibiotics; 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; BCF, beat cross frequency.*

Time	kinematics	CA	SA	CW	SW
0h	PM%	80.83±4.62	83.60±4.62	81.27±4.62	84.29±4.62
	VAP(µm/s)	93.97±3.20[a]	79.02±3.20[ac]	95.30±3.20[c]	82.13±3.20
	VCL(µm/s)	166.14±5.44[c]	137.84±5.44[c]d	163.80±5.44[d]	143.15±5.44
	VSL(µm/s)	83.75±2.86[a]	70.76±2.86[ac]	85.46±2.86b[c]	73.29±2.86[b]
	STR	0.89±0.01	0.89±0.01	0.89±0.01	0.89±0.01
	LIN	0.50±0.01	0.50±0.01	0.51±0.01	0.50±0.01
	WOB	0.56±0.01	0.56±0.01	0.57±0.01	0.57±0.01
	ALH(µm)	3.68±0.14	3.18±0.14	3.74±0.14	3.19±0.14
	BCF(Hz)	33.90±0.63	33.14±0.63	34.40±0.63	32.68±0.63
24h	PM%	49.74±4.62	59.41±4.62	52.61±4.62	57.97±4.62
	VAP(µm/s)	78.60±3.20	68.40±3.20	78.71±3.20	65.59±3.20
	VCL(µm/s)	144.83±5.44	129.60±5.44	145.28±5.44	124.82±5.44
	VSL(µm/s)	61.38±2.86[a]	52.31±2.86	61.91±2.86[b]	49.32±2.86[ab]
	STR	0.78±0.01	0.76±0.01	0.78±0.01	0.75±0.01
	LIN	0.42±0.01	0.40±0.01	0.42±0.01	0.39±0.01
	WOB	0.54±0.01	0.52±0.01	0.54±0.01	0.52±0.01
	ALH(µm)	3.91±0.14	3.62±0.14	3.84±0.14	3.45±0.14
	BCF(Hz)	31.30±0.63	29.79±0.63	31.27±0.63	29.41±0.63

continued

Time	kinematics	CA	SA	CW	SW
48h	PM%	44.20±4.62	52.28±4.62	44.92±4.62	50.94±4.64
	VAP(μm/s)	73.58±3.20^{ac}	59.66±3.20^{a}	72.58±3.20^{b}	57.39±3.26^{cb}
	VCL(μm/s)	139.63±5.44^{ac}	114.81±5.44^{ab}	138.72±5.44^{db}	111.84±5.43^{cd}
	VSL(μm/s)	56.01±2.86^{c}	44.19±2.86	55.07±2.86^{a}	41.71±2.91^{ca}
	STR	0.75±0.01	0.73±0.01	0.75±0.01	0.72±0.01
	LIN	0.39±0.01	0.38±0.01	0.39±0.01	0.37±0.01
	WOB	0.52±0.01	0.52±0.01	0.52±0.01	0.51±0.01
	ALH(μm)	3.79±0.14^{ac}	3.20±0.14^{ab}	3.82±0.14^{bd}	3.10±0.14^{cd}
	BCF(Hz)	29.11±0.63	29.27±0.63	29.53±0.63	28.61±0.63
72h	PM%	35.13±4.62	42.97±4.62	37.44±4.62	43.46±4.62
	VAP(μm/s)	67.02±3.20^{a}	55.49±3.20	65.79±3.20	53.26±3.20^{a}
	VCL(μm/s)	129.66±5.44	107.80±5.44	128.44±5.44^{a}	104.56±5.44^{a}
	VSL(μm/s)	49.33±2.86	39.17±2.86	48.40±2.86	37.87±2.86
	STR	0.73±0.01	0.70±0.01	0.73±0.01	0.71±0.01
	LIN	0.38±0.01	0.36±0.01	0.37±0.01	0.36±0.01
	WOB	0.52±0.01	0.51±0.01	0.51±0.01	0.51±0.01
	ALH(μm)	3.70±0.14^{cd}	3.04±0.14^{c}	3.56±0.14	3.00±0.14d
	BCF(Hz)	27.54±0.63	28.21±0.63	27.86±0.63	28.30±0.63
96h	PM%	25.77±4.62	34.97±4.62	28.52±4.62	35.11±4.62
	VAP(μm/s)	58.22±3.20	53.72±3.20	60.50±3.20	51.10±3.20
	VCL(μm/s)	110.36±5.44	104.95±5.44	121.38±5.44	98.54±5.44
	VSL(μm/s)	42.47±2.86	37.33±2.86	44.71±2.86	35.84±2.86
	STR	0.73±0.01^{a}	0.69±0.01^{ac}	0.73±0.01^{bc}	0.69±0.01^{b}
	LIN	0.39±0.01^{a}	0.35±0.01^{a}	0.36±0.01	0.36±0.01
	WOB	0.54±0.01	0.51±0.01	0.49±0.01	0.52±0.01
	ALH(μm)	3.56±0.14	3.12±0.14	3.42±0.14	3.00±0.14
	BCF(Hz)	25.55±0.63	27.89±0.63	27.38±0.63	27.41±0.63

Membrane Integrity

Sperm MI was improved in MSLC samples compared to controls, with significant differences between control samples (with and without antibiotics) and MSLC samples (with and without antibiotics) at all time points (Figure 3).

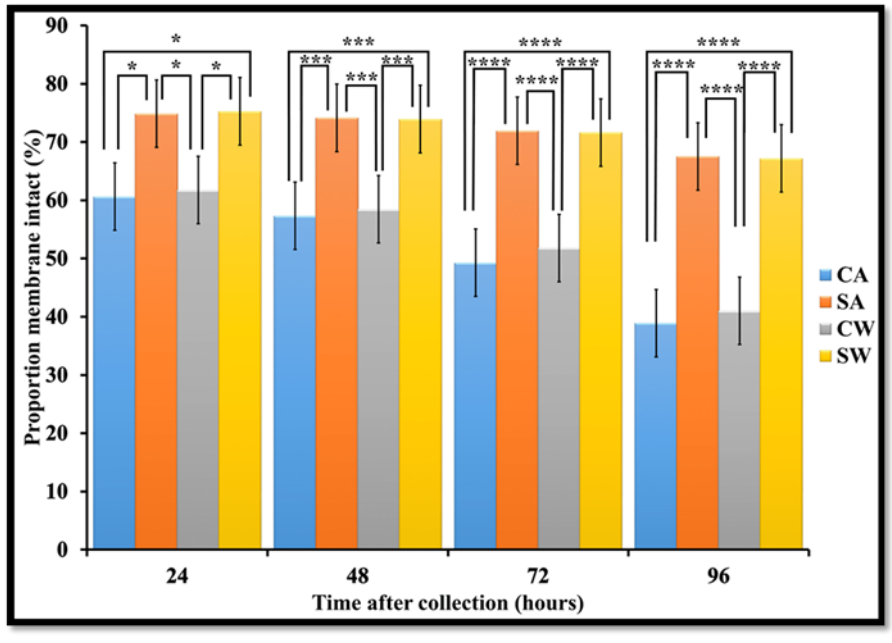

Figure 3. Membrane integrity in control and MSLC samples, with and without antibiotics, during storage for 96 h at 6 ºC. Values are Least Squares Means ± Standard Error of Mean (n = 18), * $P <$ 0.05, *** $P < 0.001$, **** $P < 0.0001$.Abbreviations: CA, control with antibiotics; SA, modified single layer centrifugation with antibiotics; CW, control without antibiotics; SW, modified single layer centrifugation without antibiotics.

Mitochondrial Membrane Potential

The MMP results are shown in Table 8. Significant differences ($P < 0.05$) were found at 24h between CA and SA in both MMP low and high, and between CA and SW at 72h for MMP low. Significant differences ($P < 0.01$) were seen between CA and SW at 24h for MMP in both MMP high and low, and at 72h in MMP high only (Table 9).

Table 9. *Mitochondrial membrane potential for samples groups, control and treatments, with and without antibiotics (Least Squares Means ± Standard Error of Mean; n=18), similar letters indicate statistical difference between columns for the same time point,* [a] *P <0.05,* [b] *P <0.01.Abbreviations: JC-1 low, low Mitochondrial Membrane Potential; JC-1 high, high Mitochondrial Membrane Potential.*

JC-1 low %	CA	SA	CW	SW
24h	48.97±6.63[ab]	34.60±6.63[a]	46.83±6.63	33.61±6.66[b]
48h	55.96±6.63	51.70±6.63	56.03±6.63	48.74±6.63
72h	69.64±6.63[a]	59.11±6.63	64.93±6.63	55.37±6.63[a]
96h	77.23±6.63	66.14±6.63	73.11±6.63	66.88±6.63
JC-1 high%				
24h	48.92±6.63[ab]	62.96±6.63[a]	50.87±6.63	64.05±6.63[b]
48h	41.85±6.63	46.65±6.63	42.22±6.63	49.36±6.66
72h	29.15±6.63[b]	39.83±6.63	34.14±6.63	43.59±6.63[b]
96h	21.83±6.63	33.16±6.63	26.27±6.63	32.40±6.63

Sperm Chromatin Structure Assay

The results for SCSA are shown in Figure 4. Control groups had significantly higher ($P < 0.0001$) levels of %DFI than MSLC on all days.

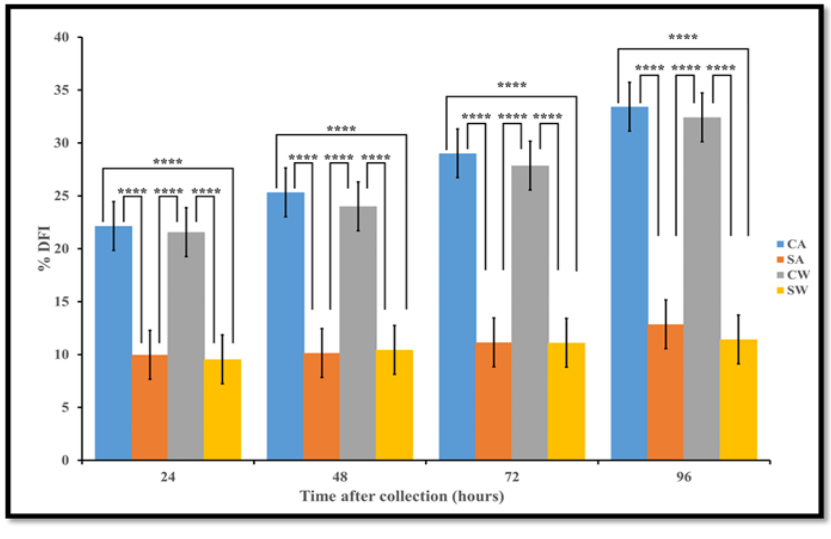

Figure 4. DNA fragmentation index for control and MSLC samples, with and without antibiotics (Least squares means ± Standard Error of Mean; n = 18), **** $P < 0.0001$. Abbreviations: CA, control with antibiotics; SA, modified single layer centrifugation with antibiotics; CW, control without antibiotics; SW, modified single layer centrifugation without antibiotics.

Bacteriology

In this experiment, CA corresponds to the sample normally used in AI. Therefore the bacterial load in all samples was compared with the "industry standard" by normalising the total bacterial colony counts relative to CA (=1 arbitrary unit) (Figure 5). The highest number of bacteria appeared in CW, and the lowest in SA; there were variations in bacterial count between stallions. There was a significant negative correlation ($r = -0.24$; $P < 0.05$) between total bacterial concentration and PM.

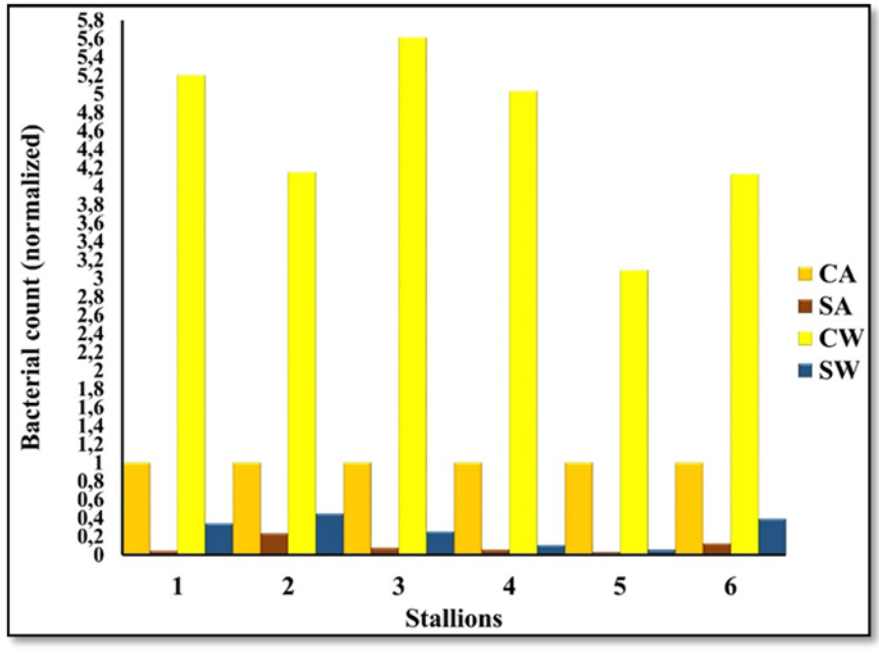

Figure 5. Total bacterial colony counts per treatment group relative to control with antibiotics (at 0 h). Control with antibiotics has been normalized to 1 arbitrary unit. Abbreviations: CA, control with antibiotics; SA, modified single layer centrifugation with antibiotics; CW, control without antibiotics; SW, modified single layer centrifugation without antibiotics.

4.4.2 Paper V:

The bacteria isolated from pony stallion semen samples in the presence or absence of antibiotics and with or without MSLC are shown in Table 10. In total, 61 species from 31 bacterial genera were identified (Tables 10 &11).

Table 10. *Bacteria (colony forming units/mL) according to treatment group, classified according to number in the original ejaculate, and the change after MSLC (Least Squares Means ± Standard Error of Mean; n=18). Note: Similar letters within rows indicate statistical difference between columns for the same parameter, [a] P <0.05. Abbreviations: CA, control with antibiotics; SA, modified single layer centrifugation with antibiotics; CW, control without antibiotics; SW, modified single layer centrifugation without antibiotics, (↓) decrease, increase (↑) and (=) no change .*

Bacteria	CA $\times 10^3$	SA $\times 10^3$		CW$\times 10^3$	SW $\times 10^3$	
Porphyromonadaceae spp.	106±45	13±45	↓(87%)	242±45[a]	52 ± 45[a]	↓(79%)
Tessaracoccus spp.	73±63	6 ±63	↓(92%)	135±63	11 ± 63	↓(92%)
Finegoldia magna	61±49	12±49	↓(80%)	219±49	59±49	↓(73%)
Mariniluteicoccus spp.	56±62	1±62	↓(98%)	111±62	4±62	↓(96%)
Porphyromonas spp.	50±135	33±135	↓(33%)	261±135	39±135	↓(85%)
Peptostreptococcus spp.	49±39	21±39	↓(57%)	99±39	49±39	↓(51%)
Propionibacterium spp.	41±40	6±40	↓(85%)	102±40	10±40	↓(90%)
Brachybacterium spp.	35±79	0.6±79	↓(98%)	319±79[a]	5±79[a]	↓(98%)
Peptococcaceae sp.	33±25	10±25	↓(70%)	33±25	16±25	↓(52%)
Peptoniphilus spp.	36±40	11±40	↓(69%)	70±40	39±40	↓(44%)
Brevibacterium spp.	18.2±22.6	1.3 ±22.6	↓(93%)	68.7±22.6	6.0±22.6	↓(91%)
Dietzia spp.	17.6±13.5	0.4±13.5	↓(98%)	18.6±13.5	5.9±13.5	↓(68%)
Pseudoclavibacter spp.	7.6±11.1	1.1±11.1	↓(86%)	30.4±11.1[a]	1.1±11.1[a]	↓(96%)
Eubacteriaceae spp.	6.1±4.1	0.6 ±4.1	↓(90%)	7.7±4.1	6±4.1	↓(22%)
Staphylococcus spp.	0.7±1	0.7±1	=(0)	3.3±1	1.7±1	↓(49%)
Dermabacter hominis	0.6±0.4	0.1±0.4	↓(83%)	0.6±0.4	0.1±0.4	↓(83%)
Campylobacter sputorum	0.6±0.4	0.06±0.4	↓(90%)	0.6±0.4	0.06±0.4	↓(90%)
Mycoplasma subdolum	0.3±2.9	0.5±2.9	↑(67%)	8.9±2.9	8.4±2.9	↓(5.6%)
Streptococcus spp.	0.3±0.3	0.06±0.3	↓(80%)	0.6±0.3	0.6±0.3	=(0)
Agrococcus citreus	0.2±0.3	0.06±0.3	↓(75%)	0.6±0.3	0.06±0.3	↓(90%)
Micrococcus spp.	0.17±0.2	0.22±0.2	↑(33%)	017±0.2	0.22±196	↑(33%)
Kocuria spp.	0.1±11	0.1±11	=(0)	22.3±11	0.1±11	↓(99.5%)
Rothia aeria	0.06±2.8	0.06±2.8	=(0)	5.6±2.8	0.06±2.8	↓(99%)
Cutibacterium acnes	0.06±1.4	0.06±1.4	=(0)	2.8±1.4	0.06±1.4	↓(98%)
Oligella urethralis	0.06±0.3	0.06±0.3	=(0)	0.6±0.3	0.06±0.3	↓(90%)
Bacteroides heparinolytica	0.06±0.8	0	↓(100%)	1.7±0.8	0.06±0.8	↓(97%)

Table 11. *Numbers of bacteria according to treatment group (colony forming units/mL) and % increase after MSLC (Least Squares Means ± Standard Error of Mean; n=18). Abbreviations: CA, control with antibiotics; SA, modified single layer centrifugation with antibiotics; CW, control without antibiotics; SW, modified single layer centrifugation without antibiotics, increase (↑) and (=) no change.*

Bacteria	CA	SA		CW	SW	
Kytococcus sedentarius	56±96	56±96	= (0)	56±96	167±96	↑(183.3%)
Mobiluncus curtisii	56±14959	56±14959	= (0)	11111±14959	27778±14959	↑(150%)
Pyramidobacter spp.	56±1390	56±1390	= (0)	56±1390	2778±1390	↑(4533%)
Bacillus spp.	0	111±280		0	61±280	

The number of genera per animal varied from 9 to 19. The most frequently isolated bacteria were *Corynebacterium* spp. (Table 12 & Figure 6), which appeared in all 18 ejaculates, representing a variable proportion of the total bacterial count (25-85%) among stallions.

Table 12. *Corynebacterium spp. (colony forming units/mL) compared with other bacteria from different animals (Least Squares Means; n=18).*

Stallion	*Corynebacterium* spp. $\times 10^4$	Others $\times 10^4$	Proportion of *Corynebacterium* total bacteria
1	298	53	85%
2	80	24	77%
3	31	42	43%
4	55	57	49%
5	72	93	44%
6	52	155	25%

There was a strong positive correlation between total bacterial concentration and *Corynebacterium* spp. (r=0.93, p <0.0001). Other bacterial genera were found in one or more ejaculates, with only 15 genera being isolated from all three ejaculates from the same stallion. Nine genera were isolated from only one ejaculate out of the three from an individual stallion. Even the total bacterial number varied among ejaculates with $214 \times 10^4 \pm 68 \times 10^4$ cfu/mL (42%) in the first, $122 \times 10^4 \pm 68 \times 10^4$ cfu/mL (24%) in the second, and $169 \times 10^4 \pm 68 \times 10^4$ cfu/mL (34%) in the third ejaculate.

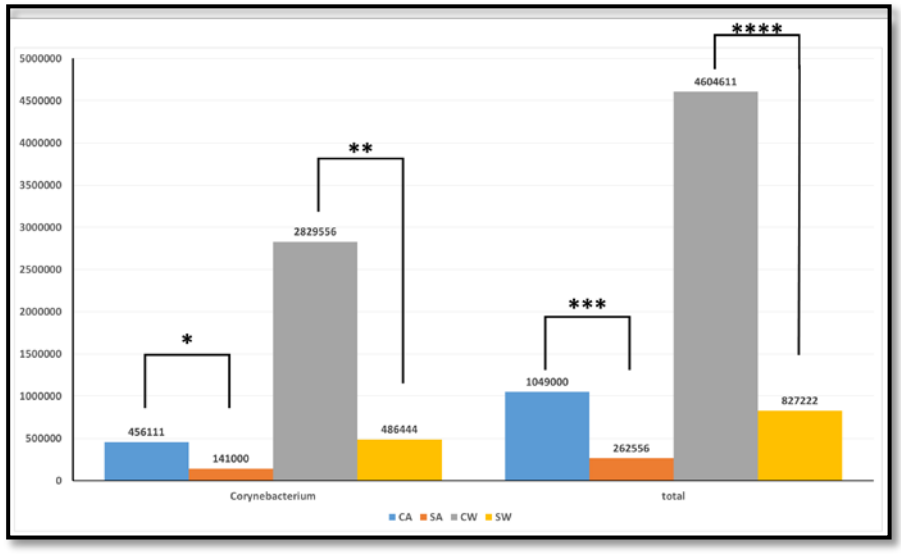

Figure 6. Total bacteria and *Corynebacterium* spp. (colony forming units/mL) according to treatments (Least Squares Means; n=18). Note: **P* <0.05, ***P* <0.01, ****P* <0.001 **** *P* <0.0001. Abbreviations: CA, control with antibiotics; SA, modified single layer centrifugation with antibiotics; CW, control without antibiotics; SW, modified single layer centrifugation without antibiotics.

There were significantly fewer bacteria (p <0.001) in CA ($10\times10^5 \pm 7.9\times10^5$ cfu/mL) than CW ($46\times10^5 \pm 7.9\times10^5$ cfu/mL colonies), also in SA ($2.6\times10^5 \pm 7.9\times10^5$ cfu/mL) compared to CA (p <0.001). Furthermore, there were fewer bacteria in SW than in CW ($8.3\times10^5 \pm 7.9\times10^5$ cfu/mL versus $46\times10^5 \pm 7.9\times10^5$ cfu/mL; p <0.0001). Thus, 75 % of the total bacteria were removed by MSLC from samples with antibiotics (p <0.001) and 82% of bacteria were removed from samples without antibiotics (p <0.0001) (Figure 6). The ability to remove bacteria depended on the genus (Tables 10 &11), but the reduction in bacterial number after MSLC varied from zero to 100% for SA, and from 5.6% to 99.5% for SW (Table 9). There was an apparent increase in *Kytococcus sedentarius*, *Mobiluncus curtisii* and *Pyramidobacter* spp. after MSLC, and *Bacillus* spp. appeared in MSLC samples only (Table 11). However, *Bacillus* spp. (3 cfu /mL) were identified from extender with antibiotics, 5 cfu/mL from extender without antibiotics, and 12 cfu/mL from extender without antibiotics exposed to the laminar air flow bench for 5 minutes and then left for 2 h at room temperature (22 ºC) before being sent for culture. There was no bacterial growth in distilled water.

5 Discussion

The project focused on bacteria present in stallion semen and their effects on sperm quality, on methods used to isolate and identify bacteria, and possibilities for reducing bacteria in semen during liquid storage. A physical method (MSLC) was used to separate spermatozoa from bacteria, and to improve sperm quality. The effect of antibiotics used in semen extenders on bacteria and sperm quality was also determined. Bacteria were identified to determine potential pathogens and the frequency with which they appeared in Sweden over a 10 year period, as well as to identify possible effects on sperm quality.

5.1 Sperm quality

Attention was focused on extending the shelf-life of semen doses beyond the 24-48h that is commonly used as an industry standard. Sperm quality was improved after MSLC, even in the absence of antibiotics. These results on improved sperm quality are in agreement with previous studies (Johannisson *et al.*, 2009; Lindahl *et al.*, 2012; Morrell *et al.*, 2016; Ortiz *et al.*, 2014; Morrell *et al.*, 2010; Morrell *et al.*, 2008b). The study by Johannisson *et al*. (2009) showed that the SLC samples were significantly better than control samples after storage for 48h, and there were increases in sperm viability, motility, morphologically normal spermatozoa, and a decrease in %DFI. In a further study, SLC samples were used for insemination after 96h storage with successful fertilization (Lindahl *et al.*, 2012). Sperm quality was also improved in donkey semen, with improvements in viability, morphology, TM, PM and sperm kinematics (Ortiz *et al.*, 2014). Therefore, using SLC enabled us to prolong the length of time for which stallion semen samples could be stored without affecting their quality, in agreement with previous studies (Morrell *et al.*, 2008b). In the present study, it was possible to compare sperm quality in samples with and without antibiotics, in addition to testing the effect of MSLC. It was interesting to note that the major

effect on sperm quality arose from MSLC, rather than from the presence or absence of antibiotics since there was little difference in sperm quality between controls with and without antibiotics, or between MSLC samples with and without antibiotics. A wider study, including other semen extenders, would be required to determine if antibiotics actually have a detrimental effect on sperm quality.

5.2 Antibiotics

Even without the addition of antibiotics to semen extenders, the sperm quality in MSLC samples was as good as in MSLC samples with antibiotics and the shelf-life of sperm samples was prolonged. These results are in contrast to those of Varner *et al.* (1998) who showed a decrease in sperm viability and motility parameters without antibiotics, and also showed differences when using different types of antibiotics. Polymixin B had a negative effect on sperm motility characteristics (Jasko *et al.,* 1993), whereas gentamicin reduced sperm motility and velocity (Aurich & Spergser, 2007). However, d-penicillamine in milk/egg yolk-based semen extender did not affect ejaculated sperm motility, viability and DNA integrity although the motility of epididymal sperm was decreased (Brogan *et al.,* 2015).

5.3 Removal of bacteria

The reduction in bacterial contamination in stallion semen after using MSLC is in agreement with previous studies (Morrell & Wallgren, 2014; Guimaraes *et al.,* 2015; Morrell *et al.,* 2014; Morrell *et al.,* 2013; Morrell & Wallgren, 2011; Nicholson *et al.,* 2000). For most bacteria identified, the bacterial load was lower for MSLC than control, in extender with or without antibiotics. Several studies showed that SLC reduced some bacterial species in semen samples, e.g. *Corynebacterium* spp. were reduced by 68%, and *Enterococcus* spp. by 100% (Morrell *et al.,* 2014). However, in another study the bacterial content of thawed samples was reduced from 5133 ± 3326 cfu/mL to 2668 ± 1239 cfu/mL (52%) in good samples, and from 24090 ± 49820 to 13930 ± 29030 cfu/mL (58%) in bad samples (Guimaraes *et al.,* 2015). Morrell & Wallgren (2011) were able to remove bacteria from six boar semen samples when the SLC was done immediately after semen collection, and bacterial contamination was reduced considerably in four samples where several hours elapsed between semen collection and preparation by SLC. By extrapolation, these results suggest that it might be possible to reduce bacterial contamination in stallion semen and possibly to reduce the use of antibiotics in semen extenders. However, since

pathogenic bacteria such as *T. equigenitalis* cannot be completely removed by SLC (Morrell *et al.,* 2014), it would still be necessary to check for their presence in stallions intended for semen collection for AI.

5.4 Identification of bacteria

Many methods are used for identifying bacteria, varying from conventional methods which are sensitive but time-consuming, to MALDI-TOF MS which is fast and accurate (Singhal *et al.,* 2015) but can only identify bacteria that are already in the database. Some bacteria need special growth conditions and are not able to grow, or grow slowly, under conventional conditions. In some cases it is not possible to differentiate bacterial strains that are very similar in appearance on the agar plate (Cash, 2009). Our results are in agreement with this previous work, since it was only possible to identify approximately 50% of isolated bacteria by MALDI-TOF MS from the first run. Therefore a more accurate method is required to be able to identify bacteria in semen samples. The methods used to identify bacteria in stallion semen include: Gram staining, automated biochemical and enzymatic essays (Corona and Cherchi, 2009), or catalase, oxidase and glucose oxidation-fermentation tests, completed by biochemical identification API20E and API 20N strips (Ortega-Ferrusola *et al.*, 2009). In contrast, Pasing *et al.* (2013) used MALDI-TOF MS, whereas Guimaraes *et al*. (2015) depended on morphological and biochemical analyses using the API identification system. None of these methods is optimal; therefore, the next step in our project was to use a more advanced method for bacterial identification. According to the scientific literature, 16S sequencing is fast, can identify fastidious bacteria that cannot be cultured easily, and even dead bacteria. However, it is expensive, needs experienced personnel and specialized equipment; the major advantage is that it does not require culturing of the bacteria first. Culture of bacteria may not provide a true representation of the total bacterial flora since species present in low numbers may be masked by other bacteria or it may be difficult to distinguish particular species on the basis of colony morphology (Singhal *et al.*, 2015; Rothberg *et al.,* 2011). After 16S sequencing, a high number of bacteria (83 genera) were identified. Only 11 genera were isolated from all seven stallions. The most frequently isolated bacteria in our study were *Corynebacterium* spp., in agreement with a number of other studies on the microbiome of stallion semen (Neto *et al.,* 2015; Pasing *et al.*, 2013; Ortega-Ferrusola *et al.,* 2009; Althouse *et al.,* 2010; Varela *et al.,* 2018). The bacteria genera and species isolated varied between studies; some bacteria may be isolated in one study but are absent in another. Even within a

study, bacteria may not be isolated from all animals or from all ejaculates from the same animal.

5.5 Pathogenic bacteria

Apart from the detrimental effects on sperm quality from bacterial metabolic byproducts and from bacteria competing with spermatozoa for nutrients in the semen extender, some bacteria can potentially cause diseases in inseminated mares. Some pathogenic bacteria could be transferred to mares during insemination, causing inflammation, abortion and infertility. In our study, pathogenic bacteria isolated from stallion semen in Sweden during the ten years from 2007 to 2017 were *T. equigenitalis*, *K. pneumoniae*, beta haemolytic streptococci, *P. aeruginosa* and a mixture of *K. pneumoniae* with *P. aeruginosa*. The last case of *T. equigenitalis* identified in Sweden in this study was one animal in 2015. The numbers had fallen progressively during the period studied, emphasizing the importance of testing stallions for pathogenic bacteria before using their semen for AI. Positive cases must be reported to the authorities and the animals treated to prevent the disease being spread. According to the Council of the European communities (European Commission, 1992), stallions must be tested 60 days before the first semen collection to determine that it is free from such viral and bacterial infection as equine infectious anemia, viral arteritis, and CEMO.

Identification of all bacteria in Study II, rather than only 64%, would provide an accurate picture of all bacterial genera appearing in semen from Swedish stallions. It would be interesting to compare the results obtained in studies II and III on material from the same ejaculates. It had been intended to use the same ejaculate in study II and III but the quality of the extracted DNA was unfortunately not sufficient for metagenomic analysis. Therefore, such a comparison has still to be done.

6 Conclusions

Regarding the results for studies I to IV, the following conclusions can be drawn:

The bacteria present in stallion semen varied among animals at the same stud and even among ejaculates from the same stallions. They were different to those found in similar studies in other countries, which may be due, in part, to the different methods used for identification. Most of the bacteria found were environmental but in some cases pathogenic bacteria were identified, such as *Klepsiella spp*., beta haemolytic streptococci and (previously) *T. equigenitalis.*

Many methods are used to isolate and identify bacteria; MALDI-TOF MS is a reliable method, but it is not able to identify all bacteria, depending on the ability to culture the bacteria and the database available. In contrast, 16 S sequencing is a more accurate method with high sensitivity that is able to identify most bacteria at the species level, even when they are present in low numbers. However, some bacteria may be missed during extraction of bacterial DNA.

Sperm quality was not affected by the presence or absence of antibiotics in semen extenders, although it was improved by MSLC. Bacterial growth was greater in the absence of antibiotics. No significant detrimental effect of antibiotics in EquiPlus extender on sperm quality was apparent in this study.

A large proportion of the bacterial population in semen can be removed using MSLC. Sperm quality was also improved, and shelf-life was increased to at least 96h.

7 Future considerations

Although vital for life, some bacteria are becoming more dangerous, especially after the appearance of many that are resistant to antimicrobials. These resistant bacteria cause high mortality in humans and animals. Apart from the economic loss due to animal death, treatment can be costly and the cost of developing new antibiotics should also be considered. Therefore, we need a better understanding of the bacteriome in general, and of the seminal bacteriome in particular. It would be interesting to use 16S sequencing on the same samples before and after colloid centrifugation to evaluate accurately which bacteria can be removed. We need to know which bacteria are present in semen doses for AI and their likely effects on sperm quality or the inseminated female. In this regard, we need to improve the MSLC technique to be able to remove more bacteria. However, sperm quality in cooled semen extended without antibiotics should be investigated and its effects on the mare after insemination determined, to optimize the use of antibiotics in semen in the equine breeding industry.

References

Aitken, R.J., Gibb, Z., Mitchell, L.A., Lambourne, S.R., Connaughton, H.S. & De Iuliis, G.N. (2012). Sperm motility is lost in vitro as a consequence of mitochondrial free radical production and the generation of electrophilic aldehydes but can be significantly rescued by the presence of nucleophilic thiols. *Biology of Reproduction*, 87(5): 110, pp. 1–11. DOI: 10.1095/biolreprod.112.102020.

Akhter, S., Ansari, M., Andrabi, S., Ullah, N. & Qayyum, M. (2008). Effect of antibiotics in extender on bacterial and spermatozoal quality of cooled buffalo (Bubalus bubalis) bull semen. *Reproduction in Domestic Animals*, 43(3), pp. 272-278. DOI: 10.1111/j.1439-0531.2007.00890.x.

Al-Essawe, E.M., Johannisson, A., Wulf, M., Aurich, C. & Morrell, J.M. (2018). Improved cryosurvival of stallion spermatozoa after colloid centrifugation is independent of the addition of seminal plasma. *Cryobiology*, 81, pp. 145-152. DOI: 10.1016/j.cryobiol.2018.01.009.

Althouse, G. C., Skaife, J.& Loomis, P. (2010). Prevalence and types of contaminant bacteria in extended, chilled equine semen. *Animal Reproduction Science*, 121S, pp. 224-225. DOI.org/10.1016/j.anireprosci.2010.04.090.

Althouse, G.C. (2008). Sanitary procedures for the production of extended semen. *Reproduction in Domestic Animals*, 43 Suppl 2, pp. 374-378. DOI: 10.1111/j.1439-0531.2008.01187.x.

Althouse, G.C., Kuster, C.E., Clark, S.G. & Weisiger, R.M. (2000). Field investigations of bacterial contaminants and their effects on extended porcine semen. *Theriogenology*, 53(5), pp. 1167-1176. DOI: 10.1016/S0093-691X(00)00261-2.

Anhalt, J.P. & Fenselau, C. (1975). Identification of Bacteria Using Mass-Spectrometry. *Analytical Chemistry*, 47(2), pp. 219-225. DOI./pdf/10.1021/ac60352a007.

Aurich, C. & Spergser, J. (2007). Influence of bacteria and gentamicin on cooled-stored stallion spermatozoa. *Theriogenology*, 67(5), pp. 912-918. DOI: 10.1016/j.theriogenology.2006.11.004.

Aurich, C., Spergser, J., Nowotny, N., Rosengarten, R. & Aurich, JE. (2003). Prevalence of venereal transmissible diseases and relevant potentially pathogenic bacteria in Austrian Noriker Draught horse stallions. *Wiener Tierarztliche Monatsschrift*, 90(5), pp. 124-130.

Aurich, J.E. (2012). Artificial insemination in horses-more than a century of practice and research (REVIEW). *Journal of Equine Veterinary Science*, 32(8), pp. 458-463. DOI.org/10.1016/j.jevs.2012.06.011

Biswas, S. & Rolain, J.M. (2013). Use of MALDI-TOF mass spectrometry for identification of bacteria that are difficult to culture (REVIEW). *Journal of Microbiological Methods*, 92(1), pp. 14-24. DOI: 10.1016/j.mimet.2012.10.014

Bjorndahl, L., Mohammadieh, M., Pourian, M., Soderlund, I. & Kvist, U. (2005). Contamination by seminal plasma factors during sperm selection. *Journal of Andrology*, 26(2), pp. 170-173. DOI.org/10.1002/j.1939-4640.2005.tb01080.x

Blanchard, T.L., Kenney, R.M. & Timoney, P.J. (1992). Venereal disease. *Veterinary Clinics of North America: Equine Practice*, 8 (1), pp.191-203. DOI: 10.1016/S0749-0739(17)30475-3

Brogan, P.T., Beitsma, M., Henning, H., Gadella, B.M. & Stout, T. A. (2015). Liquid storage of equine semen: Assessing the effect of d-penicillamine on longevity of ejaculated and epididymal stallion sperm. *Animal Reproduction Science*, 159, pp.155-162. DOI: 10.1016/j.anireprosci.2015.06.011

Brum, A. M., Sabeur, K. & Ball, B. A. (2008). Apoptotic-like changes in equine spermatozoa separated by density gradient centrifugation or after cryopreservation. *Theriogenology*, 69, pp. 1041-1055. DOI: 10.1016/j.theriogenology.2008.01.014

Buchanan, B.R., Seidel, G.E., McCue, P.M., Schenk, J.L., Herickhoff, L.A. & Squires, E.L. (2000). Insemination of mares with low numbers of either unsexed or sexed spermatozoa. *Theriogenology*, 53(6), pp. 1333-1344. DOI: 10.1016/S0093-691X (00)00276-4

Cai, Z.G., An, J.H., Liu, Y.L., Yie, S.M., Zhang, Y., Li, F.P., Chen, J.S., Wang, X., Morrell, J.M. & Hou, R. (2018). Single layer centrifugation improves the quality of frozen-thawed sperm of giant panda (Ailuropoda melanoleuca). *Animal Reproduction Science*. 195, pp. 58-64. DOI: 10.1016/j.anireprosci.2018.05.006.

Cash, P. (2009). Proteomics in the study of the molecular taxonomy and epidemiology of bacterial pathogens. *Electrophoresis*, 1, pp. 133–141. DOI: 10.1002/elps.200900059.

Chatdarong, K., Thuwanut, P. & Morrell, J.M. (2010). Single-layer centrifugation through colloid selects improved quality of epididymal cat sperm. *Theriogenology*, 73 (9), pp.1284–1292. DOI: 10.1016/j.theriogenology.2009.12.009

Colenbrander, B., Gadella, B.M. & Stout, T.A. (2003). The predictive value of semen analysis in the evaluation of stallion fertility. *Reproduction in Domestic Animals*, 38 (4), pp. 305-311.

Contri, A., Gloria, A., Robbe, D., Pio Sfirro, M. & Carluccio, A. (2012). Effect of sperm concentration on characteristics of frozen-thawed semen in donkeys. *Animal Reproduction Science*, 136, pp. 74-80. DOI: org/10.1016/j.anireprosci.2012.10.022

Corona, A. & Cherchi, R. (2009). Microbial quality of equine frozen semen. *Animal Reproduction Science*, 115(1-4), pp. 103-109. DOI: 10.1016/j.anireprosci.2008.11.016

Correa, J. R. & Zavos, P. M. (1996). Preparation and recovery of frozen-thawed bovine spermatozoa via various sperm selection techniques employed in assisted reproductive technologies. *Theriogenology*, 46(7), pp. 1225-1232.

Costa, A. L., Martins-Bessa, A., De Andrade, A. R., Guimaraes, T., Rebordao, M. R., Gamboa, S., Bravo, P. P., Correia, M. J., Colaco, J., Gsivao, I. & Rocha, A. (2012). Single Layer Centrifugation with Androcoll-E™ improved progressive motility and percentage of live spermatozoa with intact acrosome of chilled stallion semen but did not have an effect on DNA integrity. *Open Journal of Animal Sciences*, 2(3), pp. 159-165. DOI:10.4236/ojas.2012.23022.

Croxatto, A., Prod hom, G. & Greub, G. (2011). Applications of MALDI-TOF mass spectrometry in clinical diagnostic microbiology (REVIEW). *Federation of European Microbiological Societies Microbiology Reviews*, 36 (2), pp. 380-407. DOI.org/10.1111/j.1574-6976.2011.00298.x

Dell Aquila, M.E., Cho, Y.S., Minoia, P., Traina, V., Fusco, S., Lacalandra, G.M. & Maritato, F. (1997). Intracytoplasmic sperm injection (ICSI) versus conventional IVF on abattoir-derived and in vitro-matured equine oocytes. *Theriogenology*, 47, pp. 1139-1156. DOI.org/10.1016/S0093-691X(97)00095-2

Engvall, A. (1985). Survival of contagious equine metritis organisms (CEMO) in different transport media as influenced by storage time, temperature and contaminating flora. *Zentralbl veterinarmed B*, 32(6), pp. 454-459.

European Centre for Disease Prevention and Control. (2015). Surveillance Report, Antimicrobial resistance surveillance in Europe 2015. Annual Report of the European Antimicrobial Resistance Surveillance Network (EARS-Net). Available at: https://ecdc.europa.eu/sites/portal/files/media/en/publications/Publications/antimicrobial-resistance-europe-2015.pdf.

European Commission. (1992). Council Directive 92/65/EEC of 13 July 1992 laying down animal health requirements governing trade in and imports into the Community of animals, semen, ova and embryos not subject to animal health requirements laid down in specific Community rules referred to in Annex A (I) to Directive 90/425/EEC, *Official Journal L 268*, 14.9.1992, pp. 0054-0072.

Evenson, D. P., Sailer, B. L. & Jost, L.K. (1995). Relationship between stallion sperm deoxyribonucleic acid (DNA) susceptibility to denaturation in situ and presence of DNA strand breaks: implications for fertility and embryo viability. *Biology of Reproduction*, 52 (monograph_ series1), pp. 655–659. DOI: org/10,1093/biolreprod/52.monograph_series1.655

Foote, R. H. (2002). The history of artificial insemination: Selected notes and notables. *Journal of Animal Science*, 80, 1-10. DOI:org/10.2527/animalsci2002.80E-Suppl_21a

Foxcroft, G.R., Dyck, M.K., Ruiz-Sanchez, A., Novak, S. & Dixon, W. T. (2008). Identifying useable semen. *Theriogenology*, 70 (8), pp. 1324-1336. DOI: 10.1016/j.theriogenology.2008.07.015.

Frieri, M., Kumar, K. & Boutin, A. (2017). Antibiotic resistance (REVIEW). *Journal of infection and public health*, 10, pp.369-378. DOI: 10.1016/j.jiph.2016.08.007

Gibb, Z., Lambourne, S. R. & Aitken, R. J. (2014). The paradoxical relationship between stallion fertility and oxidative stress. *Biology of reproduction*, 91(3), 77. DOI:10.1095/biolreprod.114.118539

Goodla, L., Morrell, J.M., Yusnizar, Y., Stålhammar, H. & Johannisson, A. (2014). Quality of bull spermatozoa after preparation by single-layer centrifugation. *Journal of Dairy Science*, 97(4), pp. 2204-2212. DOI.org/10.3168/jds.2013-7607

Gravance, C. G., Garner, D. L., Baumber, J. & Ball. B.A. (2000). Assessment of equine sperm mitochondrial function using JC-1. *Theriogenology*, 53(9), pp.1691-1703. DOI.org/10.1016/S0093-691X (00)00308-3

Guimaraes, T., Lopes, G., Pinto, M., Silva, E., Miranda, C., Correia, M.J., Damasio, L., Thompson, G. & Rocha, A. (2015). Colloid centrifugation of fresh stallion semen before cryopreservation

decreased microorganism load of frozen-thawed semen without affecting seminal kinetics. *Theriogenology*, 83(2), pp. 186-191. DOI: 10.1016/j.theriogenology.2014.09.003

Heitmann, J., Kirchhoff, H., Petzoldt, K. & Sonnenschein, B. (1979). Isolation of acholeplasmas and mycoplasmas from aborted horse fetuses. *Veterinary Record*, 104 (15), 350.

Hossain, M. S., Johannisson, A., Wallgren, M., Nagy, S., Siqueira, A.P. & Rodriguez-Martinez, H. (2011). Flow cytometry for the assessment of animal sperm integrity and functionality: state of the art (REVIEW). *Asian Journal of Andrology*, 13 (3), pp. 406–419. DOI: 10.1038/aja.2011.15

Hurtgen, J. P. (2009). 'Semen Collection in Stallions', In Samper, J. C. (ed) *Equine Breeding Management and Artificial Insemination*. 2nd ed, USA pp. 33-39. DOI:org/10.1016/B978-1-4160-5234-0.X0001-3

Ion Reporter 2016. *Ion Reporter Metagenomics 16S algorithms overview* [pdf]. Thermo Fisher Scientific. Available at: http://tools.thermofisher.com/content/sfs/brochures/ion-reporter-16s-metagenomics-algorithms-whitepaper.pdf.

Ion torrent sequencing for all. (2018). Available at: https://ionreporter.thermofisher.com/ir/.

Ivanoff, E. I. (1922). On the use of artificial insemination for zootechnical purposes in Russia. *Journal of Agricultural Science*, 12(03), pp.244-256.

Jasko, D. J., Bedford, S. J., Cook, N. L., Mumford, E. L., Squires, E. L. & Pickett, B. W. (1993). Effect of antibiotics on motion characteristics of cooled stallion spermatozoa. *Theriogenology*, 40 (5), pp. 885-893.

Johannisson, A., Lundgren, A., Humblot, P. & Morrell, J. M. (2014). Natural and stimulated levels of reactive oxygen species in cooled stallion semen destined for artificial insemination. *Animal*, 8(10), pp. 1706–1714. DOI: 10.1017/S1751731114001499.

Johannisson, A., Morrell, J. M., Thoren, J., Jönsson, M., Dalin, A. M. & Rodriguez-Martinez, H. (2009). Colloidal centrifugation with Androcoll-E™ prolongs stallion sperm motility, viability and chromatin integrity. *Animal Reproduction Science*, 116, pp. 119–128. DOI: 10.1016/j.anireprosci.2009.01.008

Jordbruksverket 2017. Horses statistical report 2016. Available at: https://www.jordbruksverket.se/webdav/files/SJV/Amnesomraden/Statistik,%20fakta/Husdjur/JO24/JO24SM1701/JO24SM1701_inEnglish.htm#BM2.

Kirchhoff, H., Heitmann, J. & Bisping, W. (1980). Mycoplasmas isolated from the genital tract of mares. *Zentralblatt für Bakteriologie*. 246 (2), pp. 228–235.

Klug, E. & Sieme, H. (1992). Infectious agents in equine semen. *Acta Veterinaria Scandinavica*, 88S, pp. 73–81.

Kordan, W., Holody, D., Eriksson, B., Fraser, L., Rodriguez-Martinez, H. & Strzezek, J. (1998). Sperm motility inhibiting factor (SMIF) - a plasmatic peptide with multifunctional biochemical effects on boar spermatozoa. *Reproduction in Domestic Animals*, 33, pp. 347-354. DOI.org/10.1111/j.1439-0531.1998.tb01369.x

Kuster, C.E. & Althouse, G.C. (2016). The impact of bacteriospermia on boar sperm storage and reproductive performance. *Theriogenology*, 85 (1), pp. 21–26. DOI: 10.1016/j.theriogenology.2015.09.049

Lane, D. J. (1991). "16S/23S rRNA sequencing". In: Stackebrandt E, Goodfellow M, Eds. *Nucleic acid techniques in bacterial systematics*, New York, USA, pp.115–175.

Levy, S.B. & Marchall, B. (2004). Antibacterial resistance worldwide: causes, challenges and responses. *Nature Medicine*, 10 (12 Suppl), pp. 122-129. DOI: 10.1038/nm1145

Lindahl, J., Dalin, A. M., Stuhtmann, G. & Morrell, J. M. (2012). Stallion spermatozoa selected by single layer centrifugation are capable of fertilization after storage for up to 96 h at 6°C prior to artificial insemination. *Acta Veterinaria Scandinavica*, 54(1), pp.40-44. DOI: 10.1186/1751-0147-54-40

Lu, K. G. & Morresey, P. R. (2007). Infectious diseases in breeding stallions. *Clinical Techniques in Equine Practice*, 6 (4), pp. 285-290. DOI.org/10.1053/j.ctep.2007.09.008

Luo, S.M., Schatten, H. & Sun, Q.Y. (2013). Sperm mitochondria in reproduction: good or bad and where do they go? (REVIEW). *Journal of Genetics and Genomics*, 40 (11), pp. 549-556. DOI: 10.1016/j.jgg.2013.08.004

MacGowan, A. & Macnaughton, E. (2017). Prevention and control of infection, Antibiotic resistance. *Medicine*, 45 (10), pp.622-628. DOI: https://doi.org/10.1016/j.mpmed.2017.07.006

Maes, D., Nauwynck, H., Rijsselaere, T., Mateusen, B., Vyt, P., De Kruif, A. & Van Soom, A. (2008). Diseases in swine transmitted by artificial insemination: an overview. *Theriogenology*, 70 (8), pp. 1337-1345. DOI: 10.1016/j.theriogenology.2008.06.018

Malmgren, L.(1997). Assessing the quality of raw semen (REVIEW).*Theriogenology*, 48, pp. 523-530.

Michael, F. (1983). 'Gram staining technique' In: Razin, S. & Tully, J. G. (eds) *Methods in Mycoplasmology*. Academic Press, New York, 1, 39-40. ISBN.978-0-12-583801-6

Morrell, J. M., Persson, B., Tjellstörm, H., Laessker, A., Nilsson, H., Danilova, M. & Holmes, P. V. (2005). Effect of semen extender and density gradient centrifugation on the motility and fertility of turkey spermatozoa. *Reproduction in Domestic Animals*, 40 (6), pp. 522-525. DOI: 10.1111/j.1439-0531.2005.00620.x

Morrell, J.M., Rodriguez-Martinez, H. & Linde, F.C. (2008a). Single layer centrifugation on a colloid selects motile and morphologically normal spermatozoa from dog semen: preliminary results. Reproduction in Domestic Animals, 43, 61.

Morrell, J. M., Dalin, A. M. & Rodriguez-Martinez, H. (2008b). Prolongation of stallion sperm survival by centrifugation through coated silica colloids: A preliminary study. *Animal Reproduction Science*, 5, pp. 121–126.

Morrell, J. M., Johannisson, A., Dalin, A M. & Rodriguez-Martinez, H. (2009a). Morphology and chromatin integrity of stallion spermatozoa prepared by density gradient and single layer centrifugation through silica colloids. *Reproduction in Domestic Animals*, 44(3), pp. 512–517. DOI: 10.1111/j.1439-0531.2008.01265.x

Morrell, J. M., Johannisson, A., Dalin, A. M. & Rodriguez-Martinez, H. (2009b). Single-layer centrifugation with Androcoll-E can be scaled up to allow large volumes of stallion ejaculate to be processed easily. *Theriogenology* 72 (6), pp. 879-884. DOI: 10.1016/j.theriogenology.2009.05.015

Morrell, J. M., Klein, C., Lundeheim, N., Erol, E. & Troedsson, M. (2014). Removal of bacteria from stallion semen by colloid centrifugation. *Animal reproduction science*, 145 (1-2), pp. 47–53. DOI: 10.1016/j.anireprosci.2014.01.005

Morrell, J. M., Núñez-González, A., Crespo-Félez, I., Martínez-Martínez, S., Martínez Alborcia, M-J., Fernández-Alegre, E., Dominguez, J. C., Gutiérrez-Martín, C. B. & Martínez-Pastor, F.

(2019). Removal of bacteria from boar semen using a low-density colloid. *Theriogenology*, 126, pp. 272-278. DOI.org/10.1016/j.theriogenology.2018.12.028

Morrell, J. M., Rodriguez-Martinez, H. & Johannisson, A. (2010). Single layer centrifugation of stallion spermatozoa consistently selects the most robust spermatozoa from the rest of the ejaculate in a large sample size. *Equine Veterinary journal*, 42(7), pp. 579–585. DOI: 10.1111/j.2042-3306.2010.00101.x

Morrell, J. M., Timoney, P., Klein, C., Shuck, K., Campos, J. &Troedsson, M. (2013). Single-layer centrifugation reduces equine arteritis virus titre in the semen of shedding stallions. *Reproduction in Domestic Animals*, 48(4), pp. 604–612. DOI: 10.1111/rda.12133.

Morrell, J.M. & Rodriguez-Martinez, H. (2009). Biomimetic Techniques for Improving Sperm Quality in Animal Breeding (REVIEW). *The Open Andrology Journal*, 1, 1-9. DOI: 10.2174/1876827X00901010001

Morrell, J.M. & Wallgren, M. (2011). Removal of bacteria from boar ejaculates by Single Layer Centrifugation can reduce the use of antibiotics in semen extenders. *Animal Reproduction Science*, 123(1-29, pp. 64-69. DOI: 10.1016/j.anireprosci.2010.11.005

Morrell, J.M. & Wallgren, M. (2014). Alternatives to antibiotics in semen extenders: A review. *Pathogens*, 3(4), pp. 934–946. DOI: 10.3390/pathogens3040934

Morrell, J.M., Alsina, M.S., Abraham, M.C. & Sjunnsson, Y. (2016). Practical applications of sperm selection techniques for improving reproduction efficiency. *Animal Reproduction Science*, 13(3), pp. 340–345. DOI: 10.21451/1984-3143-AR876

Morrell, J.M., Lagerqvist, A., Humblot, P. & Johannisson, A. (2017). Effect of Single Layer Centrifugation on reactive oxygen species and sperm mitochondrial membrane potential in cooled stallion semen. *Reproduction, Fertility and Development*, 29, pp. 1039–1045. DOI:org/10.1071/RD15440

Neto, C. R., Da Silva, Y.F.R.S., Resende, H.L., Guasti, P.N., Monteiro, G. A., Papa, P. M., Dell´aqua Junior, J.A., Puoli Filho, J. N. P., Alvarenga, M. A.& Papa, F. O. (2015). Control methods and evaluation of bacterial growth on fresh and cooled stallion semen. *Journal of Equine Veterinary Science*, 35 (4), pp. 277-282. DOI: org/10.1016/j.jevs.2015.01.014

Nicholson, C. M., Abramsson, L., Holm, S. E. & Bjurulf, E. (2000). Bacterial contamination and sperm recovery after semen preparation by density gradient centrifugation using silane-coated silica particles at different *g* forces. *Human reproduction*, 15(3), pp. 662-666.

Nongbua, T., Johannisson, A., Edman, A. & Morrell, J. M. (2017). Effects of single layer centrifugation (SLC) on bull spermatozoa prior to freezing on post-thaw semen characteristics. *Reproduction in Domestic Animals*, 52 (4), pp. 596–602. DOI: 10.1111/rda.12954.

Ock, S.A., Lee, S.L., Jeon, B.G., Cho, S.R., Kumar, B.M., Choi, Y.S., Choe, S. Y. & Rho, G. J. (2006). Isolation and viability of presumptive spermatids collected from bull testes by Percoll density gradient. *Animal Reproduction Science*, 93(1-2), pp. 144-156. DOI: 10.1016/j.anireprosci.2005.07.011

Olsson, U. (2011). 'random and hierarchical models' In Olsson, U. *Statistics for life science1*, Holmbergs I Malmö AB, Sweden pp. 219-245.

Ortega-Ferrusola, C., González-Fernández, L., Muriel, A., Macías-García, A., Rodríguez-Martínez, H., Tapia, J. A., Alonso, J. M. & Peña, F. J. (2009). Does the microbial flora in the ejaculate

affect the freezeability of stallion sperm? *Reproduction in Domestic Animals*, 44 (3), pp. 518-522.

Ortiz, I., Dorado, J., Ramírez, L., Morrell, J.M., Acha, D., Urbano, M., Galvez, M. J., Carrasco, J. J., Gomez-Arrones, V., Calero-Carretero, R. & Hidalgo, M. (2014). Effect of single layer centrifugation using Androcoll-E-Large on the sperm quality parameters of cooled-stored donkey semen doses. *Animal*, 8 (2), pp. 308–315. DOI: 10.1017/S1751731113002097

Pagl, R., Aurich, JE., Muller-Schlosser, F., Kankofer, M. & Aurich, C. (2006). Comparison of an extender containing defined milk protein fractions with a skim milk-based extender for storage of equine semen at 5ºC. *Theriogenology*, 66(5), pp. 1115–1122. DOI: 10.1016/j.theriogenology.2006.03.006

Pasing, S.S., Aurich, C., Von Lewinski, M., Wulf, M., Kruger, M. & Aurich, JE. (2013). Development of the genital microflora in stallions used for artificial insemination throughout the breeding season. *Animal Reproduction Science*, 139 (1-4), pp. 53-61. DOI: 10.1016/j.anireprosci.2013.03.009

Perry, E. J. (1968). *The Artificial Insemination of Farm Animals*.4th ed. Rutgers University Press, New Brunswick, NJ. pp.6.

Pickett, B. W., Voss, J.L. & Jones, R.L. (1999). Control of bacteria in stallions and their semen. *Journal of Equine Veterinary Science*, 19 (7), pp. 424-441. DOI: org/10.1016/S0737-0806 (99)80254-8.

Popwell, J.M. & Flowers, W.L. (2004). Variability in relationships between semen quality and estimates of in vivo and in vitro fertility in boars. *Animal Reproduction Science*, 81(1-2), pp. 97-113.

Rodriguez-Martinez, H. (2003). Laboratory Semen Assessment and Prediction of Fertility: still Utopia? *Reproduction in Domestic Animals*, 38, pp. 312-318.

Rodriguez-Martinez, H. (2013). Semen evaluation techniques and their relationship with fertility. *Animal reproduction*, 10 (3), pp.148-159.

Rothberg, J.M., Hinz, W., Rearick, T.M., Schultz, J., Mileski, W., Davey, M., Leamon, J.H., Johnson, K., Milgrew, M.J., Edwards, M., Hoon, J., Simons, J.F., Marran, D., Myers, J.W., Davidson, J.F., Branting, A., Nobile, J.R., Puc, B.P., Light, D., Clark, T.A., Huber, M., Branciforte, J.T., Stoner, I.B., Cawley, S.E., Lyons, M., Fu, Y., Homer, N., Sedova, M., Miao, X., Reed, B., Sabina, J., Feierstein, E., Schorn, M., Alanjary, M., Dimalanta, E., Dressman, D., Kasinskas, R., Sokolsky, T., Fidanza, J.A., Namsaraev, E., McKernan, K.J., Williams, A., Roth, G.T. & Bustillo, J. (2011). An integrated semiconductor device enabling non-optical genome sequencing. *Nature*, 475, pp. 348–352. DOI: org/10.1038/nature10242

Samper, J. C. & Tibary, A. (2006). Disease transmission in horses. *Theriogenology*, 66 (3), pp.551-559. DOI: 10.1016/j.theriogenology.2006.04.019

Schulze, M., Junkes, C., Muller, P., Speck, S., Ruediger, K., Dathe, M. & Mueller, K. (2014). Effects of Cationic Antimicrobial Peptides on Liquid- Preserved Boar Spermatozoa. *PLoS One*, 9 (6),| e100490. DOI: 10.1371/journal.pone.0100490

Singhal, N., Kumar, M., Kanaujia, P. K. & Virdi, J. S. (2015). MALDI-TOF mass spectrometry: an emerging technology for microbial identification and diagnosis (REVIEW). *Frontiers in Microbiology*, 6,791. DOI: org/10.3389/fmicb.2015.00791.

Sterbenc, N., Morrell, J.M., Kosec, M., Rath, D., Klein, S. & Klinc, P. (2019). Single layer colloid centrifugation technique improves motility, viability and chromatin integrity of ram spermatozoa after thawing. *Cryobiology*, 86, pp. 77-83. DOI: 10.1016/j.cryobiol.2018.12.001.

Tibary, A., Rodriquez, J. & Samper, J. C. (2009). 'Microbiology and Diseases of Semen'. In: Samper, J. C. (ed) *Equine Breeding Management and Artificial Insemination*. 2nd ed, USA pp. 99-112. DOI:org/10.1016/B978-1-4160-5234-0.X0001-3

Varela, E., Rey, J., Plaza, E., Munoz de Propios, P., Ortiz-Rodriguez, J.M., Avarez, M., Anel-Lopez, L., Anel, M. C., Morrell, J. M. & Ortega-Ferrusola, C. (2018). How does the microbial load affect the quality of equine cool-stored semen? *Theriogenology*, 114, pp. 212-220. DOI: org/10.1016/j.theriogenology.2018.03.028

Varner, D. D., Scanlan, C. M., Thompson, J.A., Brumbaugh, G. W., Blanchard, T. L., Carlton, C.M. & Johnson, L. (1998). Bacteriology of preserved stallion semen and antibiotics in semen extenders. *Theriogenology*, 50(4), pp. 559-573.

Wang, Z & Goonewardene, L.A. (2004). The use of MIXED models in the analysis of animal experiments with repeated measures data. *Canadian Journal of Animal Science*, 84(1), pp.1-11.DOI: org/10.4141/A03-123.

Yaniz, J. L., Marco-Aguado, M. A., Mateos, J. A. & Santolaria, P. (2010). Bacterial contamination of ram semen, antibiotic sensitivities, and effects on sperm quality during storage at 15 ◦C. *Animal Reproduction Science*, 122(1-2), pp.142–149. DOI: 10.1016/j.anireprosci.2010.08.006

Yoon, S. H., Ha, S. M., Kwon, S., Lim, J., Kim, Y., Seo, H. & Chun, J. (2017). Introducing EzBioCloud: A taxonomically united database of 16S rRNA and whole genome assemblies. *Int J Syst Evol Microbiol*. 67(5):1613-1617. DOI: 10.1099/ijsem.0.001755.

Popular science summary

Artificial insemination in horses is most frequently carried out with cooled semen rather than frozen semen. To achieve good results, good sperm quality is required, i.e. spermatozoa with high viability, normal morphology and mitochondrial activity, and with an intact acrosome. In addition, the sample should be free of pathogenic and non-pathogenic bacteria. Contamination of semen with bacteria causes a reduction in the time that the spermatozoa remain viable, thus reducing conception rates, and may lead to infection in mares. Therefore, antibiotics are added to semen extenders; however, this practice may lead to the development of antibiotic resistance. In commercial AI centers, liquid stallion semen is used for insemination mostly 24 to 48h after collection; unused semen doses are discarded. Therefore being able to extend the "shelf-life" of semen doses would be of considerable benefit to equine breeding, to maximise use of available material without compromising pregnancy rates.

Many different methods are used to identify bacteria, each with advantages and disadvantages. Many require the bacteria to be cultured first, which is time consuming and may not be successful for all bacteria. Thus, there are differences in results when using different identification methods for bacteria. The ability to identify bacteria accurately is especially important where potentially pathogenic bacteria are concerned.

Bacteria in semen represent a big challenge. Controlling the growth of these bacteria is important for health, especially as some of them can be pathogenic for humans as well as animals. Most attempts to control their growth rely on the use of antibiotics. A method of removing bacteria by physically separating them from spermatozoa in semen is by colloid centrifugation, particularly Modified Single Layer Centrifugation (MSLC) through a colloid. This method has also been used to select the most robust spermatozoa from the rest of the ejaculate, and to enhance cryosurvival. The benefits of using this MSLC technique for semen samples for insemination would be to select robust spermatozoa that

remain functional over a prolonged period, combined with a potential reduction in the use of antibiotics. The latter would, in turn, have benefits for health in human and animal populations by helping to slow the development of antibiotic resistance and allowing existing antibiotics to be used to combat bacterial infections for a longer time before they become ineffective. In addition, there is the “opportunity cost” of not being able to use existing antibiotics in the future, and the very high cost of trying to develop new antibiotics to treat infections where the bacteria are already resistant. It is hoped that this study will contribute to decreasing the reliance on antibiotics, as well as contributing to an understanding of the factors affecting the bacteriome of stallion semen.

Populärvetenskaplig sammanfattning

Artificiell insemination hos hästar utförs oftast med kyld snarare än frusen sperma. För att uppnå goda resultat krävs god spermiekvalitet, dvs spermier med hög livskraft, normal morfologi och mitokondriell aktivitet samt med en intakt akrosom. Dessutom bör provet vara fritt från patogena och icke-patogena bakterier. Förorening av sperma med bakterier medför en minskning av tiden då spermierna förblir livskraftiga, vilket minskar befruktningsgraden och kan leda till infektioner i stona efter insemination. Därför tillsätts antibiotika till spädningsvätskor Men denna användning av antibiotika kan leda till utveckling av antibiotikaresistens. I kommersiella stuterier används kyld hingstsperma för insemination, mestadels 24 till 36 h efter spermasamling. Oanvända spermadoser kasseras. Att kunna förlänga "hållbarheten" hos kylkonserverade sperma skulle därför vara till stor nytta för hästaveln, då det skulle bidra till maximerad användning av tillgängligt material utan att medföra lägre dräktighetsresultat.

Olika metoder används för att identifiera bakterier, var och en med fördelar och nackdelar. Många kräver att bakterierna odlas först, vilket är tidskrävande och kanske inte lyckas för alla bakterier. Sålunda finns skillnader i resultat vid användning av olika identifieringsmetoder för bakterier. Möjligheten att korrekt identifiera bakterier är särskilt viktig när det är frågan om potentiellt patogena bakterier.

Bakterier i sperma utgör en stor utmaning. Att förhindra tillväxten av dessa bakterier är viktigt för hälsan, särskilt när det gäller bakterier som kan vara patogena för både människa och djur. Bakterietillväxten kontrolleras med hjälp av antibiotika. En metod att avlägsna bakterier genom att fysiskt separera dem från spermier i sperman är genom kolloidcentrifugering, särskilt så kallad ”Single Layer ” centrifugering dvs centrifugering genom ett modifierade enkelt kolloidskikt (MSLC). Denna metod har också använts för att välja de mest robusta spermierna från ejakulatet och för att förbättra överlevnad efter frysning Fördelarna med att använda denna MSLC-teknik för sperma avsedd för

insemination skulle vara möjligheten att välja robusta spermier som förblir funktionella under en längre tid kombinerat med en potentiell minskning av användningen av antibiotika. Detta skulle i sin tur ha fördelar för hälsan i människo- och djurpopulationer genom att bidra till att bromsa utvecklingen av antibiotikaresistens och låta befintliga antibiotika användas för att bekämpa bakterieinfektioner under en längre tid innan de blir ineffektiva. Dessutom finns det ekonomiska konsekvenser av att inte kunna använda befintliga antibiotika i framtiden. Försök att utveckla nya antibiotika för att behandla infektioner där bakterierna redan är resistenta medför mycket höga kostnader. Denna studie kan förhoppningsvis bidra till att minska användning av antibiotika, samt bidra till en förståelse för de faktorer som påverkar bakterier i hingstsperma.

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