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List of Abbreviations

Å	Ångström
ACN	Acetonitrile
AGC	Automatic gain control
AI	Artificial Insemination
AMPs	Antimicrobial peptides
BSA	Bovine serum albumin
CASA	Computer Assisted Sperm Analysis
CRISPs	Cysteine rich secretory proteins
GI	Growth Inhibtion
Ca	Calcium
CFU	Colony forming units
Cl	Chlorine
Cu	Copper
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
E. coli	Escherichia coli
eCATH	Equine cathelicidin
e.g.	exempli gratia; for example
ELISA	Enzyme-linked Immunosorbent Assay
eNAP	Equine neutrophil antimicrobial peptide
et al.	et alii
Fig.	Figure
Fn-2	Fibronectin type II
GI	Growth Inhibition
HCD	High energy collision dissociation
HPLC	High Performance Liquid Chromatography
HSP	Horse seminal plasma protein
IAA	Iodoacetamide
K+	Potassium ion
kDa	Kilo daltons
LC- MS	Liquid Chromatography Mass Spectrometry
Mg	Magnesium
M	Molar
MIT	Maximum injection time
mRNA	Messenger ribonucleic acid
MS	Mass Spectrometry

mQ-H_2O	Milli-Q Water
Na+	Sodium ion
NCE	Normalized collision energy
NK- lysin	Natural killer- lysin
OD	Optical density
P	Phosphor
PBD	Porcine β- defensin
PBS	Phosphate-buffered saline
PCR	Polymerase Chain Reaction
ppm	parts per million
PSM	Peptide Spectrum Match
rcf	Relative Centrifugal Force
RT	Room Temperature
S. aureus	Staphylococcus aureus
Sc. zooepidemicus	Streptococcus equi subsp. zooepidemicus
Se	Selenium
SP	Seminalplasma
TFA	Trifluoroacetic acid
TSB	Tryptic Soy Broth
V-AL	Vacuum- alcoholic
ZnS	Zinc sulfide

1. Abstract

Artificial Insemination has become a commonly used technique in horse breeding. To prolong the storage life of sperm cells, semen extenders include antibiotics to inhibit bacterial growth. A bacterial load in collected semen is inevitable and originates from the stallion's genital mucosa as well as semen collection. The prophylactic use of antibiotics can contribute to the development of multiresistant bacteria. Due to this reason, the demand for finding alternatives to the use of antibiotics is increasing. One strategy might be the use of antimicrobial peptides (AMPs), which occur naturally and have an inhibiting effect on the growth of diverse bacteria. The aim of our study was to examine if equine seminal plasma includes naturally occurring equine AMPs, if equine seminal plasma has an antibacterial effect at all, and finally, to check if synthetic lysozymes would be an alternative to antibiotics in semen extenders.

We could determine low concentrations of psoriasin and equine lysozymes via mass spectrometry and ELISA. Equine seminal plasma reduced growth of *Sc. zooepidemicus*. This was analysed via optical density (OD) and colony forming units (CFU). This bactericidal effect might not be induced by the low concentrations of the equine AMPs determined in this study. The results suggest that equine seminal plasma includes additional proteins which have a bactericidal activity against *Sc. zooepidemicus*.

When lysozyme isolated from chickens' egg white was supplemented to horse semen instead of antibiotics, concentration of 1 mg/ml lysozyme decreased bacterial growth of *Sc. zooepidemicus* significantly without detrimental effects on semen characteristics.

2. Zusammenfassung

Die künstliche Besamung beim Pferd hat sich mittlerweile als weitverbreitete Technik in der Pferdezucht etabliert. Zur Verlängerung der Lebenszeit der Samenzellen wird ein Samenverdünner verwendet, der meistens prophylaktisch Antibiotika enthält. Eine Keimbelastung des gewonnenen Samens ist unausweichlich und stammt von der Genitalschleimhaut des Hengstes sowie von der Samengewinnung und -aufbereitung in einer nicht-sterilen Umgebung. Die prophylaktische Verwendung von Antibiotika kann zur Enstehung von multiresistenten Bakterien beitragen. Aufgrund dessen steigt die Nachfrage nach Alternativen zu Antibiotika zunehmend. Eine mögliche Strategie ist die Verwendung von antimikrobiellen Peptiden, die natürlich vorkommen und das Wachstum von diversen Bakterien verringern können.

Ziel dieser Studie war es, zu untersuchen, ob antimikrobielle Peptide in Seminalplasma von Hengsten vorkommen, ob equines Seminalplasma überhaupt über antibakterielle Effekte verfügt und ob synthetische Lysozyme eine mögliche Alternative für Antibiotika in Samenverdünnern sind.

Wir konnten niedrige Konzentrationen von Psoriasin und equinen Lysozymen durch Massenspektrometrie und ELISA ermitteln. Das Seminalplasma verursachte aber eine Reduktion des Bakterienwachsum von *Sc. zooepidemicus*, welche durch Messung der optischen Dichte, sowie durch die koloniebildenden Einheiten festgestellt wurde. Dieser bakterizide Effekt wurde jedoch wahrscheinlich nicht durch die niedrigen Konzentrationen der antimikrobiellen Peptiden verursacht. Die Ergebnisse lassen vermuten, dass equines Seminalplasma andere Proteine enthält, die eine bakterizide Aktivität gegen *Sc. zooepidemicus* aufweisen.

Unsere Untersuchungen zeigen weiter, dass aus Hühnereiweiß isolierte Lysozyme sich als Alternative zu Antibiotika in Pferdesamenverdünner eignen. Bei Verwendung von Lysozymen in einer Konzentration von 1 mg/ml wurde das Bakterienwachstum von *Sc. zooepidemicus* reduziert und gleichzeitig kein negativer Einfluss auf die Samenqualität festgestellt.

3. Introduction

3.1. Artificial Insemination

Artificial insemination (AI) is the manual deposition of sperm into the female's reproductive tract. AI includes the collection of semen with an artificial vagina, often on a dummy, the preparation of the semen with an extender to increase the storage life of sperm, the manipulation of the female's estrous cycle, induction of ovulation as well as insemination. Nowadays, AI is a very popular method in animal breeding and is used in various domestic species (Morrell, 2011).

AI in horses started to develop at the end of the 19th century to overcome infertility. In Russia, Elia Iwanoff conducted equine AI and was the first scientist, who performed systematic studies. These experiments and data were fundamental for the beginning of AI in horses (Aurich, 2012). Since the beginning of the use of AI in animal breeding, several advantages over natural mating could be demonstrated. One benefit and the most important reason for the development of AI is the prevention of spreading diseases. The transport of animals for mating and therefore the transmission of infectious diseases is decreased. Moreover, no physical contact between the mare and stallion is necessary, whereby the risk of infection and breeding injuries is avoided (Morrell, 2011).

Another advantage of AI is the possibility to divide an ejaculate into several insemination doses, which is in horse breeding especially of interest for stallions, who are in high demand. Thus, more mares can be inseminated with only one ejaculate and therefore the availability and reproductive efficiency of a stallion is improved. Additionally, the semen quality can be checked before insemination and preparation of the semen enables transportation over long distances and therefore increases the breeding opportunities because the choice of a stallion is not restricted to any location (Pagl et al., 2006) and the range of available stallions increases. This fact leads to a higher genetic input and further succession to genetic process. The processing of semen with a semen extender leads to a longer storage life of sperm which facilitates determination of the optima time point for insemination.

Because of these benefits, AI has become a common and successful used practice which is operated worldwide (Aurich, 2012). The frequency of using AI in horse breeding is even increasing, and the development of new technologies like cryopreservation and sperm sexing is in progress (Morrell, 2011).

3.2. Semen processing

After semen collection with an artificial vagina, the ejaculate is filtered to remove smegma, epithelial cells, dirt, and other constituent material. Thereafter, the ejaculate is analysed for volume, color, consistency, pH, sperm concentration, motility, and viability. For an immediate use, it is not necessary to dilute the semen with an appropriate extender, i.e. it can be used as raw semen. When the semen has to be transported or has to be stored for the next few days, the ejaculate must be processed with an extender to ensure a longer storage life. Most extenders, which are used for equine semen, are either egg yolk- or skim milk-based and the majority of extenders contain antibiotics to limit bacterial growth in the ejaculate (Pagl et al., 2006). Among others, polymixin B sulfate, crystalline penicillin, gentamicin sulfate, amikacin sulfate, and ticarcillin are commonly used antibiotics in equine semen extenders (Varner, 1991).

One insemination dose for a mare should imply 300- 500 x 10^6 progressive motile sperm (Allen, 2005). Hence, the dilution factor of semen to extender depends on the density of the ejaculate. For optimal longevity, the final dilution should be between 25 and 50 x 10^6 sperm/ml. After diluting the semen, it is cooled to 4-6 °C for storage, and at this temperature, the motility of the spermatozoa should be more or less constant for at least 72 hours to ensure a high semen quality and consequently a good pregnancy rate (Pagl et al., 2006).

3.3. Seminal Plasma Proteins

In general, semen can be defined as a fluid, composed of spermatozoa and other cells like epithelial cells from the excurrent ducts, epididymis or accessory glands, leukocytes, and spermatogenic cells as well as cell vesicles like epididymidosomes and prostasomes. Semen can be divided into cellular and acellular components. Seminal plasma (SP) belongs to the acellular components (Beyler et al., 1982) and is secreted by the testes, epididymis and accessory sex glands (Kareskoski et al., 2008).

Stallion ejaculates can be classified into six to nine different fractions. Approximately 70% of the spermatozoa are secreted in the first three ejaculatory jets (Kosiniak, 1975). The pre-ejaculate is produced by the bulbourethral gland and is secreted first. The initial fraction of the sperm-rich ejaculate derives from the epididymis and ampulla, and the last fraction is formed by seminal vesicle fluid (Magistrini et al., 2000).

Seminal plasma acts as transport fluid for semen (Mann, 1978) and provides components which are necessary for spermatozoal function, metabolism, and survival (Edwards et al., 1981; Miller et al., 1990). Mráčková et al. (2015) studied the components of equine seminal plasma, and they found the following biochemical substances: proteins, creatinine, urea, alkaline phosphatase, alanine aminotransferase, aspartate- aminotransferase, creatinekinase, gamma-glutamyl-transferase, lactate-dehydrogenase, Na+, K+, Ca, P, Cl, ZnS, Cu, Mg, Se and vitamin E. Moreover, seminal plasma plays an important role in the sperm's ability to interact with the secretions and epithelia of the female genital tract and even carries signals for the female's immune system (Rodriguez-Martinez et al., 2009; Rodriguez-Martinez et al., 2010).

The proteins which are included in seminal plasma, are secreted by the epididymis and accessory sex glands (Töpfer-Petersen et al., 2005). The seminal plasma proteins mainly belong to proteins carrying fibronectin type II (Fn-2) modules, spermadhesins or cysteine-rich secretory proteins (CRISPs) (Kelly et al., 2006). Proteins with the Fn-2 structure recognize choline phospholipids, which are located on the sperm surface and interact with the spermatozoa (Ekhlasi-Hundrieser et al., 2005). Spermadhesins are multifunctional glycoproteins that have multiple effects on sperm, including capacitation and sperm-zona pellucida interaction (Calvete et al., 1997). Proteins, which can be found exclusively in horse seminal plasma, are called horse seminal plasma proteins (HSP-1 to HSP-8). All seminal plasma proteins, beside HSP-4, have sperm-binding abilities. HSP-1 and -2 as well as HSP-5 to -8 have heparin-binding properties (Calvete et al., 1994). HSP-1 is the most frequent protein in the seminal plasma of horses and constitutes 70% of all proteins (Ekhlasi-Hundrieser et al., 2005; Guasti et al., 2020). It is also called SP-1, belongs to the short Fn-2 type proteins, and is associated with capacitation (Töpfer-Petersen et al., 2005). HSP-1 acts as a molecular chaperone and is therefore important for sperm motility and viability (Plante et al., 2016). It plays an important role in sperm preservation and DNA stabilization (Guasti et al., 2020).

HSP-3 (also called CRISP-3) is a major component of seminal plasma, is positively correlated with fertility (Hamann et al., 2007), and regulates the elimination of dead and abnormal sperm from the female reproductive tract (Doty et al., 2011). HSP-6 and HSP-8 are mainly contained in the first fractions of the stallion's ejaculate. All other HSPs are present in the other the fractions. HSP-1, 2, and 4 are present in all ejaculate fractions (Kareskoski et al., 2011). HSP-7 belongs to the family of spermadhesin and shows zona pellucida binding activity (Reinert et al., 1996). HSP-2 and HSP-3 have a significant relationship with semen freezability (Jobim et al., 2011).

3.4. Bacteria, antibiotics and alternatives

During semen collection, contamination with bacteria which are located in the reproductive tract and on the penis (Rota et al., 2011; Pasing et al., 2013) is inevitable, but with appropriate hygienic measures, the bacterial load can be kept low. The genital mucosa of stallions harbors a variety of bacteria, which can also be found in the semen (Aurich et al., 2003; Pasing et al. 2013; Picket et al., 1999). A huge amount of different bacterial taxa is present, but the distribution of the most abundant bacteria varies among stallions (Al-Kass et al., 2020) and even the same stallion presents changes over time. Moreover, in stallions who suffer from bacteremia microorganisms can cross the blood-testis barrier and occur in their semen. Another source of microbial contamination might be incorrect handling of semen during processing and storage (Thibier and Guerin, 2000).

According to metagenomic analysis, the most frequent bacterial genera in horse semen are *Corynebacterium*, *Porphyromonas*, and *Finegoldia*. Even in frozen semen, which was stored between 3 and 17 years in liquid nitrogen, bacteria and fungi were detected (Corona and Cherchi, 2009). The most abundant bacteria in equine ejaculates are *Streptococcus equi* ssp. *zooepidemicus* (*Sc. zooepidemicus*), *Klebsiella pneumoniae*, *Escherichia* (*E.*) *coli*, *Pseudomonas aeruginosa* (Cerny et al., 2014), and mycoplasmas (Spergser et al., 2002). These bacteria can be detrimental to semen characteristics during storage (Aurich & Spergser, 2007) or can cause infectious endometritis in the mare after insemination, which can lead to subfertility (Pickett, Voss and Jones, 1999). Gram-negative bacteria harbor lipopolysaccharides (endotoxins) and porins in their cell wall, which can be released during growth and are resistant to proteolytic enzymes (Garten and Henning, 1974; Gorga et al., 1992). Additionally, they can bind and damage sperm which results in decreased fertility (Paulson and Polakoski, 1977).

A prevalent procedure is the use of antibiotics in semen extenders to avoid bacterial growth and therefore maintain semen quality and prevent bacterial transmission by artificial insemination. Commonly used antibiotics for equine semen are gentamicin or penicillin G, which are included in semen extenders in different concentrations, starting from 100 µg/mL to 1 mg/mL (Santos & Silva, 2020). The prophylactic use of antibiotics in extenders can contribute to a decrease in their efficiency and the development of multiresistant bacteria (Althouse and Lu, 2005; Steckbeck, 2013). Bacteria that were isolated from raw semen and processed with an extender including antibiotics demonstrated resistance to diverse commonly used antibiotics in 90% of cases (Bolarín, 2011; Bussalleu et al., 2013). Hence, the current trend is to find alternatives to antibiotics (Santos & Silva, 2020). Strategies for alternatives include physical methods like single-layer centrifugation (Al-Kass et al. 2019; Morell et al. 2014), microfiltration (Barone et al., 2016), or the use of diverse substances, for example aloe vera gel (Brito et al., 2014; Farias et al., 2019), iodine methionine (Fang et al., 2017) or sodium

alginate (Kumar et al., 2019). In stallions, removal of seminal plasma also leads to a decreased bacterial load (Ramires Neto et al., 2015). Another possibility would be the use of antimicrobial peptides (AMPs) instead of antibiotics. In boar semen, promising results with diverse AMPs were demonstrated (Puig-Timonet et al., 2018; Shaoyong et al., 2019a). The most important requirement when considering antibiotic alternatives is that they are not detrimental to semen quality during storage for at least 72 hours to ensure fertility when used for insemination in mares.

3.5. Antimicrobial peptides in horses

Antimicrobial peptides are composed of 12 to 100 amino acids, have a cationic charge with an amphipathic characteristic, and interact with the negatively charged parts of the bacterial cell wall (Jenssen et al., 2006). They have antimicrobial activity towards diverse pathogens and affiliate with the innate immune system. The most frequent mechanisms contributing to antimicrobial activity are membrane destabilization, blockage of the DNA and membrane proteins synthesis, disruption of single-stranded DNA, induction of bacteria autolysis, and triggering apoptosis in eukaryotic cells (De Smet et al., 2005). The production of AMPs is induced by inflammatory stimuli, like the lipopolysaccharides of the bacterial cell wall (Cunliffe and Mahida, 2004). AMPs have been detected in diverse animals, plants, insects, as well as in single celled organisms (Wang and Wang, 2004). Since the 1990s, intensive studies in horses identified antimicrobial peptides also in horses in a huge variety (Bruhn et al., 2011):

Equine lysozyme was first isolated in 1972 from mare milk (Jáuregui-Adell et al., 1972), consists of 129 amino acids, and belongs to the c-type lysozymes like human and chicken lysozymes (Mc Kenzie and Shaw, 1985). There is evidence that equine lysozymes show similarities to human and hen lysozymes according to their structural characterization (Morozova-Roche, 2007). Moreover, it could be shown that equine lysozyme has antimicrobial activity against gram-positive as well as gram-negative bacteria (Bruhn et al., 2011). in horses, lysozyme was detected in ceruminous glands, the small intestine, uterine fluid, synovial fluid, myeloid cells as well as on the skin. During infection, increased concentrations of lysozymes were described (Torbeck and Prieur, 1979; Wiśniewski and Kuźma, 1987), also in uterine fluid isolated from mares with bacterial endometritis (Pycock and Allen, 1990).

Natural killer-lysin (NK-lysin) was first described in 2005 (Davis et al., 2005) and consists of 83 amino acids. It can be found in the lymphoid cells and includes seven cysteine residues, which might explain their antimicrobial activity (Bruhn et al., 2011).

Equine neutrophil antimicrobial peptide eNAP-1 has similar characteristics to granulins, whereas eNAP-2 is similar to equine microbial protease inhibitors. Equine NAP-1 has 10 conserved cysteine residues, has at least weak antimicrobial activity against *Sc. zooepidemicus*, *E. coli*, and *Pseudomonas aeruginosa* (Couto et al., 1992), and can be detected in uterine exudates together with eNAP-2 (Bruhn et al., 2011). Equine NAP-2 consists of five highly conserved cysteine residues, and its antimicrobial activity is comparable to eNAP-1.

Psoriasin (S100A7) belongs to the group of calcium-binding proteins. Equine psoriasin 1 transcripts could be found in the skin, esophagus, intestine, trachea, and vulva. Weak antimicrobial activity was examined against *E. coli* (Bruhn, 2005). Psoriasin reduces iron and zinc concentrations and therefore restricts bacterial growth (Lee & Eckert, 2007).

Cathelicidins are peptides harbor an antimicrobial domain (Zanetti, 2004). In horses, three different cathelicidins could be identified in myeloid bone marrow cells mRNA via reverse transcription- PCR-based analysis: eCATH-1, eCATH-2, and eCATH-3 (Socci et al., 1999). In equine neutrophils, eCATH-2 and eCATH-3 could be detected in their uncleaved form (Skerlavaj et al., 2001). According to functional tests with synthetic eCATH peptides, they have antimicrobial activity, but the activity of eCATH-3 was strongly dependent on the salt concentration.

Defensins are built up of 18 – 45 amino acids and six conserved cysteine residues. They have antimicrobial activity against fungi, enveloped viruses, gram-positive and gram-negative bacteria, whereby this activity is triggered by electrostatic interaction with the microbial cytoplasmatic membrane. In mammals, there are three subfamilies: α-, β-, and θ-defensins. These subfamilies differ in structure, genetic anatomy, and biological characteristics like synthesis (Bruhn et al., 2011). The first equine β-defensin was described in 2004 (Davis et al., 2004), is now known as DEFB1, and could be verified in the lung, spleen, kidney, heart, liver, small intestine, and lymph node (Bruhn et al., 2011). Via sequencing analysis more β-defensins could be examined (DEFL2, DEFL3, DEFB103) (Looft et al., 2006), which can be ascertainable in tongue, meninges, trachea, lung, small intestine, colon, uterus, and vulva (Bruhn, 2009). Looft et al. (2006) also discovered the transcript of one equine α-defensin in their sequencing experiment, which leads to the mature antimicrobial peptide DEFA1. DEFA1 showed a broad spectrum of antimicrobial activity against gram-positive as well as gram-negative bacteria. In 2009, Bruhn et al. detected via genome screening transcripts for 38 α-defensins in the equine intestine, but only 20 coded for functional peptides.

Hepcidins are antimicrobial peptides with eight conserved cysteine residues, which regulate iron absorption and release. They show antimicrobial activity against gram-negative and gram-positive bacteria as well as fungi (Verga Falzacappa and Muckenthaler, 2005) and appear mainly in the liver, but can also be found in the heart, brain, lung, and body fluids like urine (Park et al., 2001). Equine hepcidin consists of 25 amino acids and was identified via sequencing analysis using real-time PCR. It was detected at a high concentration in the liver, additionally at lower concentrations in the cervical spinal cord, cerebral cortex, lung, duodenum, stomach, spleen, kidney, skeletal muscle, and bladder (Oliveira et al., 2010). The specificity of the antimicrobial activity has not been tested yet.

3.6. Use of antimicrobial peptides instead of antibiotics in semen

A lot of different equine AMPs have already been identified and sequenced in diverse tissues, and their antimicrobial potential has been studied (Bruhn et al., 2011). So far, there is no research with regard to AMPs in equine seminal plasma, nor trials to use synthetic AMPs instead of antibiotics.

Antibacterial activity of ejaculates has been described in diverse taxa and with effectivity against a wide range of bacterial species (Chitnis et al., 1987; Otti et al., 2009; Poiani, 2006; Rowe et al., 2011; Yenugu et al., 2006). Several substances have been identified that may account for this activity, including some AMPs as cause for the antibacterial activity, as well as lysozymes. In the ejaculate of wild passerines, lysozymes have been detected as well as bactericidal activity (Rowe et al., 2013).

In general, studies for assessment of the antimicrobial activity of AMPs have mainly been performed *in vitro* and only a few experiments have been executed *in vivo*. The reason for that might be that AMPs are prone to proteolytic degradation and are sensitive to salt concentrations and pH. Due to these reasons, the handling of AMPs as an antibiotic alternative is difficult and requires caution. There are some studies, however, in which suitable conditions to use AMPs were achieved. In boar semen, the use of PBD-1 and PBD-2 (porcine β- defensin 1 and 2) (Puig-Timonet et al., 2018) as well as ε-polylysine (Shaoyong et al., 2019) could decrease bacterial load after supplementation to semen extenders.

3.7. Aim of the study

The aim of the study was to examine the presence of AMPs in the ejaculate of stallions in general. Moreover, we assessed the overall antimicrobial activity of equine seminal plasma. Additionally, we determined if synthetic AMPs can be used as an alternative to antibiotics. We therefore investigated if addition of synthetic AMPs to extended horse semen reduced the bacterial load during cooling preservation without having a negative influence on semen characteristics.

Our hypothesis for this study was that equine seminal plasma includes AMPs and consequently presents antimicrobial activity. Furthermore, we hypothesized that synthetic AMPs might be effective as alternatives to antibiotics in semen extenders.

4. Material and Methods

4.1. Proteomic analysis of equine seminal plasma

4.1.1. Animals and semen collection

Semen was collected from 5 different stallions of different breeds (2 Warmblood, 1 Pony, 1 Thoroughbred, and 1 Icelandic Horse), aged from 6 to 29 years. At the time of semen collection, all stallions were housed at the Centre for Artificial Insemination and Embryo Transfer, Vetmeduni Vienna, Austria. Semen collection was performed on a dummy in presence of a teaser mare. An artificial vagina (Hannover model, Minitube, Tiefenbach, Germany) was used, which included a sterile plastic liner (Minitube) and a sterilized glass bottle with a semen filter (Minitube) to remove the gel fraction immediately.

A volume of 5ml of raw semen from each ejaculate was centrifuged (10 min, 1800xg, 4 °C) to separate seminal plasma from sperm. Subsequently, three aliquots (1ml each) of seminal plasma were frozen at -80 °C until further analysis. The remaining ejaculate was used for semen analysis.

4.1.2 Semen analysis

Semen was evaluated according to the following parameters:

For determining the volume, the ejaculate was transferred into a measuring cylinder and simply read off. Color and consistency were assessed empirically.The pH was identified using indicator paper (Merck, Darmstadt, Germany).

Sperm concentration was measured with the NucleoCounter SP-100 Sperm Cell Counter, Reagent S100, and SP1 Cassette (Chemometec, Allerod, Denmark) as described.

Motility was assessed by CASA (Computer Assisted Sperm Analysis) with the Sperm Vision 35 machine (Minitube, Tiefenbach, Germany). For analysing motility by CASA, raw semen was diluted with a skim-milk extender to a final concentration of 25-50 million sperm / ml and was evaluated via a phase contrast microscope (Optiphot-2, Nikon, Düsseldorf, Germany). CASA detected the motility of the semen cells via a camera, which was connected to the microscope. The camera recognized the sperm cells and analyzed the movement of the sperms according to various parameters, e.g. percentage of motile and progressive semen cells, velocity, linearity… via 30 frames/s.

Membrane integrity was also evaluated with CASA “Sperm Vision 35”. The used samples had a concentration of 25- 50 million cells / ml. 100µl of the diluted semen were added to 2.5 µl viability kit, which contained propidium- iodide and SYBR 14 for staining. The mixture had to incubate for at least 7 minutes at room temperature, analysis was performed with a fluorescence microscope (AX70, Olympus, Vienna, Austria).

For the evaluation of sperm morphology, semen was added to a Hancock’s solution (62.5 ml formalin (37%), 150 ml saline solution, 150 ml buffer solution, and 500 ml double distilled water) dependent on the concentration of the semen (10 – 50 µl ejaculate to 200 µl Hancock solution). Hancock’s solution induces immobility of sperm thus enabling the assessment of morphological abberations. Therefore, 200 cells were analyzed for morphological

abnormalities of the head, midpiece, and tail under a phase contrast microscope with an oil immersion objective at 1000x magnification.

4.1.3. Extraction of peptides

For extraction of peptides from seminal plasma, 4 different extraction methods were performed and afterwards evaluated via SDS page according to their effectiveness and accuracy.

1. C18 SPE columns (Phenomenex, Strata C18-E (55 µm, 70 Å), 1 g / 6 ml) were used. The column was washed 2x with methanol, equilibrated with 0.1% TFA and 1ml of the sample was loaded on the column with 0.1% TFA. Afterwards the column was washed again twice with 0.1% TFA, eluted with 80% Acn/ 0.1% TFA and eluate was finally lyophilized.
2. Most of the antimicrobial peptides are cationic, so we adopted the method of Bourgeon et al. (2004) and extracted these with the weak cationic exchange matrix Macro-Prep CM (Bio-Rad, Ivry-sur-Seine, France). Macro-Prep CM beads were added to seminal plasma samples (diluted 1:30 in PBS) at a ratio of 1:60. The mixture was incubated overnight at 48 °C with gentle stirring, then centrifuged at 1000 x g for 5 min and washed 3 times, each time for 5 min with 80 matrix volumes of 25 mM ammonium acetate (pH 7.5). Peptides bound to the matrix beads were eluted once with 4 matrix volumes of 10% acetic acid in water for 30 min, and twice with 5% acetic acid in water. Eluates were pooled and lyophilized.
3. Ultrafiltration was performed with Omega membrane 3 kDa from Pall Life Science:
   500 µl 8M urea/ 50 mM TRIS were added to the filter column and centrifuged for 20 min (10 000 x g). This preparation step was repeated and afterwards, the filter was washed with 300 µl 8M urea/ 50 mM TRIS and centrifuged for 15 min (10 000 x g). 1 ml of seminal plasma sample was loaded on the membrane and centrifuged for 85 min (10 000 x g). Afterwards, the filtered flow-through sample was cleaned with C18 Thermo Scientific Pierce Micro-Spin Columns (Cat. No. 89870/11814141) to remove remaining buffer and salts. The retentate, which remained on the top of the filter, was diluted with 300 µl 0.1% TFA and afterwards lyophilized.
   Briefly, the C18 cleanup started with column preparation. The column was placed into a 1.5 ml receiver tube. 200 µl of activation solution (50% Acn/ 50% mQ-H_2O) were added, centrifuged at 1500 x g for 1 minute and the flow-through was discarded. Then the whole step with activation solution, centrifugation and discarding the flow-through was repeated. Afterwards 200 µl of equilibration solution (5% Acn/ 0.5% TFA) were added, centrifuged at 1500 x g for 1 minute, flow through was discarded, and the whole equilibration step had to be done for a second time. The sample was loaded on the top of the resin bed and centrifuged at 1500 x g for 1 minute.The flow through was again pipetted on the top of the column, centrifuged again for 1 minute to ensure complete binding and after that, the flow through was discarded. This was followed by a washing step with 200 µl washing solution (5% Acn/ 0.5% TFA), centrifuged at 1500 x g for 1 minute and repeated again.
   For elution, 20 µl of elution buffer (70% Acn/ 0.1% TFA) was placed on the top of the resin bed, centrifuged at 1500 x g for 1 minute and the eluate was pipetted into a fresh 0,5 ml Eppendorf tube. The elution step was done twice. The eluates were dried in a vacuum centrifuge (45 °C, 60 min, V-AL).

4. Ultrafiltration with Omega membrane 10 kDa from Pall Life Science and Amicon membrane 10 kDa from Millipore were done in the same way as ultrafiltration with the 3 kDa filter, followed also by a C18 sample cleanup as described in method 3.

4.1.4. Tricine-SDS-PAGE

For visualization of proteins and comparison of the four different methods of extraction, a Tricine-SDS-PAGE was conducted. Tricine-SDS-PAGE is an electrophoretic method especially for proteins smaller than 30 kDa like AMPs.

Proteins were separated using 16.5% Tris-tricine gels run in Tris Tricine buffer and visualized via Coomassie blue-staining. Original untreated seminal plasma was used as control in comparison to samples of all 4 different methods of protein extraction. The retentates of the ultrafiltration samples were also visualized on a gel.

4.1.5. Determination of protein concentration

For determining the protein concentration of the samples, the Pierce 660 nm Protein Assay (Thermo Fisher Scientific, Rockford, IL, USA) was used. Bovine Serum Albumin was used as protein concentration standard. Samples were measured according to absorbance values at 660 nm on the NanoDrop spectrophotometer and were compared to the BSA standard curve.

4.1.6. Orbitrap LC-MS/MS

Firstly, a filter aided sample preparation was performed. Therefore, 30 µg of the protein were filled up to 500 µl with 8 M Urea in 50 mM TRIS and were loaded on to a Pall 10 kDa filter. The solution was centrifuged 2 x 20 min at 10 000 rcf. The proteins were reduced with 200 mM DTT (37 °C, 30 min) and alkylated with 500 mM IAA (37 °C, 30 min) on the filter. After two washes with 100 µl 50 mM TRIS, digestion was carried out using Trypsin/LysC Mix in a ratio of 1:25 (Protease:Protein) overnight. Digested peptides were recovered with 3 times 50 µl of 50 mM TRIS and acidified with 1 µl concentrated TFA. Before LC-MS analysis, peptide extracts were desalted and cleaned up using C18 spin columns (Pierce) according to the manufacturer's protocol as described above (4.1.3). The dried peptides were redissolved in 300 µl 0.1%TFA, of which 3 µl were injected to the LC-MS/MS system.

For the Orbitrap LC-MS/MS analysis, peptides were separated on a nano-HPLC Ultimate 3000 RSLC system (Dionex). Sample pre-concentration and desalting was accomplished with a 5 mm Acclaim PepMap µ-Precolumn (300 µm inner diameter, 5 µm particle size, and 100 Å pore size) (Dionex). For sample loading and desalting, 2% ACN in ultrapure H_2O with 0.05% TFA was used as a mobile phase with a flow rate of 5 µl/min.

Separation of peptides was performed on a 25 cm Acclaim PepMap C18 column (75 µm inner diameter, 2 µm particle size, and 100 Å pore size) with a flow rate of 300 nl/min. The gradient started with 4% B (80% ACN with 0.08% formic acid) for 7 min, increased to 31% in 60 min and to 44% in additional 5 min. It was followed by a washing step with 95% B. Mobile Phase A consisted of ultrapure H_2O with 0.1% formic acid. For mass spectrometric analysis, the LC was directly coupled to a high resolution Q Exactive HF Orbitrap mass spectrometer.

MS full scans were performed in the ultrahigh-field Orbitrap mass analyzer in ranges m/z 350−2000 with a resolution of 60 000, the maximum injection time (MIT) was 50 ms and the automatic gain control (AGC) was set to 3e^6. The top 10 intense ions were subjected to Orbitrap for further fragmentation via high energy collision dissociation (HCD) activation over a mass range between m/z 200 and 2000 at a resolution of 15 000 with the intensity threshold at 4e^3. Ions with charge state +1, +7,+8 and >+8 were excluded. Normalized collision energy (NCE) was set at 28. For each scan, the AGC was set at 5e^4 and the MIT was 50 ms. Dynamic exclusion of precursor ion masses over a time window of 30 s was used to suppress repeated peak fragmentation.

4.1.7. Database Search ID

Database search was performed using the Proteome Discoverer Software 2.4.0.305 (Thermo Fisher Scientific) with the following settings:

- Protein Database: AMPs.fasta, UP_horse_tx9796_210421.fasta, crap.fasta (https://www.thegpm.org/crap/)

- Enzyme Name: Trypsin (Semi)

- Max. Missed Cleavage Sites: 2

- Precursor Mass Tolerance: 10 ppm

- Fragment Mass Tolerance: 0.02 Da

- Dynamic Modification: Oxidation / +15.995 Da (M)

- Dynamic Modification: Deamidation / +0.984 Da (N,Q)

- N-Terminal Modification: Gln->pyro-Glu / -17.027 Da (Q)

- N-Terminal Modification: Acetyl / +42.011 Da (N-Terminus)

- N-Terminal Modification: Met-loss / -131.040 Da (M)

- N-Terminal Modification: Met-loss+Acetyl / -89.030 Da (M)

- Static Modification: Carbamidomethyl / +57.021 Da (C)

Decoy Database Search:

- Target FDR (Strict): 0.01

- Target FDR (Relaxed): 0.05

- Validation based on: q-Value

4.2. Lysozyme analysis

4.2.1. Animals, semen collection and semen analysis

Semen was collected from 16 different stallions of several breeds (6 Warmbloods, 5 Shetland ponies, 1 Thoroughbred, 1 Icelandic Horse and 3 Quarter Horses), aged from 3–29 years. All stallions were housed at the Centre for Artificial Insemination and Embryo Transfer, Vetmeduni Vienna, Austria. From each stallion, 2 ejaculates were collected within 10 days. Semen collection, aliquot preparation and semen analysis were performed as mentioned in 4.1.1. and 4.1.2.

4.2.2. Lysozyme ELISA Kit

For determining lysozyme concentration with a lysozyme ELISA kit (Immundiagnostik AG, Bensheim, Germany), samples were analysed as described in the manual. Briefly, 100 µl of each sample were added into the wells and incubated for 1 hour at RT. Afterwards, the content was discarded and each well was washed 5 times with 250 µl washing buffer. A total of 100 µl conjugate was added into each well and incubated for 1 hour at RT. After incubation, the content was discarded and the wells were washed 5 times with 250 µl washing buffer. A total of 100 µl substrate was added and incubated for 10 to 20 min at RT in the dark. The reaction was stopped with 100 µl stop solution and absorption was measured with an ELISA reader (EL808, Bio-Tek Instruments Inc., Winooski, VT, USA) at 450 nm against 620 nm.

4.3. Microbiological analysis of equine seminal plasma

4.3.1. Animals, semen collection and semen analysis

Semen was collected from 5 different stallions (2 Warmbloods, 3 Shetland ponies) aged from 5 to16 years. At the time of semen collection, all stallions were housed at the Centre for Artificial Insemination and Embryo Transfer, Vetmeduni Vienna, Austria. From each stallion, 2 ejaculates were collected within 10 days. One day before semen collection and on the same day before collection, the penis was washed with chlorhexidine and dried with a towel to prevent additional bacterial contamination. Semen collection was performed as described before with a dummy, a teaser mare and an artificial vagina (Hannover model, Minitube, Tiefenbach, Germany), which included a sterile plastic liner (Minitube) and a sterilized glass bottle with a semen filter (Minitube) to remove the gel fraction immediately.

A total of 5 ml raw semen of each ejaculate were centrifuged (10 min, 1800 x g, 4 °C) to separate seminal plasma from sperm. Afterwards, an additional centrifugation step was performed (10 min, 15000 x g, 4 °C) to remove the remaining bacteria. The 500 µl aliquots of the seminal plasma were frozen at -80 °C until further analysis. The remaining ejaculate was used for semen analysis. Semen was analyzed as described before for volume, pH, as well as sperm concentration, motility and membrane integrity.

4.3.2. Bactericidal effect of seminal plasma: Growth inhibition assay

For testing bactericidal and bacteriostatic effects of equine seminal plasma, bacterial isolates of *Sc. zooepidemicus* and *E. coli* were used. These bacterial isolates were cultured on Columbia agar (Oxoid, Basingstoke, UK) with 5% sheep blood and incubated at 37 °C for 24h. Bacterial colonies were transferred into Tryptic Soy Broth (TSB) and aliquots were stored at -80 °C until use. One aliquot was used for determining the bacterial concentration in the samples. For this purpose, serial dilutions were plated onto Columbia agar, incubated at 37°C for 24h, and colonies counted to determine the CFU (colony forming units)/ml. Only plates presenting between 15 and 150 colonies were examined and considered as valid. The bacterial concentration was then calculated considering the dilution factor.

Unprocessed seminal plasma was thawed and 100 µl were plated on Columbia agar and incubated at 37°C for 24h to check if the samples were free from any bacteria to avoid any cross-reaction.

For determination of a possible antimicrobial effect of the seminal plasma, we conducted a liquid growth inhibition assay as described by Otti et al. (2009), Rowe et al. (2011) and Schulze et al. (2019) with some modifications. Briefly, the seminal plasma samples were diluted 1:1 with TSB. A total of 100 µl of the diluted seminal plasma was mixed with 800 µl bacterial suspension in TSB (defined CFU: $1x10^5$). Each sample was tested in triplicate. As controls, TSB with seminal plasma and bacterial suspension in TBS were used, respectively. All samples were added to a 96-well plate. Optical density (OD) was measured at 595 nm using a spectrophotometer (SpectraMax M3; Medical Devices, San Jose, CA, USA) as basic value. After a 24h incubation step at 37°C, OD was determined again. Serial dilutions of each sample and controls were plated onto Columbia agar and incubated at 37°C for 24h. Afterwards CFU were determined as described above.

Growth inhibition (GI) in seminal plasma for each microbial strain was defined as

GI (%) = (1−R) × 100, where R= ODsample/ ODcontrol bacterial suspension

Samples with R < 1 show GI. Cases of R ≥ 1 show no GI and these samples were documented as 0% GI.

A second run of this experiment was performed with another start concentration of bacteria (defined CFU: $1x10^3$).

4.4. Effect of lysozymes on sperm

4.4.1. Animals, semen collection and semen analysis

Semen was collected from 2 different stallions (1 Pony, 1 Warmblood), which were 4 and 6 years old. Both stallions were again housed at the Centre for Artificial Insemination and Embryo Transfer, Vetmeduni Vienna, Austria. From each stallion, 1 ejaculate was collected and examined as described in 4.1.1. Semen samples were diluted with Equiplus extender (Minitube) to a final concentration of 25 million/ml and synthetic lysozyme (Carl Roth, Karlsruhe, Germany) derived from chicken protein dissolved in 12.5 mM Tris was added at different concentrations (0.5 mg/ml; 1 mg/ml; 2 mg/ml). One sample without lysozyme served

as control. All samples were stored at 5 °C and analyzed according to motility and membrane integrity via CASA over 72 hours.

4.5. Synthetic lysozymes as alternative to antibiotics

4.5.1. Animals, semen collection and semen analysis

The same seminal plasma samples were used as described in 4.3.1. Briefly, the samples derived from 5 different stallions (2 Warmblood, 3 Pony) aged from 5–16 years. From each stallion, 2 ejaculates were used. One day before semen collection and on the same day directly before semen collection, the penis was washed with chlorhexidine and dried with a towel. Semen collection was performed as mentioned before. The 5 ml of raw semen of each ejaculate were centrifuged twice (10 min, 1800 x g, 4 °C; 2nd centrifugation: 10 min, 15000 x g, 4 °C). The 500 µl aliquots were frozen at -80 °C for further analysis. The remaining ejaculate was used for semen analysis.

4.5.2. Growth inhibition assay with synthetic lysozymes

The same *Sc. zooepidemicus* and *E. coli* isolates were used as described in 4.3.2. Seminal plasma samples were diluted 1:1 with TSB. An amount of 200 µl of the diluted seminal plasma samples were mixed with 1600 µl bacterial suspension in TSB (defined CFU: 1x10^5). Each sample was examined with *E. coli* and with *Sc. zooepidemicus,* respectively. The seminal plasma-bacterial suspension samples were mixed with lysozyme (Carl Roth) dissolved in 12.5 mM Tris at different concentrations (0.5 mg/ml; 1 mg/ml; 2 mg/ml). Each sample was tested in triplicate. Control groups were TSB with seminal plasma, bacterial suspensions in TBS and seminal plasma-bacterial suspension mixture without lysozyme. All samples were added to a 96-well plate. The OD was measured at 595 nm with a spectrophotometer before and after 24h incubation at 37°C. The GI was calculated as described in 4.3.2. Serial dilutions of each sample and controls were plated onto Columbia agar and incubated at 37°C for 24h. Afterwards CFU values were determined as described before.

4.5. Statistical analysis

Data analysis was performed with the SPSS statistics program (version 29.0.1.0, IBM-SPSS, Armonk, NY, USA). With regard to OD values, two-way repeated measures ANOVA with both incubation time (0 and 24h) and treatment (control, *E. coli, Sc. zooepidemicus*) and lysozyme supplementation as within subject factors was used. This approach takes into account that the same horses and ejaculates were used for all treatments within the different subprojects. Data distribution was normal and variances were homogenous. With regard to CFU values, non-parametric tests for repeated measures (Friedman test) was used. For all tests, the significance threshold was set at $p < 0.05$. All values given are means ± standard error of the mean (SEM).

5. Results

5.1. Semen analysis

After semen collection, semen was analyzed according to volume, pH, density, motility, membrane integrity and morphology.

All collected ejaculates were in a pH range of 6.8- 7.2, which corresponds to the reference values of equine semen. The volume varied between 2- 135 ml. This fluctuation is also quite normal with regard to the diverse breeds of the used stallions, e.g. the volume of a Shetland pony stallion is smaller than a warmblood stallion. The concentration of semen cells in the ejaculates was between 37.7 and 596.7 mio/ml and the total number of sperm cells in the whole ejaculate was between 4.11 and 23.68 billion, which also conforms to the reference values. Only a few exceptions were below 4 billion total number of semen cells, which belonged to pony breeds and Icelandic Horse.

The semen samples included into the study had a total sperm motility between 81.3 – 94.3%, a progressive motility between 70.0– 88.3% and membrane integrity between 75.0 – 93.6%. Hence, the motility of all stallions met the minimal requirements for semen of fertile stallions.

5.2. Proteomic analysis of equine seminal plasma

5.2.1. Extraction of peptides checked on Tricine-SDS-PAGE

According to the Tricine- SDS-PAGE (Figure 1), the sample extracted with the C18 SPE column, showed a lot of smear between the bands. This smear derived either from a very large protein concentration, beginning of protein degradation already soon until semen collection, contamination with nucleic acids or other compounds, which would impede further analysis with mass spectrometry. On the contrary, samples processed with Macro Prep CM beads presented only a few bands which appeared very pale. This suggests that protein concentration was much lower than after C18 SPE column extraction, but the protein extraction of variant 2 may also be cleaner. Nevertheless, with both methods, the existence of peptides in a lower kDa- range than 5 kDa could not be proven. The unprocessed seminal plasma sample looked quite similar to the sample of variant 1. This strengthens the assumption that the peptide extraction with the C18 SPE column was not very efficient.

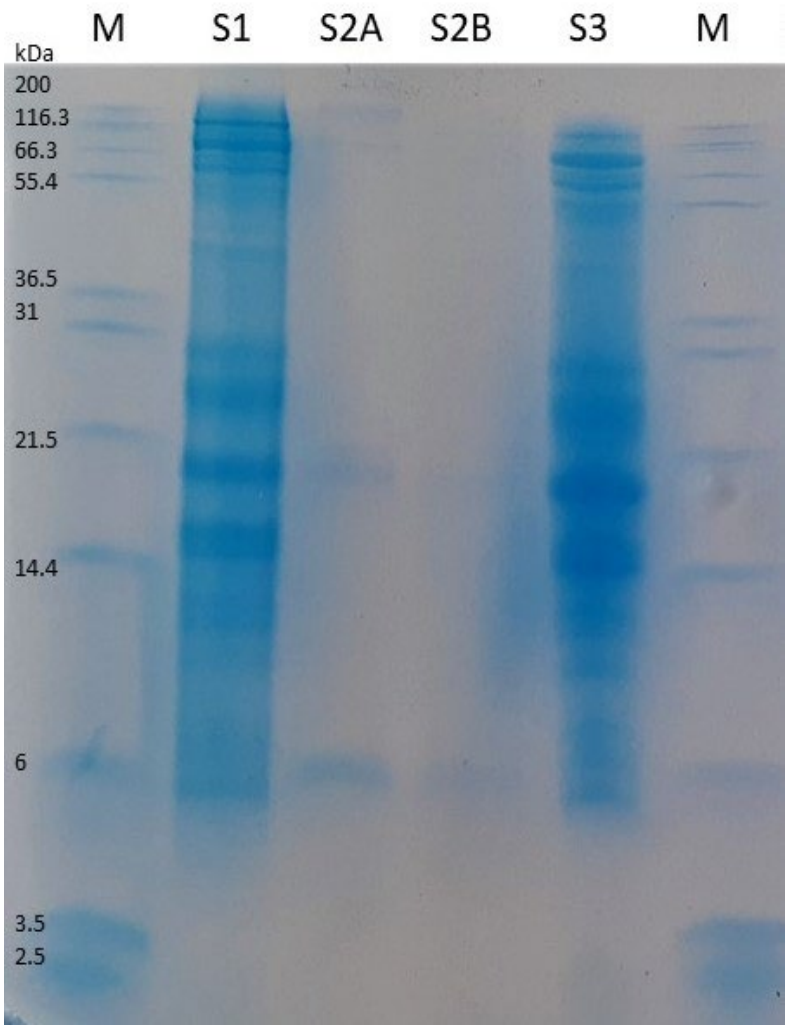

Fig. 1: Tricine-SDS-PAGE of extracted peptides via different variants. Sample 1 (S1) was extracted via C18 SPE column. Sample 2A (S2A) was extracted via Macro-Prep CM beads, whereat the seminal plasma samples, which were used for extraction, were diluted 1:30 with PBS before extraction like in the published method of Bourgeon et al. (2004). Sample 2B (S2B) was also extracted via Macro-Prep CM beads, but the used seminal plasma sample was undiluted. Sample 3 (S3) corresponds to an unprocessed seminal plasma sample. The used Marker (M,Invitrogen) is more precisely in the range of 2.5 – 55.4 kDa. In this range we would expect AMPs.

Protein extraction via ultrafiltration with a 3 kDa and 10 kDa filter were also checked on a Tricine- SDS-PAGE. Firstly, the retentates (Fig. 2) were analyzed to show all proteins, which were retained with the ultrafiltration devices. These retentates were compared to an unprocessed original seminal plasma sample. All four samples presented the same bands and therefore the same proteins.

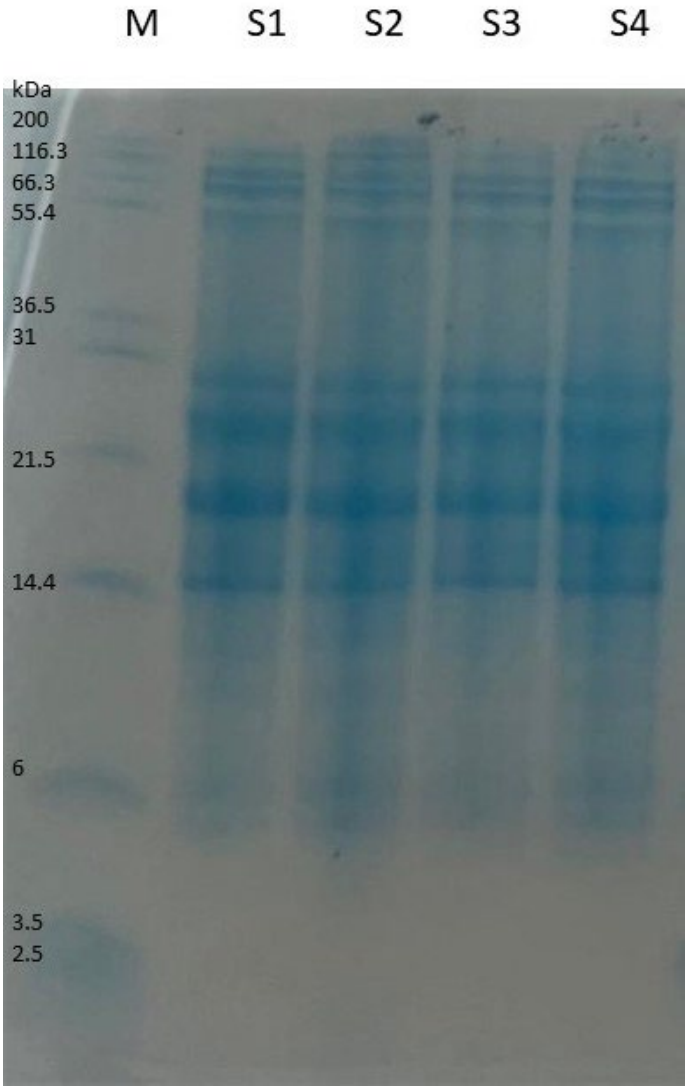

Fig. 2: Tricine-SDS-PAGE of the retentates obtained after ultrafiltration (variant 3 and 4). Sample 1 (S1) is an original unprocessed seminal plasma sample. Sample 2 (S2) is the retentate of the 3 kDa filter (Pall). Sample 3 (S3) shows the bands of the retentate of the 10 kDa filter (Pall) and sample 4 (S4) is the retentate of the 10 kDa filter (Amicon).The used Marker (M,Invitrogen) is more precisely in the range of 2.5 – 55.4 kDa.

Secondly, we compared the flow through samples of the 3 kDa and 10 kDa filter (variant 3 and 4) on a Tricine- SDS-PAGE (Fig. 3). Interestingly, the sample of the 3 kDa ultrafiltration included proteins, which were in a range higher than 3 kDa. In contrast, both 10 kDa filters contained no clear visible proteins neither above nor beneath 10 kDa.

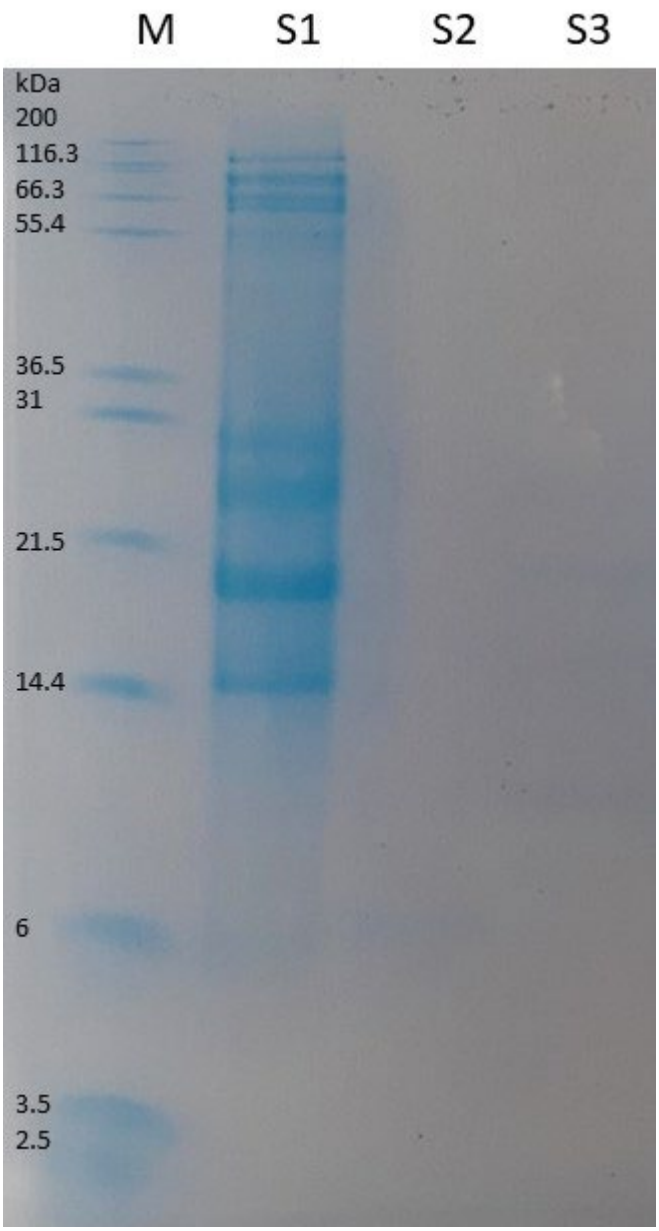

Fig. 3: Tricine-SDS-PAGE of the flow through obtained after ultrafiltration (variant 3 and 4). Sample 1 (S1) is the flow through of the 3 kDa filter of Pall. Sample 2 (S2) is the flow through of the 10 kDa filter (Pall) and sample 3 (S3) corresponds to the flow through of the 10 kDa filter (Amicon). The used Marker (M,Invitrogen) is more precisely in the range of 2.5 – 55.4 kDa.

5.2.2. Determination of protein concentration

For determining the protein concentration, we used seminal plasma of one ejaculate of one stallion for simply comparing the different extraction methods.

By determination of protein concentrations of the extracted seminal plasma samples, it could be shown that the extraction with the Macro-Prep CM beads had the lowest protein concentration (Fig. 4), which corresponds to the results of the SDS PAGE (Fig. 1). The retentate of the 3 kDa ultrafiltration had lower amounts of proteins compared to the original seminal plasma sample. In contrast to that, the 10 kDa ultrafiltration had a much higher protein concentration than the untreated seminal plasma sample. Hence, the retentates of the 10 kDa filter resulted in the most concentrated protein samples.

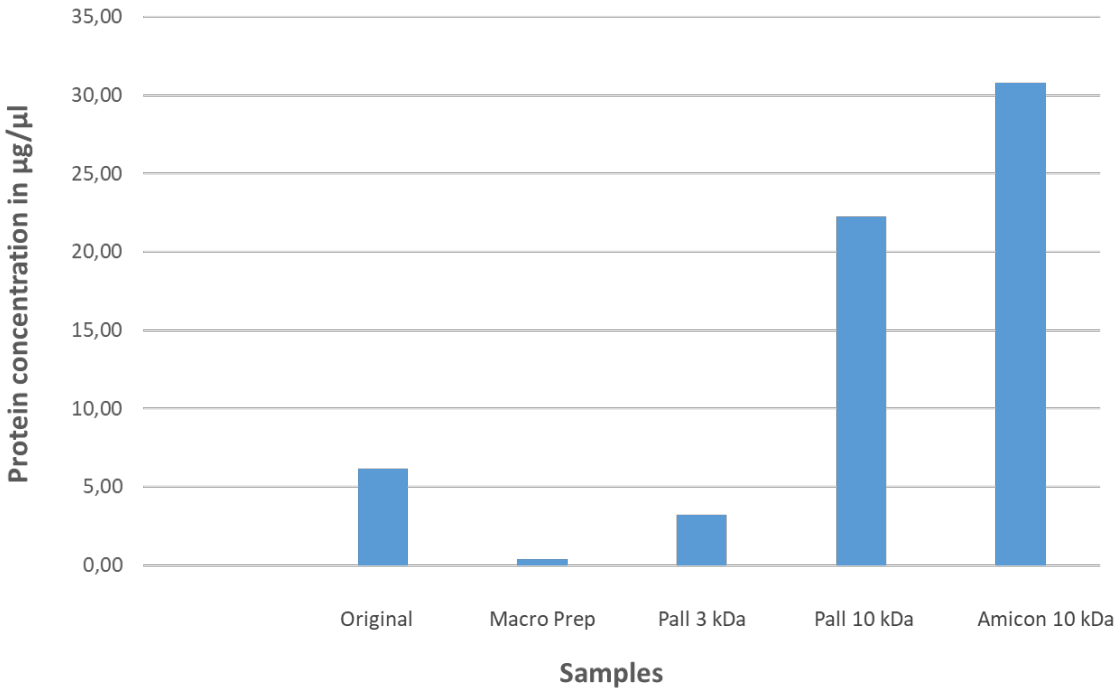

Fig. 4: Protein concentrations of a seminal plasma sample determined via the Pierce 660nm Protein Assay after protein extraction via different methods. An untreated seminal plasma sample was used as control. Protein extraction was operated with the Macro- Prep CM beads, with a 3 kDa membrane ultrafiltration, ultrafiltration with a 10 kDa membrane of Pall and ultrafiltration with an Amicon 10 kDa membrane. The samples of the ultrafiltration corresponds to the retentates of the filters.

5.2.3. Mass spectrometry analysis of biological replicates

The retentates of the Pall 10 kDa filter samples were used for protein digestion, followed by nano HPLC- MS/MS analysis to identify the proteins contained in equine seminal plasma. In order to show variability, five biological replicates of seminal plasma were examined for their protein composition.

According to database search, between 575 and 741 proteins were identified, whereas 0 to 2 AMPs were included (Table 1). The AMPs, which could be discovered across these 5 seminal plasma samples were equine lysozyme (Lysozyme C, milk isozyme OS=Equus caballus OX=9796 GN=LYZ PE=1 SV=1) and Psoriasin (S100A7; Protein S100-A7 OS=Equus caballus OX=9796 GN=s100A7 PE=3 SV=1). However, the corresponding PSM values of these two AMPs were between 2 and 4, indicating low concentrations.

Table 1: Orbitrap LC-MS/MS analysis of seminal plasma samples of 5 different stallions. 0 to 2 AMPs were identified.

Sample	AMPs	Total number of identified proteins
Seminalplasma_1	0	**586**
Seminalplasma_2	1	**716**
Seminalplasma_3	2	**741**
Seminalplasma_4	2	**592**
Seminalplasma_5	1	**575**

The comparison showed that in total 514 proteins were detected in all 5 seminal plasma samples (Fig. 5). In general, there is a broad abundance overlap of all identified proteins, just 101 proteins were only present in one stallion.

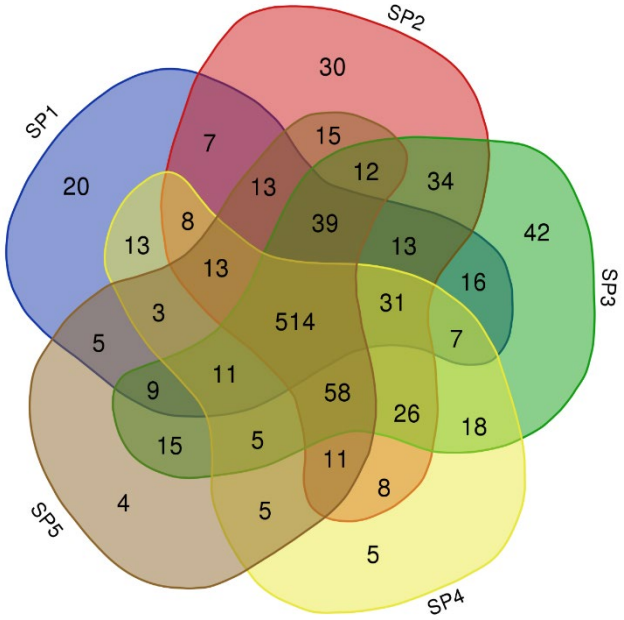

Fig. 5: Abundance and overlap of identified proteins: The Venn diagram show all proteins, which could be identified via Orbitrap LC-MS/MS analysis in seminal plasma samples 1-5 (SP1-5). 514 proteins were shared by all 5 stallions while 101 proteins appeared only in one stallion.

Additionally, flow-through fractions from ultrafiltration were analyzed by LC-MS as well as exemplarily for one replicate each. According to the SDS PAGE gels, low abundance of proteins and peptides in these molecular weight range was assumed. This presumption was confirmed: no AMPs could be detected and 64 to 160 proteins and peptides were identified in the flow through samples of the ultrafiltration (Table 2). Most of these 64 to 160 proteins belonged to fragments of diverse proteins. Thymosin beta-4 (5.1 kDa) was the protein with the lowest molecular weight, which could be identified.

Table 2: Orbitrap LC-MS/MS analysis of the flow through, which we received after ultrafiltration via a 3 kDa filter of Pall Life Science, a 10 kDa filter of Pall Life Science and a 10 kDa filter of Amicon. No antimicrobial peptides could be detected and 64 to 160 different proteins were identified in these samples.

Sample	Identified proteins	AMPs
3 kDa_Pall_FT	143	0
10 kDa_Pall_FT	64	0
10 kDa_Amicon_FT	160	0

In general, a broad spectrum of proteins could be determined in equine seminal plasma samples, but no proteins or peptides below 5 kDa. Across all equine seminal plasma samples, 2 AMPs were examined, but abundance was assumed to be very low.

5.3. Lysozyme analysis

Lysozyme concentration was determined via a lysozyme ELISA kit. In one pony stallion, no lysozyme was detected in both ejaculates (Fig. 6). This 4-year-old pony stallion had a very poor semen quality: low volume and density, 17.47% and 23.43% total motility, and 76% pathomorphological sperm. In two other stallions (one Quarter Horse and one pony), lysozyme could not be detected in one ejaculate each although these ejaculates fulfilled the minimal requirements and had good semen quality.

Seminal plasma samples of all other stallions contained lysozymes in both ejaculates in a concentration of 0.2 – 3.5 ng/ml. In general, the concentration was below 1 ng/ml, except for one pony stallion, which had a concentration of 3.5 ng/ml in one ejaculate. This one ejaculate had only 2 ml volume and a concentration of 600 mio sperm cells/ ml. Motility was quite good and within the minimal requirements (~ 80% total motility).

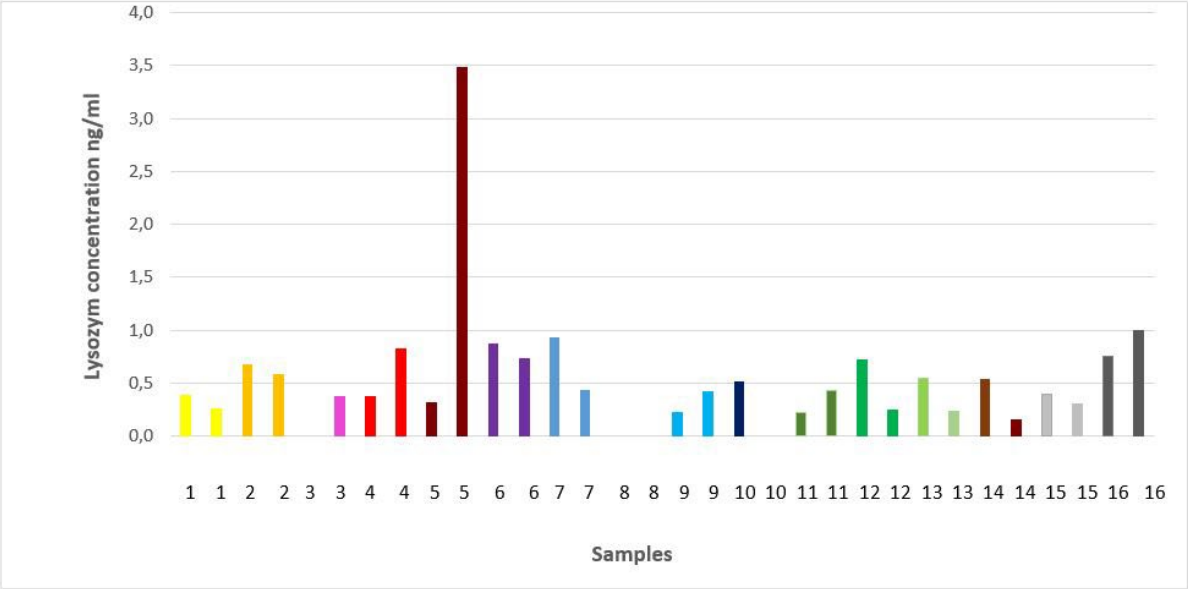

Fig. 6: Lysozyme concentration in seminal plasma collected from 16 stallions. From each stallion, two ejaculates were included.

5.4. Bactericidal effect of seminal plasma: Growth inhibition assay

Seminal plasma samples were incubated with *Sc. zooepidemicus* and *E. coli* at 37 °C for 24h using a bacterial starting dose of $1x10^5$ CFU in the first trial and in the second experiment a defined CFU of $1x10^3$. OD was measured at 595 nm before and after incubating and GI was calculated. Afterwards, CFU values of each sample were determined. Control samples were seminal plasma without any bacteria as well as *E.coli* and *Sc. zooepidemicus* without seminal plasma.

No changes of the OD values of seminal plasma samples without bacteria could be observed. *E. coli* with and without seminal plasma had quite similar OD values. In contrast, measured OD values of *Sc. zooepidemicus* with seminal plasma were lower compared to *Sc. zooepidemicus* without seminal plasma (Fig.7).

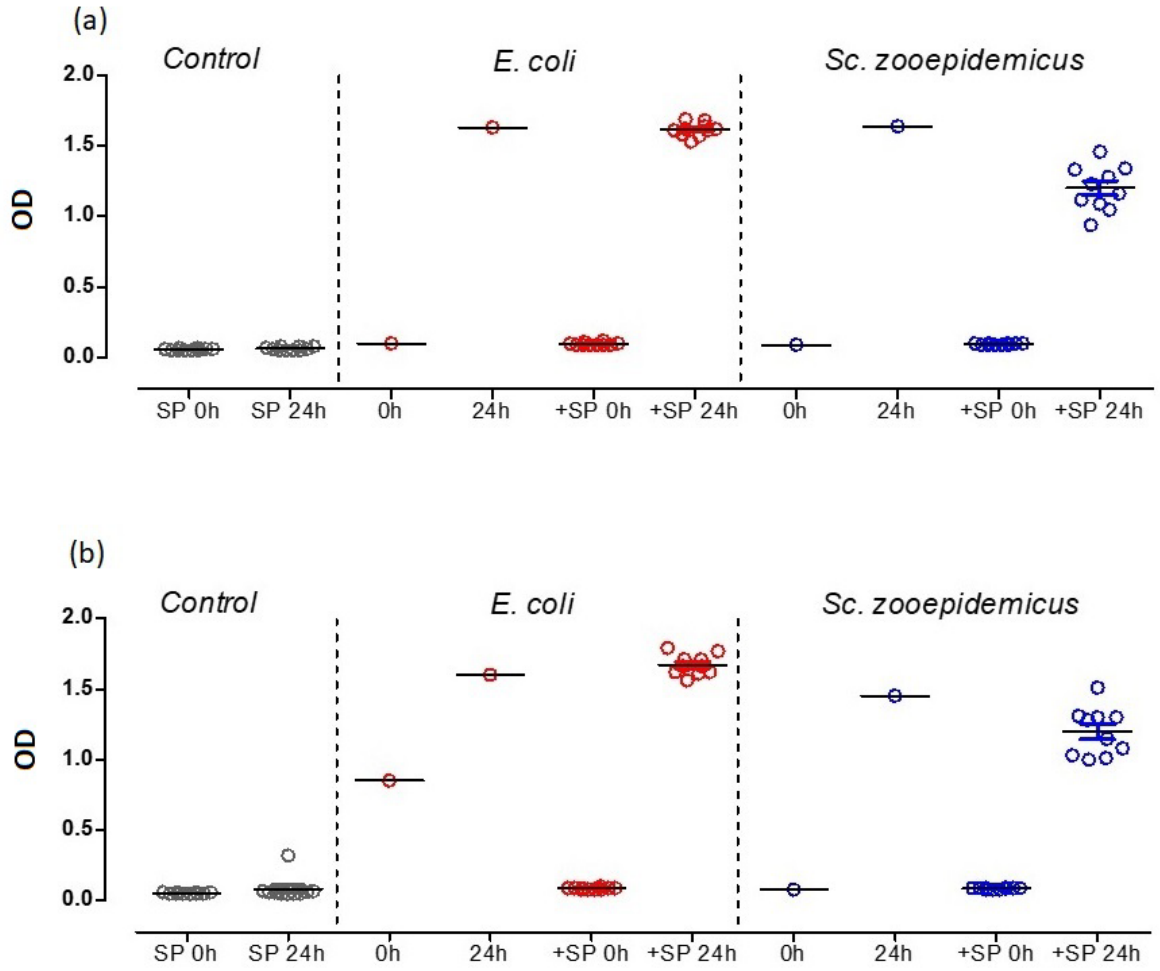

Fig. 7: Bactericidal effect of equine seminal plasma – results from the growth inhibition assay: Seminal plasma samples (SP) of five different stallions with two ejaculates per stallion were incubated with either *E.coli* or *Sc. zooepidemicus* for 24h at 37°C. The OD was measured before and after 24h incubation. Controls were seminal plasma samples without bacteria as well as *E.coli* and *Sc. zooepidemicus* without seminal plasma. In the first experiment (a), we used a defined starting concentration of bacteria with a defined CFU of $1x10^5$. In the second experiment (b), the CFU was lower with of $1x10^3$. Statistical analysis for a and b: time $p<0.001$, treatment $p<0.001$, time x treatment $p<0.001$.

In 27 of 60 equine seminal plasma samples, there was no growth inhibition effect on *E. coli*. In the remaining 33 samples, the average growth inhibition was 1.76 % (SD ± 2.51). In contrast, in 58 of 60 equine seminal plasma samples, growth inhibition of *Sc. zooepidemicus* could be detected. The average growth inhibition was 23.59 % (SD ± 11.24).

Additionally, we determined the CFUs in all samples and compared the resulting CFUs in the positive controls, which included only bacteria, with those in samples including bacteria and equine seminal plasma (Tab. 3). In both trials, CFU values of seminal plasma samples with *E.coli* were decreased compared to CFU values of the control, including pure samples of *E.coli* (Fig. 8). Moreover, *Sc. zooepidemicus* with equine seminal plasma had also less CFU compared to *Sc. zooepidemicus* without seminal plasma.

Table 3: Growth inhibition assay: CFU values of seminal plasma samples (S) of five different stallions, two ejaculates per stallion. These seminal plasma samples were incubated with either *E. coli* or *Sc. zooepidemicus* for 24h at 37°C. Positive controls (+C) were *E. coli* and *Sc. zooepidemicus* without seminal plasma. Moreover, we used two different initial concentrations (IC) of bacteria with a defined CFU of $1x10^5$ and $1x10^3$.

Bacteria	IC	+C	S
E.coli	10^5	22.5×10^{13} ($\pm 2.5 \times 10^{13}$)	8.7×10^{13} ($\pm 6.4 \times 10^{13}$)
E.coli	10^3	22.5×10^{13} ($\pm 2.5x\ 10^{13}$)	10.8×10^{13} ($\pm 16.6 \times 10^{13}$)
Sc. zooepidemicus	10^5	300×10^{12} ($\pm 200 \times 10^{12}$)	1×10^{12} ($\pm 2.4 \times 10^{12}$)
Sc. zooepidemicus	10^3	20×10^9 ($\pm 0 \times 10^9$)	3.6×10^9 ($\pm 1.4 \times 10^9$)

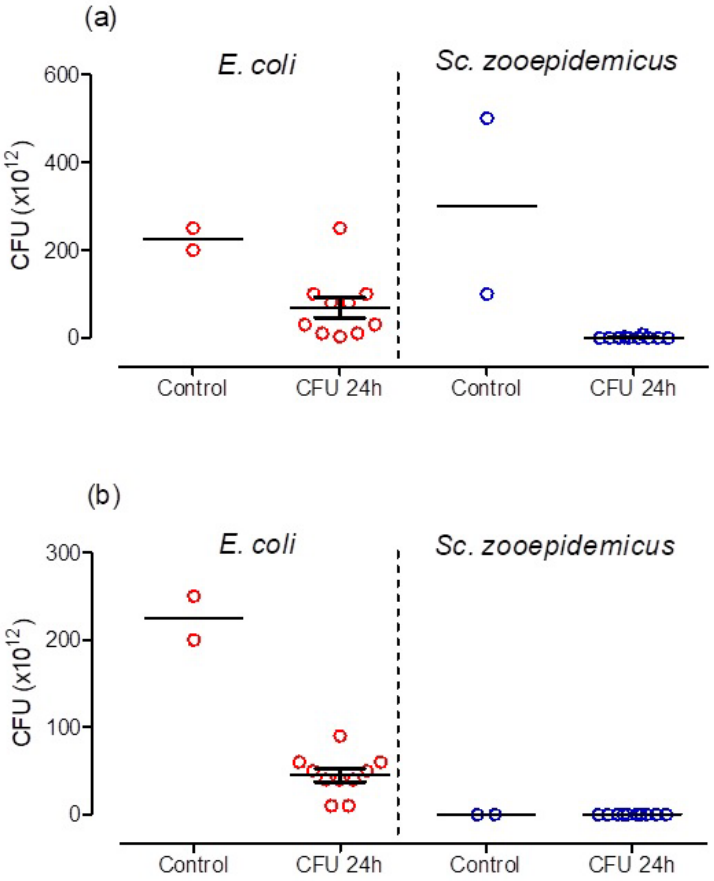

Fig. 8: Bactericidal effect of equine seminal plasma – colony forming units: Seminal plasma samples (SP) of five different stallions with two ejaculates per stallion were incubated with either *E.coli* or *Sc. zooepidemicus* for 24h at 37°C. Afterwards, CFU was determined. Controls were *E.coli* and *Sc. zooepidemicus* without seminal plasma. In the first experiment (a), we used a defined starting concentration of bacteria with a defined CFU of $1x10^5$. In the second experiment (b), the CFU was lower with of $1x10^3$. Statistical analysis for a and b: time within treatment $p<0.001$

Thus, taking into account OD values, while there was a clear bactericidal effect of seminal plasma on growth of *Sc. zooepidemicus*, only minor to no effects of seminal plasma on growth of *E. coli* were detected. Interestingly, taking into account CFU values, equine seminal plasma had an impact on *Sc. zooepidemicus* as well as *E.coli.*

5.5. Effect of lysozyme on sperm characteristics

In samples containing lysozymes, sperm motility (Fig. 9) showed a more pronounced decrease in comparison to controls in a dose-dependent manner. In samples with 2 mg/ml lysozyme, motility was lowest. An even more pronounced effect of lysozyme supplementation on sperm viability (Fig. 10) was demonstrated.

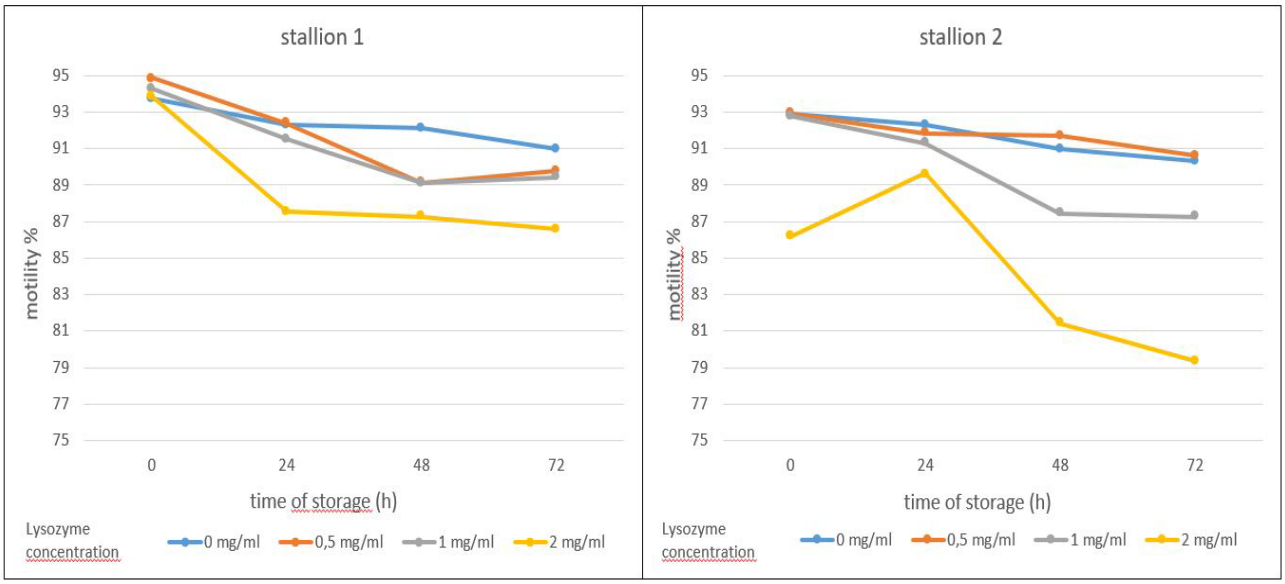

Fig. 9: Effect of different lysozyme concentrations on sperm motility: ejaculates of 2 different stallions were diluted to a sperm cell concentration of 25 mio/ml and different lysozyme concentrations (control = 0 mg/ml, 0.5 mg/ml; 1 mg/ml; 2 mg/ml) were added. The samples were stored at 5°C and motility was analyzed over 72 hours via CASA..

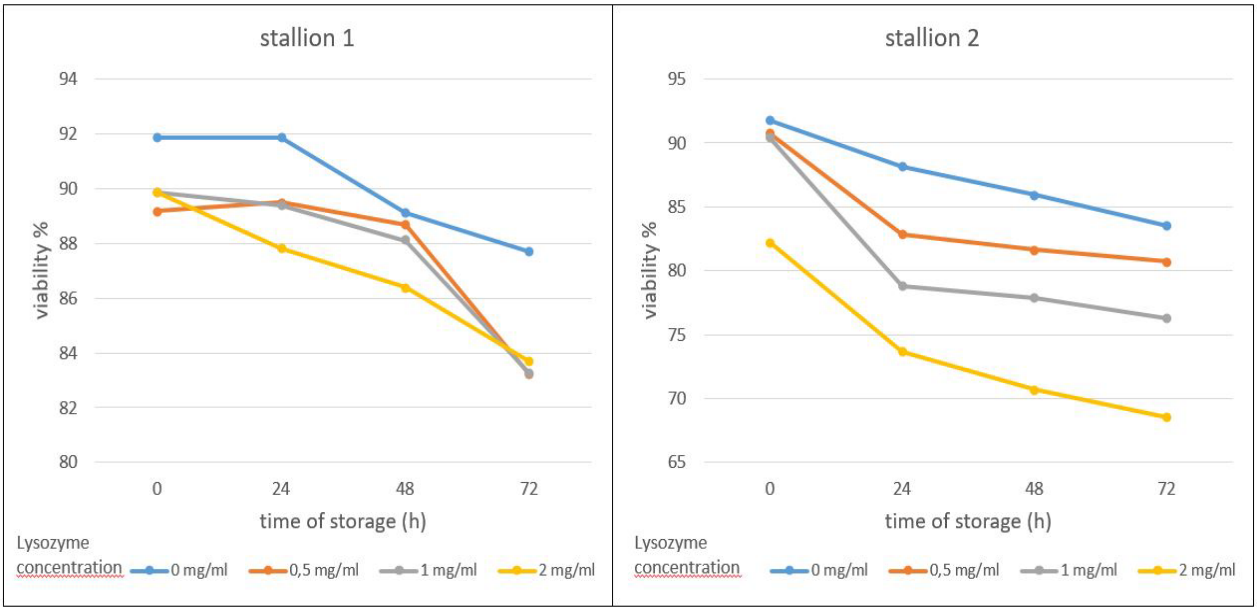

Fig. 10: Effect of different lysozyme concentrations on sperm viability: ejaculates of 2 different stallions were diluted to a sperm cell density of 25 mio/ml and mixed with different lysozyme concentrations (control = 0 mg/ml, 0.5 mg/ml; 1 mg/ml; 2 mg/ml). The samples were stored at 5°C and their viability was analyzed over 72 hours via CASA.

5.6. Synthetic lysozymes as alternative to antibiotics

5.6.1. Growth inhibition assay with synthetic lysozymes

For testing the effectiveness of synthetic lysozymes as an alternative to antibiotics, we incubated all seminal plasma samples with either *Sc. zooepidemicus* or *E. coli* (defined CFU: $1x10^5$) and additionally with different lysozyme concentrations (control = 0 mg/ml, 0.5 mg/ml; 1 mg/ml; 2 mg/ml). The OD was measured before and after 24h incubating at 37 °C. Subsequently, GI was calculated and CFU values were determined.

No differences in OD values after incubation with *E. coli* were detected in comparison to controls. In contrast, significant inhibition of growth of *Sc. zooepidemicus* in the presence of lysozyme was detected by OD values in a dose-dependent manner, OD was lowest in the presence of the highest lysozyme concentration. (Fig. 11).

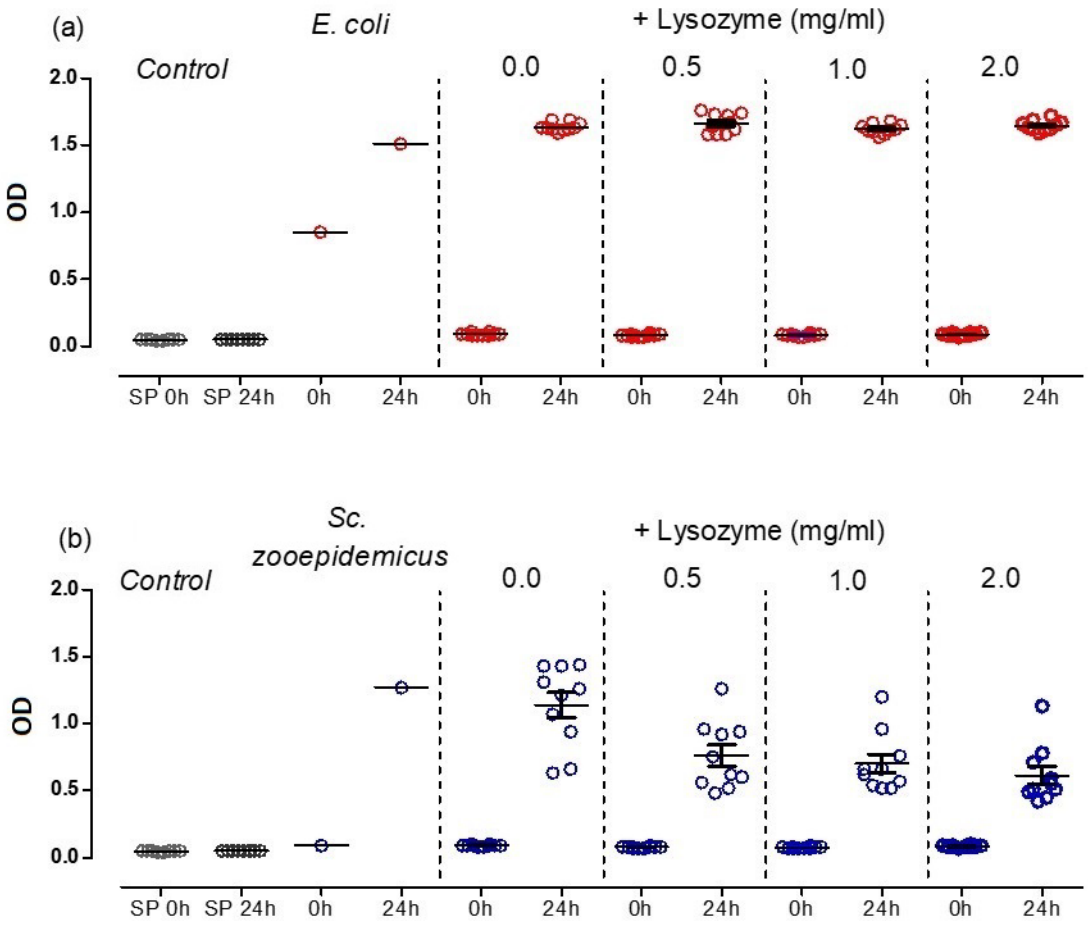

Fig. 11: Growth inhibition assay with synthetic lysozymes: Seminal plasma samples (SP) of five different stallions, two ejaculates per stallion, were incubated with either *E. coli* (a) or *Sc. zooepidemicus* (b) for 24h at 37°C. Different lysozyme concentrations (control = 0 mg/ml, 0.5 mg/ml; 1 mg/ml; 2 mg/ml) were added. The OD was measured before and after 24h incubation. Controls were pure seminal plasma samples as negative control and *E. coli* and *Sc. zooepidemicus* as positive controls. Statistical analysis for a and b: no differences.

Afterwards, GI was determined for the seminal plasma samples. In all samples with *E. coli*, seminal plasma and different concentrations of lysozymes, no GI was detected. In comparison, equine seminal plasma with *Sc. zooepidemicus* led to an average bacterial growth inhibition of 28.11 % (SD ± 19.47), which fits to the results of the first growth inhibition test. However, additional lysozymes with the concentration of 0.5 mg/ml had a GI of 38.47 % (SD ± 18.33), samples including 1 mg/ml lysozymes showed a GI of 39.99 % (SD ± 16.49) and 2 mg/ml lysozymes had even 49.04 % GI (SD ± 16.25).

Moreover, CFU values were determined for all samples (Fig. 12). Bacterial growth inhibition could be determined in samples with equine seminal plasma with both *E.coli* and *Sc. zooepidemicus*. Additionally, the higher the lysozyme concentration, the lower the CFU.

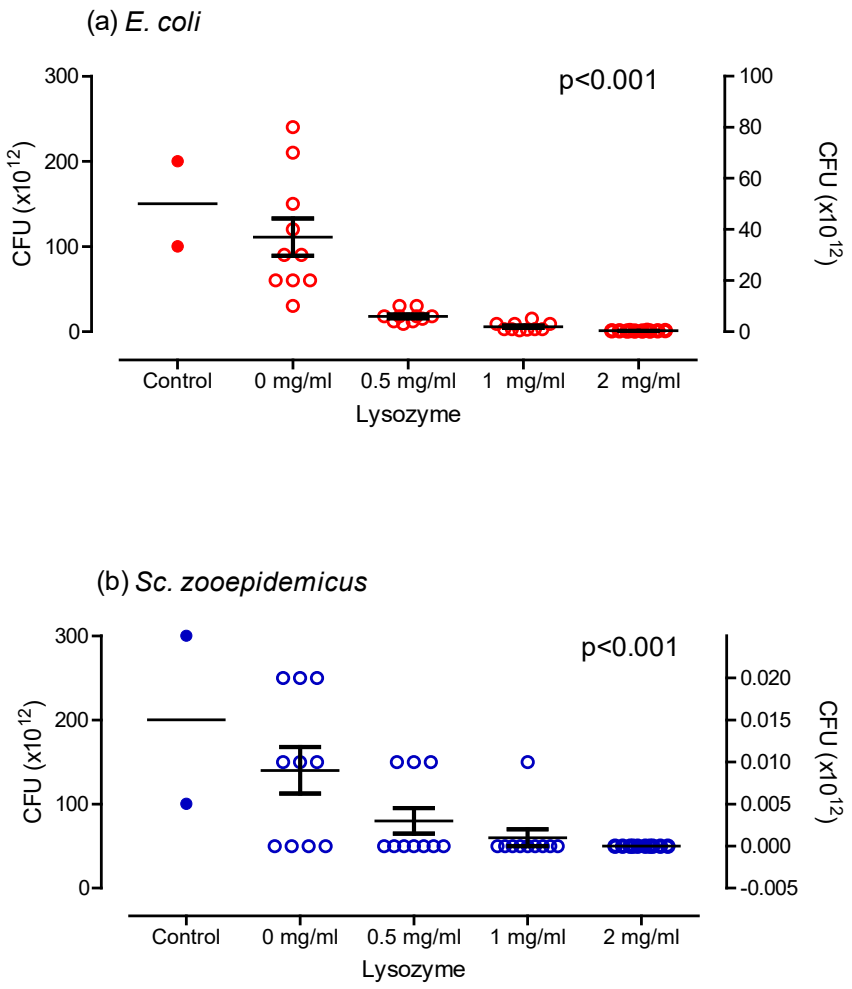

Fig. 12: Growth inhibition assay with synthetic lysozymes – results from the colony forming units: Seminal plasma samples (SP) of five different stallions with two ejaculates per stallion were incubated with either *E.coli* or *Sc. zooepidemicus* for 24h at 37°C. Afterwards, CFU was determined. Controls were *E.coli* and *Sc. zooepidemicus* including neither seminal plasma nor lysozymes. Different lysozyme concentrations (control = 0 mg/ml, 0.5 mg/ml; 1 mg/ml; 2 mg/ml) were added. In the first experiment (a), we used a defined starting concentration of bacteria with a defined CFU of $1x10^5$. In the second experiment (b), the CFU was lower with of $1x10^3$. The control results refer to the left y-axis, however the samples with seminal plasma and lysozyme refer to the right y-axis. Statistical analysis for a and b: lysozyme supplementation $p<0.001$.

To sum up, synthetic lysozymes decreased bacterial growth of *Sc. zooepidemicus*, which could be examined via OD and CFU values. In contrast, there was no difference between pure *E. coli*, *E. coli* with equine seminal plasma and *E. coli* with seminal plasma and additionally different lysozyme concentrations according to analyzed OD values. But when determining CFU, growth inhibition of *E. coli* was obvious and was increasing with rising lysozyme levels.

6. Discussion

6.1. Presence of AMPs in stallion seminal plasma

Protein extraction was performed with 4 different extraction methods, which were tested according to their effectiveness via SDS PAGE and protein concentration measurement. According to the gels (Fig. 1), variant 1 seemed to extract a large amount of proteins, but might include additional contamination. Contrary, the sample of variant 2 included a lower amount of proteins, but possibly with little contamination. The flow through of the 3 kDa filter (Pall) (Fig. 3) had clear bands on the Tricine- SDS-PAGE above 3 kDa. Hence, this filter did not separate the proteins very precisely. Both 10 kDa filters (Pall, Amicon) seemed to filter very accurately. Due to the fact that samples of variant 1 showed a lot of smear on the SDS-PAGE, we did not use these C18 SPE columns anymore. Moreover, we only used the retentates of ultrafiltration for further analysis because no proteins below 5 kDa were visible on the gel.

Hence, ultrafiltration with a 10 kDa filter crystallized as the most accurate protein extraction. In general, no proteins or peptides below 5 kDa could be detected in all 4 extraction methods, as well as in the unprocessed seminal sample, either via SDS PAGE or via mass spectrometry. The absence of proteins in seminal plasma of stallions below 5 kDa is consistent with other studies (Druart et al., 2013; Jobim et al., 2011). It can be concluded that equine seminal plasma does not include any proteins or peptides below 5 kDa, consequently the majority of AMPs that were characterized in horses (Bruhn, 2005) cannot be present in equine seminal plasma.

In our study, a huge variety of proteins could be identified, but only two known equine AMPs were determined: equine lysozyme and psoriasin. According to the PSM values, these AMPs appeared only occasionally suggesting that their concentration is very low. An antibacterial effect in stallion seminal plasma is therefore not feasible.

The average lysozyme concentration in stallion seminal plasma was less than 1 ng/ml when measured via an ELISA Kit (Immundiagnostik AG) that we validated for this fluid. This ELISA Kit was originally developed for human lysozymes. Equine lysozymes belong to the c-type lysozymes and are consequently similar to chicken and human lysozymes, but with a divergent calcium binding mechanism (Mališauskas et al., 2003). Hence, the sensitivity of the human lysozyme ELISA kit could be reduced, but results were confirmed by mass spectrometry analysis. In contradiction to the results of our study, Jakob et al. (2023) detected a lysozyme concentration of approximately 1 µg/ml in equine seminal fluid when using the lyso-plate method (Schulze et al., 2020). This method can, however, be biased by salts like sodium chloride (Peeters & Vantrappen, 1977). Equine lysozyme has a molecular mass of 14.7 kDa. When using SDS-PAGE, a clear band in this area was visible in all stallion seminal plasma samples. Mass spectrometry results, however, demonstrated that several other proteins could also trigger a band in this area. We therefore assume that equine lysozymes is present in stallion seminal plasma in the small concentrations determined in our samples.

6.2. Bactericidal effect of seminal plasma: Growth inhibition assay

To determine possible antibacterial effects of stallion seminal plasma, growth inhibition assays with *E. coli* and *Sc. zooepidemicus* were performed. According to the OD values and the associated GI, we could not determine a pronounced bactericidal effect of equine seminal plasma against *E. coli.* On the other hand, CFU values of *E. coli* with equine seminal plasma were decreased. The determination of bacteria via CFU only considers bacteria which are alive

and growing (Davey, 2011; Kerstens et al., 2013). Culturable and alive bacteria are the crucial factors in this study. Consequently, they influence the quality of the semen. OD measurement is counting bacterial cell debris of life as well as of dead bacteria. Hence, it might be that there is no visible difference according to optical density, but the concentration of living culturable bacteria is decreased. It might be that the growth of *E. coli* is impeded by equine seminal plasma, which can be observed when examining the CFU, but the cell wall of *E. coli* stays intact whereby the OD is not influenced.

Nevertheless, the study demonstrated a clear bactericidal effect of stallion seminal plasma against *Sc. zooepidemicus*, which was determined both via decreased OD and CFU values with an average growth inhibition of 23.6%. These results suggest that equine seminal plasma contains substances that have an antibacterial effect at least against *Sc. zooepidemicus.* This antibacterial effect might be caused by proteins or peptides. In human seminal plasma, such bactericidal effect is, however, zinc dependent (Edstrom et al., 2008).

In the study of Schulze et al. (2019), growth inhibition of boar semen was tested in 119 boar ejaculates. Bacteria included were *Staphylococcus aureus* (*S. aureus*) and *E. coli*. Growth inhibition differed among ejaculates with decreased growth (tested by OD) of both bacteria in 38 samples (*E. coli*: 22.6 %; *S. aureus*: 35.9 %), decreased growth of only *E. coli* in 13 ejaculates (39.5 %), and decreased growth of only *S. aureus* in 46 semen samples (24.8 %). In 22 semen samples, no bacterial growth inhibition was detected. This study thus shows a wide variation of bacterial growth inhibition by porcine seminal plasma, which might be suggested as age related variance. Further studies for determining the specific mediators of the observed antimicrobial effect are necessary. The number of stallions (n=5) included into the present study is, however, too small to investigate such differences.

Rowe et al. (2011) determined growth inhibition of *E. coli* and *S. aureus* by semen from 11 mallard ducks. Bactericidal activity was 51% against *E.coli* and 40% against *S. aureus*, i.e. more consistent results than in pigs and horses were detected.

The mechanism of action of the antibacterial effect of horse seminal plasma against *Sc. zooepidemicus* remains unclear. It is, however, unlikely that the low concentration of equine AMPs demonstrated in this study are responsible and other mechanisms have to be assumed. One approach for further experiments might be to identify bacteria binding proteins or peptides, which affect the stability or composition of the bacterial cell wall. Therefore, a mass spectrometry analysis of bacteria which were incubated with equine seminal plasma, would be a possible approach to identify proteins, which bind to the bacterial cell wall.

6.3. Synthetic lysozymes as alternative to antibiotics

Searching for an adequate AMP, which can be used as an alternative to antibiotics in semen is a complex process. It is difficult to find an AMP, which shows a distinct reduction of bacterial growth, similar to antibiotics, and additionally does not affecting the semen quality. Examples of tested AMPs, which failed these requirements are PR-39, PMAP-36 and PMAP-37 in boar semen (Bussalleu et al., 2017). These AMPs could reduce bacterial load in higher concentrations, but with these concentrations, sperm motility, progressivity and viability were significantly impaired. Shaoyong et al. (2019) demonstrated that ε-polylysine in boar semen decreased bacterial concentration without consirable effects on sperm characteristics. Similarly, Puig-Timonet et al. (2018) evaluated successfully porcine beta defensins-1 (PBD1)

and -2 (PDB2) as possible replacements for antibiotics in semen extenders. A concentration of 3 µM of both AMPs could reduce bacterial growth without impairing sperm quality. Both approaches might also be tested for horse semen. In the present investigation, lysozymes in concentrations of 0.5 and 1 mg/ml did not impair sperm motility and viability. When semen was stored for 24 hours, OD values and CFU of *Sc. zooepidemicus* decreased significantly, whereas no effects on *E. coli* were detected. Because lysozymes hydrolyze 1,4- beta linkages in the peptidoglycan cell wall of bacteria, a greater effect on gram positive bacteria can be explained. Their thick cell wall largely consists of peptidoglycan whereas in gram negative bacteria a thin peptidoglycan cell wall is surrounded by an outer membrane containing lipopolysaccharide (Gutiérrez & Briers, 2021). The effects of lysozymes on bacterial growth in horse semen may be further improved by optimizing for example pH, storage temperature and extender composition.

Seminal plasma including 2 mg lysozymes had the most pronounced effect on growth of *Sc. zooepidemicus*, but this concentration had a clear detrimental effect on the quality of sperm and thus questioning its use during cooled-storage. A lysozyme concentration of 1 mg/ml might be worth considering as a possible alternative for antibiotics in semen extenders. because of simultaneous effects on bacterial growth of at least *Sc. zooepidemicus* and no detrimental effects on semen quality.

7. Conclusion

Equine seminal plasma includes only low concentrations of the so far isolated equine AMPs psoriasin and equine lysozymes. Itself has antibacterial effects on *Sc. zooepidemicus*. Seminal plasma therefore causes bacterial growth reduction, which can be measured via OD values and CFU. This bactericidal effect might not be induced by the low concentrations of the determined equine AMPs. The results suggest that seminal plasma might include other substances which impact the growth of *Sc. zooepidemicus*. This requires further investigations.

Hen egg white lysozyme was identified as a possible alternative to antibiotics in semen extender used for cooled-stored use of horse semen. A concentration of 1 mg/ml could decrease bacterial growth of *Sc. zooepidemicus* and was not detrimental to semen quality. Contrary, *E. coli* was not influenced at all by the lysozymes.

More research is required to determine which bacteria are influenced by lysozymes in this special concentration. Moreover, the identification of the protein or complex which induces an antibacterial effect on *Sc. zooepidemicus*, would be another perspective for future studies, as well as the identification of this bactericidal effect and if it is effective against other bacteria.

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