THE INTERACTION BETWEEN SEMEN, SEMINAL PLASMA, AND ENDOMETRIUM IN INTER- AND INTRASPECIES BREEDING IN HORSES

BY

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DISSERTATION

Submitted in partial fulfillment of the requirements
for the degree of Doctor of Philosophy in VMS-Comparative Biosciences
in the Graduate College of the
University of Illinois Urbana-Champaign, 2024

Urbana, Illinois

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ABSTRACT

Endometritis is the leading cause of subfertility in horses. Endometritis often starts as a physiological inflammatory response to sperm and bacteria contained in the semen, which self-resolves within 48 hours; in 20% of the mare population, this condition is associated with an unregulated uterine inflammatory response that leads to an unfavorable environment for an embryo and predisposes to chronic infectious endometritis and the development of fibrosis. Semen factors such as concentration and motility can affect post-breeding endometritis; anecdotally donkey sperm causes lower uterine inflammation and higher pregnancy rates than horse sperm in subfertile mares. The role of bacteria in uterine healthy including endometritis has been re-invented over the past few years due to significant changes in the techniques that allow the detection of a core uterine microbiota, without the necessity of performing standard microbiological techniques. The first systematic description of the core uterine microbiome in horses was recently published in 2022, a multicentric study conducted in the US and Australia. A core uterine microbiome characterized by high richness and low diversity is thought to be necessary for women to modulate inflammation, establish a pregnancy, and prevent uterine infections from pathogenic bacteria. In domesticated animals, there is scarce information on the functional role of the uterine microbiome. To compare the uterine inflammatory response in the presence of intraspecies and interspecies sperm and seminal plasma, markers of uterine inflammation (uterine fluid, uterine edema, cytokines) were assessed before and after the infusion with donkey and horse semen and seminal plasma; next, changes in the uterine microbiome over time, across treatments and based on the embryo recovery outcome were evaluated. The results showed that donkey seminal plasma has an anti-inflammatory composition and role on the mare endometrium and that the horse uterine

microbiome fluctuates over time and in relation to the presence of an embryo. Collectively, the present findings suggest that the male seminal proteome, metabolome, and microbiome alter the mare uterine response and may have further trickle-down effects on physiological processes such as the response to semen or the early stages of embryonic development.

ACKNOWLEDGMENTS

I am grateful to my advisor, Dr. Igor Canisso, and doctoral committee, Drs. Jay Ko, Indrani Bagchi, and David Miller, for the knowledge, support, and guidance received during this program; it is one of the most significant achievements of my career. I would also like to thank my family, my father, Pierluigi, and my sisters, Martina and Matilde, for their unwavering presence and encouragement.

To the girl of GRIT who dreamt of this day since childhood,

To the family who walked in GRACE supporting her to this day,

To a heart filled with GRATITUDE, which turned adversities into opportunities

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CHAPTER 1: Overview

Endometritis is the leading cause of subfertility, it decreases the pregnancy rate and increases pregnancy loss; noteworthy, it is also the third most common disease in horses (Traub-Dargatz et al., 1991, Canisso et al., 2016, Canisso et al., 2020). Endometritis often starts as a physiological inflammatory response to sperm and bacteria contained in the semen which self-resolves within 48 hours; in 20% of the mare population, this condition is associated with an unregulated uterine inflammatory response that leads to an unfavorable environment for an embryo and predisposes to chronic infectious endometritis and the development of fibrosis (Canisso et al., 2016, Canisso et al., 2020). The uterine inflammatory response is orchestrated by innate and adaptive immunity, involving several molecules and enzymatic cascades. Factors regarding sperm, such as concentration, motility, and the presence and amount of seminal plasma, affect the amplitude and duration of the uterine response (Troedsson et al., 2000, Troedsson et al., 2001, Fiala et al., 2007). Donkey sperm causes lower uterine inflammation and higher pregnancy rates than horse sperm in subfertile mares; however, this has not been thoroughly evaluated. The role of bacteria in endometritis and other uterine conditions has been re-evaluated over the past few years due to significant improvements in the investigation techniques that allow the detection of a uterine microbiota. The use of sequencing technologies enabled the discovery of a core microbiome in almost any location of the human body starting in 2007 with the launch of the National Institute of Health Human Microbiome Project (Turnbaugh et al., 2007); those techniques became progressively more available also in veterinary medicine and the core microbiome of different locations of the animal body was described in multiple species (Yang et al., 2017, Ault et al., 2019, Barba et al., 2020, Comizzoli and Power, 2019, Koziol et al., 2022, Lyman et al.,

2019, Murga Valderrama et al., 2023). The first systematic description of the core uterine microbiome in horses was published in 2022 with a multicentric study conducted in the United States and Australia (Holyoak et al., 2022). In women, the functional roles of the core microbiome was investigated, it is now recognized that a core uterine microbiome characterized by high richness and low diversity is necessary to modulate inflammation, establish a pregnancy, and prevent uterine infections from pathogenic bacteria (Giudice, 2016). Certain strains of bacteria seems pivotal in certain reproductive conditions. *Lactobacillus* spp. bacteria decreased pain and lesions in women affected by endometriosis and improved epithelial cell barrier function in response to the infection of human immunodeficiency virus-1 (Somigliana et al., 1999, Itoh et al., 2011, Dizzell et al., 2019, Khodaverdi et al., 2019). To date, there is scarce information on the functional role of uterine microbiome in domestic species. Researchers from Japan have confirmed that a uterine microbiome with a high abundance of *Lactobacillus spp*. is associated with fewer inflammatory cells in the uterus of postpartum dairy cows (Wu et al., 2021). Given the demand for alternative treatments to decrease antibiotic use for common animal diseases and the pressure for antimicrobial resistance, there is a critical need to investigate the core microbiome and explore ways to manipulate it to improve uterine health.

This dissertation details studies exploring the interaction between the endometrium, sperm, and seminal plasma using interspecies breeding to evaluate the impact of the seminal metabolome, proteome, and microbiome on the uterine inflammatory response and its microbiome. This dissertation is divided into five chapters, including figures, tables, and references at the end of the main text. Chapter 2 includes a review of the pertinent literature regarding intra- and interspecies breeding in horses, the post-breeding uterine inflammatory response, the composition and role of seminal plasma, and the mare uterine microbiome. Chapter 3 describes the changes in the uterine

inflammatory markers after intra- and interspecies breeding and also details the embryo and nuclear morphometry of mule embryos compared to horse embryos. Chapter 4 explores the mare uterine microbiome and its changes after the uterine infusion with semen or seminal plasma. The infusion of donkey semen and seminal plasma added further opportunities to elucidate the impact of the uterine microbiome on the mare uterine inflammatory response. Finally, conclusions and future directions are presented in Chapter 5.

CHAPTER 2: Introduction and review of pertinent literature

INTERSPECIES BREEDING IN HORSES

The earliest human-bred hybrid animal was produced in ancient Syria and Mesopotamia 4,500 years ago; it was a cross between two equids referred to as "Kunga" (Bennett et al., 2022). The kunga was obtained by mating domestic female donkeys (*Equus africanus asinus*) to Syrian wild male donkeys (*Equus hemionus hemippus*). Archeological records report that kungas were prized animals, with values exceeding 5-6 times that of one donkey and used to pull chariots of "nobility and gods" (Heimpel, 1994). The speed and agility given by the sire's side were essential in war zones; in one of the first representations of military expeditions that was depicted in mosaics of "The Standard of Ur", the Sumerian warriors were seen on a wagon pulled by a type of equid whose identity remained unknown until recently when it was confirmed that they were kungas **(Figure 2.1)** (Bennett et al., 2022). The use of kungas in the Mesopotamian regions faded over the centuries due to the introduction of the domestic horse (*Equus ferus caballu*s) that presented the advantages of being more tamable and having less reproductive challenges. In addition, the notion of the sterility of the kungas was immediately clear, and the young kunga foals were almost exclusively produced in Nagar, a village in the northern Mesopotamian in a single breeding farm, which limited its spreading across the region (Maekawa, 2006).

In the following centuries, horse-donkey hybrids became more common, with the mule (♂ *Equus ferus caballus* × ♀ *Equus africanus asinus*) being the most prevalent. The mule was initially bred in the Mediterranean regions and then spread to other territories with the Army and soldiers; its presence was confirmed in Roman forts starting from 160AD (Berger et al., 2010). Over the centuries, mules were used in anything from harness racing by ancient Greeks to agriculture during

the Medieval ages, especially in France, Italy, and Spain (Mann and Scharff, 2020, Lepetz et al., 2021). The size, power, and endurance of mules made them prized animals; the use of specific breeds of donkeys, like the Poitou or the Catalonian, was considered the key to obtaining large-size, strong mules that would be able to endure hard work in the fields (Mitchell and Mitchell, 2018). Noteworthy, two donkey jacks were imported from the Zamora province in Spain by George Washington in 1785 to supply quality sires to the shallow mule population present in the United States at that time (Washington, 1994). The mules and horses enhanced transportation and colonization of the west of the United States and Brazil (Canisso et al., 2019). Nowadays, mules are used as working animals in large cattle operations both in North and South America, but in many cases, they are used for leisure such as trail riding and in several Western and English disciplines, packing and trail riding (Canisso et al., 2019).

The Equus Genus includes three subgenera and seven living species with a wide array of morphological and karyotypical features **(Table 2.1.)**. Although donkey-horse hybrids are more common, hybrids between the other species of the Equus genus have also been reported: ♂ *Equus africanus asinus* × ♀ *Equus grevyi*, donkra; ♂ *Equus ferus caballus* × ♀ *Equus africanus asinus*, hinny; ♂ *Equus ferus caballus* × *Equus grevyi*, hebra; ♂ *Equus grevyi* × ♀ *Equus ferus caballus*, zorse; ♂ *Equus grevyi* × ♀ *Equus africanus asinus,* zonkey (King, 1967, Benirschke, 1967, Benirschke et al., 1964, Benirschke et al., 1962, Gray, 1972).

Studies assessing the interactions between these species have been focused on evaluating the behavioral interactions of donkey jacks and horse mares during natural mating or semen collection (Lodi et al., 1995, Canisso et al., 2009, Canisso et al., 2010), fertility of fresh, cooled, and frozen semen of donkey jacks in horse mares (Rota et al., 2012, De Oliveira et al., 2016, Oliveira et al., 2014, Vidament et al., 2009, Gobato et al., 2022, Segabinazzi et al., 2021),

physiology of early pregnancy (17 days onward) and losses (Boeta and Zarco, 2005, Boakari et al., 2019, Carluccio et al., 2020, Paolucci et al., 2012). Noteworthy, donkey semen appeared more successful when used to breed mares rather than jennies (**Table 2.2**); however, the interaction between the donkey semen and the mare endometrium has not been thoroughly described. Anecdotally, problem mares have higher fertility when mated to a donkey than to a horse; however, the reasons are still unknown and an objective evaluation of the interaction between the mare endometrium and the jack semen and seminal plasma has yet to be conducted.

A research group from Brazil presented preliminary data in 2023 that showed that after 6 h from the insemination with donkey frozen semen, both mares and jennies showed a marked uterine inflammatory response, but the number of inflammatory cells in the uterine cytology decreased in mares compared to jennies beyond 12 h from the insemination (Lopes Costa et al., 2023). The uterine inflammatory response to semen is complex, and numerous markers could be used to assess it as described in the following subheadings.

UTERINE INFLAMMATORY RESPONSE IN THE HORSE

The uterus's mucosal immune system is activated after sperm and bacteria contained in the semen interact with the endometrium; the resulting inflammation (i.e., post-breeding endometritis) normally resolves within 48 hours after exposure (Kotilainen and Huhtinen, 1994, Troedsson et al., 1995, Canisso et al., 2020). The mucosal immune system's innate and adaptive components orchestrate the defense of the reproductive tract with the goal of eliminating seminal plasma, debris, bacteria, and excess sperm from the uterine lumen to prepare an appropriate environment for the embryo (Troedsson et al., 1995, Troedsson et al., 2001, Zent et al., 1998, Canisso et al., 2020). Based on the ability of the mare to clear the uterus after breeding and modulate the

inflammatory response, mares are classified as resistant or susceptible to persistent breeding-induced endometritis (PBIE) if the inflammation resolves within 48 hours or not, respectively (Christoffersen and Troedsson, 2017, Canisso et al., 2020). Mares susceptible to PBIE present an exacerbated inflammatory response, with defects in the mechanisms that normally modulate the inflammatory response, or both (Woodward et al., 2013a, Woodward et al., 2013b, Woodward and Troedsson, 2013, Woodward and Troedsson, 2014, Canisso et al., 2020). Both sperm and bacteria are detected by epithelial and sentinel cells in the endometrium via Toll-like receptors (TLR) that bind to invariant molecular structures present on their surface, which are also known as pathogen-associated molecular patterns (PAMPS) (Quayle, 2002, Kotilainen and Huhtinen, 1994). Other pattern recognition receptors (PRRs) may contribute to the mucosal defense; NOD-like receptors can detect intracellular pathogens but their role in horse endometritis has not been cleared (Franchi et al., 2009). The activation of TLR functions as a trigger for the inflammatory cascade; nuclear factor kappa beta (NF-κβ) is then activated by a series of enzymatic reactions that activate one of its 5 subunits (ReIA, ReIB, ReL, p50, and p52) (Lawrence, 2009). Next, the activation of NF-κβ upregulates the expression of genes coding for pro-inflammatory pro-cytokines (interleukin 1, IL1; interleukin 6, IL6; tumor necrosis factor-alpha, TNFα) that are activated by a variety of enzymes, including caspases, elastase, cathepsins, metalloproteinases, and trypsin. Cyclooxygenase-2 (COX-2) is also a product of the NF-κβ activation that causes the production of prostaglandins, another important mediator of inflammation. Inflammatory cytokines and prostaglandins trigger the activation of the vascular endothelial cells resulting in local uterine edema. Noteworthy, mares susceptible to PBIE have a greater endometrial expression of pro-inflammatory molecules such as IL1β, chemokine ligand 8 (CXCL8), and TNFα (Fumuso et al., 2006, Fumuso et al., 2007). The activation of the vascular endothelial cells induces chemotaxis, and neutrophils start accumulating

in the equine uterine lumen within 30 minutes from exposure to the semen (Kotilainen T and Huhtinen M, 1994, Katila, 2018). Monocytes and mast cells are also parts of the innate immune system, but their role in the mare uterus has not been elucidated (Skarzynski et al., 2020). Once in the lumen, neutrophils start phagocytizing pathogens, releasing lytic enzymes, and also forming neutrophil extracellular traps (NETs). NETosis or NETs are an extrusion of DNA, nuclear, and cytoplasmic proteins that trap bacteria to facilitate phagocytosis by macrophages (Rebordão et al., 2014); molecules present in the seminal plasma regulate NETs formation in the donkey and the horse, but not in swine (Alghamdi and Foster, 2005, Alghamdi et al., 2009, Wei et al., 2020, Mateo-Otero et al., 2022). Uncontrolled inflammation leads to tissue damage and degenerative changes in the endometrium; mechanisms to modulate the uterine inflammatory response are present and come into action between 2 to 6 hours after breeding in the mares resistant to PBIE (Woodward et al., 2013a), whereas they are delayed in mares susceptible to PBIE. Anti-inflammatory cytokines (interleukin 10, IL10; 1R antagonist, IL1RN; interleukin 4, IL4; interleukin 13, IL13) are produced and act in different ways, for example, by reducing the expression of pro-inflammatory cytokines in monocytes, by competing for the binding with their receptors, or by inducing cell death (Christoffersen and Troedsson, 2017, Christoffersen et al., 2012, Fiorentino et al., 1991, Opal and DePalo, 2000, Couper et al., 2008).

This section focused on the aspects of the mare uterine inflammatory response that were pertinent to the subjects contained in this dissertation; a comprehensive description of the immunopathogenesis, pathobiology, and clinical implications of endometritis in the mare is enclosed in recently published reviews (Morris et al., 2020, Canisso et al., 2020). To date, no study has objectively assessed the endometritis of mares in response to exposure to sperm and seminal plasma from donkeys and horses.

SEMINAL PLASMA

While sperm is responsible for triggering the uterine inflammatory response, the role of seminal plasma in endometritis is more complex. Seminal plasma is the extracellular component of the ejaculate and represents up to 98% of its volume. It is formed by the epididymal fluids and secretions from the accessory sex glands during sexual stimulation and ejaculation. Ejaculation in stallions occurs in 6 to 9 jets; each portion of the reproductive tract contributes differently; fluid in the first portion is mainly from the bulbourethral glands, then the sperm-rich fraction comes from the ampullae, the epididymides, and the prostate, and the last portion is produced by the seminal vesicles (Kosiniak, 1975). Each segment of the male reproductive tract contributes to the seminal plasma with specific molecules; for example, the epididymides release carnitine, glyceryl phosphorylcholine, choline, and alkaline phosphatase; the ampullae release glycoproteins with N-acetyl residues, the bulbourethral glands produce acetic acid; the seminal vesicles contribute citric acid and the prostate secretes lactic acid (Magistrini et al., 1987, Magistrini et al., 1995). The first and the last jets have the highest concentration of chloride (Kareskoski et al., 2005), whereas calcium and magnesium are in higher concentrations in the pre-ejaculatory fraction and the first jets of the sperm-rich fraction (Kareskoski et al., 2005). Proteins are approximately 10 mg/mL with a molecular weight that ranges from 12 to 30 kDa (Töpfer-Petersen et al., 2005); the most abundant ones are cysteine-rich secretory proteins (CRISP), spermadhesins and proteins carrying two to four fibronectin type II modules (Fn-2 type proteins) (Töpfer-Petersen et al., 2005). Horse seminal plasma has a high number of proteins, but for most, the function is still unknown (Töpfer-Petersen et al., 2005). Several enzymes have been identified in horse seminal plasma, such as alkaline phosphatase, lactate dehydrogenase, and aspartate aminotransferase, which are correlated with semen volume, sperm concentration, and sperm morphology (Pesch et al., 2006). Further, β-

glucuronidase is at a high concentration in the sperm-rich fraction of the ejaculate and is thought to be involved in the acrosome reaction of spermatozoa (Brandelli et al., 1994). The most abundant bound carbohydrate in horse seminal plasma is galactose; glucose and mannose are also present; overall, the amount of carbohydrate is lower than in bulls (Gebauer et al., 1974, Katila et al., 2006). Phospholipids and glycerylphosphorylcholine are present in lower concentrations in seminal plasma, 28.4 μmol/100 mL and 55.8 mg/100mL, respectively (Chow et al., 1986). Despite the lower presence, phospholipids play major roles in capacitation and the acrosome reaction; stallions with a high ratio of cholesterol-to-phospholipid in the seminal plasma have impaired capacitation (Brinsko et al., 2007). Prostaglandins are well represented in seminal plasma and are produced by the prostate and seminal vesicles. The stallion semen has higher concentrations of prostaglandin F2α than other domestic species (boar, bull) (Claus et al., 1992). No information is available regarding the types and concentrations of prostaglandins in donkey semen. Prostaglandins are involved in sperm function but also influence the female reproductive tract; different types of prostaglandins are present in the stallion seminal plasma, such as PGE and PGF2α (Cohen et al., 1977). In humans, PGE, together with TGFβ, is deemed responsible for the regulation of Treg cells in the endometrium to allow tolerance toward the paternal alloantigens during the first stages of embryo development; PGE seemed to act on the transcription factor forkhead box protein P3 (FOXP3) which is essential in the differentiation and activity of Treg cells (Lu et al., 2017, Baratelli et al., 2005, Robertson, 2005). The presence of Treg cells proved essential for the development of "pregnancy tolerance" in domestic species, including pigs and cattle (Jalali et al., 2014, Oliveira and Hansen, 2008). In horses, Treg cells are also present early in gestation, but their role and their regulation by prostaglandins have not been studied (De Mestre et al., 2010).

Macro- and microelements are present and have a functional role in sperm metabolism. Elements are present in the following decreasing order Na > K > Ca > Zn > Mg > Fe > Cu > Pb > Hg > Cd (Tirpák et al., 2021); however, their concentration is highly variable between stallions (Amann et al., 1987). Zinc, manganese, and copper are cofactors in all three families of superoxide dismutase, which are the most abundant antioxidant enzymes in the seminal plasma; due to their type of metabolism, stallion sperm are prone to the production and damage by exposure to reactive oxygen species (Papas et al., 2019). The composition of donkey seminal plasma has not been thoroughly investigated. Results from the nuclear magnetic resonance showed that donkey and horse seminal plasma share 28 metabolites, such as amino acids (alanine, aspartate, glutamate, glycine, isoleucine, leucine, phenylalanine, taurine, tyrosine, and valine) and amino acid derivatives (carnitine, creatine, creatine phosphate, hypotaurine, *o*-acetylcarnitine, trimethylamine N-oxide, and Ƭ-Methylhistidine); salts (acetate, benzoate, citrate, formate, fumarate, and lactate), alcohols (ethanol, methanol, and myo-inositol), saccharides (glucose), and lipids (glycero-3-phosphocholine); overall donkey seminal plasma seems to have lower abundance of salts and higher abundance of common amino acids and amino acid derivatives (Catalán et al., 2023). The abundance of protein in donkey seminal plasma is four to ten times higher than in horses (Talluri et al., 2017). A seminal study in donkeys revealed that sperm viability was positively correlated with the abundance of methanol and *o*-acetylcarnitine, and the proportion of progressively motile sperm was positively correlated with the abundance of aspartate, glucose, hypotaurine, methanol, o-acetylcarnitine, and taurine (Catalán et al., 2023). Those findings seemed to suggest a difference in sperm metabolism and source of energy between the donkey and the horse sperm; however, it remains unclear whether the different metabolite profile affects the endometrium.

Seminal plasma has a plethora of roles in relation to sperm functions and the female reproductive tract. One of the main ones is the immunomodulator function (Alghamdi et al., 2004, Troedsson et al., 2005, Troedsson et al., 2000). Seminal plasma arrives in the uterus and the oviduct together with spermatozoa within 1-2 hours from mating in the horse (Mann et al., 1956). Seminal plasma suppresses chemotaxis and phagocytosis of neutrophils starting at 2 hours from the arrival into the uterus (Fiala et al., 2007, Alghamdi et al., 2004, Troedsson et al., 2001). In mice and men, the molecule involved in the chemotaxis regulation seems to be the transforming growth factor-beta family (TGF-β), but its presence is not yet verified in stallion seminal plasma. An early study evaluating the protein fractions of stallion seminal plasma concluded that the fraction whose molecular weight was between 50 and 100kDa had the strongest suppressive effect on PMN-chemotaxis (Troedsson et al., 1999). Other studies also hypothesized that the molecules responsible were glycoproteins, peptides, proteins, or lipoproteins (Alghamdi et al., 2004, Robertson, 2005). Over the years, a variety of proteins were identified as relevant to the modulation of the post-breeding inflammatory response, including the cysteine-rich secretory protein 3 (CRISP3), lactoferrin, insulin-like growth factor 1 (IGF-1), insulin-like growth factor-binding protein 2 (IGFBP-2), and insulin-like growth factor-binding protein-5 (IGFBP-5). Cysteine-rich secretory proteins are present in several tissue and cell types in mammals and share 50-80% of homology throughout the entire protein family; CRISP3 was the first member of the CRISP family described in the horse (Schambony et al., 1998). The epididymis secretes CRISP3, and it is one of the main proteins of stallion seminal plasma at about 1 mg/mL of concentration (Calvete et al., 1994); an *in vitro* study demonstrated that CRISP3 decreased the binding between sperm and neutrophils (Doty et al., 2011). Lactoferrin is a protein present in a variety of tissues and secretions, including seminal plasma, colostrum, epididymis, endometrium, neutrophilic

granules, and breast milk (Inagaki et al., 2002, Kolm et al., 2006, Kolm et al., 2007). Reports in other species and humans describe its antimicrobial and anti-inflammatory properties, more recently a study demonstrated that the uterine infusion of human recombinant lactoferrin after breeding in susceptible mares was able to mitigate the post-breeding inflammation characterized by a reduction in the number of PMNs, the expression of pro-inflammatory cytokine IFNγ, and an increase in the expression of IL1RN (Inagaki et al., 2002, Kolm et al., 2007, Li et al., 2015, Fedorka et al., 2018, Luo et al., 2015). Additionally, lactoferrin suppressed the expression of the tumor necrosis factor (TNF)-α, confirming its role in the modulation of post-breeding inflammation in the mare (Fedorka et al., 2016). An *in vitro* study on donkeys confirmed that exposure to seminal plasma results in a lower number of neutrophils being recruited (Miró et al., 2020). A follow-up study done a year later by the same research group in Spain revealed that in donkey, seminal plasma also increases NETs formation in a dose-dependent manner (Miró et al., 2021). The exact molecule responsible is unknown, but it seemed that proteins with a molecular weight between 30 to 50kDa and 50 to 100kDa were able to modulate the neutrophil's interaction with sperm in donkeys (Miró et al., 2021).

UTERINE MICROBIOME

The first pieces of evidence of a mare core uterine microbiota started more than 40 years ago (Scott et al., 1971, Hinrichs et al., 1988, Purswell et al., 1989); due to the use of culture-based techniques of analyses, the results of the first studies were not consistent, and it was difficult to undoubtfully confirm that bacteria reside in the uterus of healthy fertile mares. Between 30 and 40% of samples collected from the uterus of healthy fertile mares presented bacterial growth after the aerobic culture; *Arcanobacterium spp.*, *Staphylococcus spp.*, and *Streptococcus spp.* were the most commonly isolated bacteria, only 4% of the samples showed a heavy growth (>10 colonies)

(Hinrichs et al., 1988). In 1989 another research group focused on the uterine microbiome of postpartum mares, but no bacteria were isolated after the second day of the second postpartum estrous cycle (Purswell et al., 1989). Progress in this area of research stalled until new sequencing technologies became more available, the Human Microbiome Project was launched in 2007 and aimed to characterize the human microbiome using genome sequencing technologies. The "sterile uterus" dogma was immediately challenged and proved not accurate (Vitale et al., 2021, Baker et al., 2018), the interest in studies focused on women's uterine microbiome grew exponentially since that moment; over 150 publications have been listed in Pub Med only in the last three years (Last accessed August, 8th 2024; keywords "women", "uterus", "microbiome"). In veterinary medicine, the uterine microbiome has now been characterized in both domestic and wild species, including cattle (Moore et al., 2017, Santos et al., 2011), dogs (Lyman et al., 2019), giant pandas and some species of primates (Yang et al., 2017). In the horse, the first two studies to describe bacteria colonizing the uterus of healthy mares were published in 2017 using 16S sequencing (Schnobrich, 2017, Schnobrich et al., 2018, Sathe, 2017). A more comprehensive description of the healthy uterine microbiome was provided in 2022, where more than fifty mares from three different geographical locations (Oklahoma, Louisiana, and Australia) were studied (Holyoak et al., 2022). The study confirmed that the location has a significant effect on the microbiome composition; this assumption was previously suggested by other studies that showed a consistent variability in the relative abundance of the phyla of bacteria present (Heil et al., 2024, Gil-Miranda et al., 2024, Sathe, 2017, Jones, 2017, Schnobrich, 2017, Schnobrich et al., 2018) (**Table 2.3**). The most abundant phyla in the healthy mare's uterus are Firmicutes, Bacteroidetes, Proteobacteria, and Actinobacteria; the relative abundances of each phylum in the different studies are reported in (**Table 2.3**). The composition of the uterine microbiome is similar to other species; in the dog,

Proteobacteria, Bacteroidetes, and Firmicutes are the most abundant phyla; in cows, Firmicutes, Bacteroidetes, and Proteobacteria. There are no predominant bacteria at the genus level; the most common ones are Streptococcus, Pseudomonas, Klebsiella, Campylobacter, and Mycoplasma (Heil, 2018, Heil et al., 2024, Krekeler et al., 2023, Roach et al., 2016, Schnobrich, 2017, Schnobrich et al., 2018, Holyoak et al., 2022, Jones, 2017, Sathe, 2017, Hinrichs et al., 1988). Peculiar genera, like the Lonsdalea, are isolated in specific geographical locations (Holyoak et al., 2022).

In women and cows, the microbiome composition is influenced by the hormones of the estrous cycle, the diet, the presence of diseases, and the composition of the vaginal microbiome. The luteal phase with high progesterone is linked to a predominance of Firmicutes (~60%) that significantly decreases over time once the cow enters estrus (Ault et al., 2019). To this moment, there are no similar studies done in horses, and little is known about the microbiome fluctuations during the estrous cycle, disease, or other physiological events. The effect of estrous cycle has only been evaluated in respect of the vaginal microbiome by a single study on Arabian horses; the study demonstrated that the vaginal microbiome remained stable during the different phases of estrus (Barba et al., 2020). A recent study investigated the uterine microbiome in anestrus mares and found that the relative composition of bacteria followed the same pattern as in estrus, with Proteobacteria, Firmicutes, and Bacteroidota as the most prevalent phyla present (Heil et al., 2024).

The role of the uterine microbiome has been thoroughly investigated in women, and it is now believed that the uterine microbiome plays an important role in regulating reproductive physiological processes and reproductive health. A uterine microbiome without the prevalence of *Lactobacillus* spp. was associated with a decreased implantation rate, pregnancy establishment, and live birth weight; metabolites secreted by *Lactobacillus rhamnosus* GR-1 inhibit the

production of pro-inflammatory cytokines in *in vitro* human cell culture (Kim et al., 2019, Moreno et al., 2016). In veterinary medicine, this type of study is still in its infancy. In cows, a link between the uterine microbiota and uterine disease has been identified; dairy cows that had metritis have a dysbiotic microbiota with a reduced bacterial richness and an increase in the abundance of Bacteroidetes and Fusobacteria (Galvao et al., 2019). The role of uterine microbiome and endometritis has not been investigated yet in mares, as the studies have mainly focused on the different stages of the estrous cycle and geographical locations.

The *overall objective* of this dissertation was to characterize the mare uterine inflammatory response to inter- and intraspecies sperm and seminal plasma and its interplay with the uterine microbiome. The *central hypothesis* was that the uterine, semen, and the seminal plasma proteome, microbiome, and metabolome are intertwined and have trickle-down effects on the uterine immune response to inflammation and infections. The *rationale* behind this research was that studying the uterine inflammatory response to interspecies breeding provides fundamental knowledge on the impact of a different semen microbiome, proteome, and metabolome on the uterine inflammatory response. Such knowledge is paramount for developing improved, novel diagnostic and therapeutic approaches for endometritis. The following aims and sub-aims were developed and completed:

Aim 1. To evaluate the interaction between sperm, seminal plasma, and endometrium in intra- and inter-species breeding.

A) To evaluate uterine inflammatory response to the donkey and horse semen and seminal plasma.

B) To assess embryo and nuclear morphology of mule and horse embryos.

Aim 2. To assess the shift in the uterine microbiome in intra- and interspecies breeding.

A) To assess changes in the uterine microbiome composition overtime and after the exposure to semen and seminal plasma.

B) To determine if the presence of an embryo influences the uterine microbiome composition.

FIGURE AND TABLES

Figure 2.1. The first-ever hybrid animal created by humanity was a cross between a domestic donkey and a wild Syrian donkey. The kungas were prized animals used in peace and in war. (Source: The British Museum London, #121201)

Table 2.1. Extant species assigned to the Equus genus

Subgenus	Common name	Scientific name	Chromosome (2n)
Asinus	African wild ass	*Equus africanus*	62
	Onager	*Equus hemionus*	56
	Kiang	*Equus kiang*	52
Hippotigris	Grévy's zebra	*Equus grevyi*	46
	Plains zebra	*Equus quagga*	44
	Mountain zebra	*Equus zebra*	32
Equus	Domestic horse	*Equus ferus caballus*	64
	Przewalski's horse	*Equus ferus przewalski*	66

Table 2.2. Pregnancy rate obtained after the use of donkey semen to inseminate jennies or mares.

Type of semen	Use with jennies	Use with mares	Reference
Frozen		33%	(Vieira et al., 1985)
Frozen		44%	(Arruda et al., 1989)
Frozen	0%	40%	(Oliveira et al., 2006)
Frozen	36%	11%	(Vidament et al., 2009)
Frozen	13%		(Trimeche et al., 1998)
Frozen		50-53%	(Canisso et al., 2011)
Frozen	0%	54%	(Lopes Costa et al., 2023)
Frozen	22-60%		(Fanelli et al., 2022)
Cooled	45%	45%	(Vidament et al., 2009)
Cooled	52.4%	42.9%	(Fanelli et al., 2023)
Cooled		86.9%	(Carluccio et al., 2020)
Cooled		62.5-71.4%	(Paolucci et al., 2012)
Cooled		33-89%	(Gobato et al., 2022)

Table 2.3. Most abundant phyla of bacteria present in the uterus of healthy mares

Most abundant phyla	Location	Number of mares	Sampling technique	Reference
Bacteroidetes, Proteobacteria,	USA	20	Low-volume lavage	(Sathe, 2017)
Proteobacteria (40%), Firmicutes, (30%), Bacteroidetes, (12%), Actinobacteria (5%)	Australia,	14	Low-volume lavage	(Holyoak et al., 2022)
Firmicutes (52%), Proteobacteria, Bacteroidetes	LA, USA	12	Low-volume lavage	(Holyoak et al., 2022)
Proteobacteria (36%), Firmicutes (36%)	OK, USA	9	Low-volume lavage	(Holyoak et al., 2022)
Proteobacteria (70%), Firmicutes (21%), Bacteroidetes (8%)	Chile	21	Double-guarded swab	(Thomson et al., 2022)
Firmicutes, Proteobacteria	India	15	Low-volume lavage	(Virendra et al., 2024)
Bacteroidetes, Firmicutes, Actinobacteria, Proteobacteria	TN, USA	16	Low-volume lavage	(Jones, 2017)
Proteobacteria (54%), Firmicutes (23%), Bacteroidetes (6%),	LA, USA	16	Uterine biopsy	(Heil, 2018)
Proteobacteria (58%), Firmicutes (14%), Bacteroidetes (8%),	LA, USA	16	Low-volume lavage	(Heil, 2018)
Proteobacteria (48%), Firmicutes (22%), Bacteroidetes (8%), Actinobacteria (6%)	LA, USA	16	Double-guarded swab	(Heil, 2018)
Preoteobacteria (49%), Firmicutes (20%), Bacteroidetes (14%), Verrucomicrobiota (6%)	LA, USA	16 (anestrus)	Double-guarded swab	(Heil et al., 2024)

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CHAPTER 3: Post-breeding inflammatory response in mares during inter- and intraspecies breeding[1]

ABSTRACT

Anecdotal experience suggests that horse mares have less post-breeding inflammation and better fertility when bred with donkeys. This study aimed to compare the post-breeding inflammatory response of mares exposed to donkey and horse semen and seminal plasma and evaluate the proteome and metabolome of donkey and horse sperm and seminal plasma. Uterine edema, intrauterine fluid accumulation, PMNs on cytology, progesterone, and pro- and anti-inflammatory cytokines concentrations (IL1α, IL1β, IL4, IL6, CXCL8, IL10) were assessed pre- and post-infusion of semen and seminal plasma (donkey and horse). The metabolome and proteome were analyzed by LC-MS/MS. Mares bred with horse semen had a greater progesterone concentration than those cycles bred with donkey semen at 8 days post-ovulation (P=0.046). At 6 h post-infusion, the inflammatory response due to the donkey semen tended to be lower (P=0.074). Donkey seminal plasma had an anti-inflammatory property compared to horse semen and seminal plasma as determined by fewer of neutrophils on uterine cytology (P<0.05). Horse semen resulted in a greater concentration of IL6 and lesser concentration of IL1β (P<0.05). Concentrations of PGE_1, PGE_3, and lactoferrin were significantly more abundant in donkey sperm and seminal plasma. Prostaglandins play an important role in immunomodulation and might contribute to the

[1] This work has been previously published and is modified for inclusion here with the permission of the authors: Giorgia Podico, João H. Bittar, Shavahn C. Loux, Fabiana F. Souza, Igor F. Canisso. The interaction between seminal plasma, sperm, and endometrium in inter- and intra-species breeding in equids. *Reproduction* 2024;167(6).

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response triggered in inter-species breeding. In conclusion, breeding horse mares with donkey semen induces a similar post-breeding endometritis to horse semen. Donkey seminal plasma results in a lower post-infusion inflammatory response than other combinations in the immediate post-breeding.

INTRODUCTION

The ability of equids to successfully crossbreed has fascinated farmers and reproductive physiologists for several centuries (Allen et al., 1993, Allen and Short, 1997, Allen et al., 2010, Antczak et al., 2010, Canisso et al., 2010, Desta et al., 2022). Studies assessing the interactions between these species have focused on evaluating the behavioral interactions of donkey jacks and horse mares during natural mating or semen collection (Lodi et al., 1995, Canisso et al., 2009, Canisso et al., 2010), the fertility of fresh, cooled, and frozen semen of donkey jacks in horse mares (Vidament et al., 2009, Rota et al., 2012, De Oliveira et al., 2014, De Oliveira et al., 2016, Segabinazzi et al., 2021, Gobato et al., 2022,), and the physiology of early pregnancy and losses (Boeta and Zarco, 2005, Boeta and Zarco, 2010, Paolucci et al., 2012, Boakari et al., 2019, Carluccio et al., 2020). However, despite extensive research and centuries of interspecies breeding, the post-breeding inflammatory response of horse mares bred to donkeys has yet to be evaluated. Anecdotally, subfertile mares are thought to have better fertility when bred with donkey semen rather than horse semen; however, this has not been critically assessed. Clinical experiences suggest that mares bred with donkey semen have lower levels of persistent breeding-induced endometritis than mares bred with horse semen; however, this has not been studied in a controlled setting.

Post-breeding endometritis is a transitory physiological reaction to sperm and bacteria contained in the semen; the innate immunity is activated by the interaction with the Toll-like receptors 1 and 4 and causes the release of pro-inflammatory cytokines (IL1α, IL1ß, IL4, IL6, and CXCL8) that translates into polymorphonucleates (PMNs) recruitment and endothelial activation (Canisso et al., 2016, Canisso et al., 2020). Sperm concentration affects the post-breeding inflammatory response in horse mares, possibly due to the lower amount of seminal plasma in semen with high concentration (Kotilainen and Huhtinen, 1994). Although donkey semen (DS) typically has greater sperm concentration than horse semen (Canisso et al., 2019, Magalhaes et al., 2021), it is thought that donkey seminal plasma has anti-inflammatory properties and, therefore, it is effective in modulating post-breeding endometritis; however, the effect of donkey seminal plasma has not been critically assessed in mares (Miró et al., 2013, Miró et al., 2021).

The seminal plasma is the acellular liquid portion of the ejaculate; it is produced by the efferent ducts and the epididymides; however, the bulk of the volume is produced by the accessory sex glands (Juyena and Stelletta, 2012). Seminal plasma contributes to essential functions like the formation of sperm reservoirs in the female reproductive tract, the timing of capacitation, sperm transport, and uterine contractility (Juyena and Stelletta, 2012). Studies in horses have shown that seminal plasma has immunomodulatory properties; more specifically, the presence of horse seminal plasma in the mare reproductive tract after insemination decreases PMNs-chemotaxis, PMNs-sperm binding, and improves pregnancy rate (Troedsson et al., 2001, Troedsson et al., 2005). Similarly, *in vitro* studies showed that donkey seminal plasma also decreases PMNs-sperm binding (Miró et al., 2013); the putative immunomodulatory role of donkey seminal plasma was attributed to proteins ranging between 30 and 100 kDa that were characterized in a recent study (Miró et al., 2021). There is scant data regarding the effects of donkey seminal plasma *in vivo;* in

one study, the pregnancy rates of jennies bred with frozen-thawed semen tended to improve after the extension of frozen-thawed semen with donkey seminal plasma before breeding (Panzani et al., 2020). Additionally, uterine infusion of donkey seminal plasma after artificial insemination with frozen-thawed semen in jennies resulted in a lower expression of COX2 receptors than when the same jennies were bred with frozen-thawed semen but not infused with seminal plasma and lower recruitment of PMNs in the uterus after breeding (Rota et al., 2012, Vilés et al., 2013). The present study was set forth to compare the uterine inflammatory response of horse mares exposed to donkey and horse semen and seminal plasma and to identify potential anti-inflammatory molecules in the proteome and metabolome of donkey and horse sperm and seminal plasma. We hypothesized that sperm results in a higher inflammatory response than seminal plasma regardless of species and that donkey seminal plasma induces a lower post-breeding inflammatory response than horse seminal plasma due to the higher presence of molecules with anti-inflammatory properties.

MATERIALS AND METHODS

This study was performed from September to December 2020 at the University of Illinois at Urbana-Champaign, IL, USA. The Institutional Animal Care Unit Committee (protocol #19141) approved all procedures in the present experiment.

Animals and study design

Twenty-two light-breed mares (14 ± 5 years old, range 4 – 23 years old) were enrolled in the study. To enroll in the study, each mare had negative uterine cytology (i.e., less than 1 PMNs/high-power field [HPF]) and no bacterial growth after 48 h of aerobic incubation of the

uterine culture. Conversely, mares with signs of endometritis were treated as appropriate and had an additional washout cycle (Canisso et al., 2016, Canisso et al., 2020). Mares were randomly assigned to a uterine infusion with i) donkey raw semen, ii) donkey seminal plasma, iii) horse raw semen, iv) horse seminal plasma, and v) saline in a crossover design **(Figure 3.1).** Mares had a washout cycle between each group of assignments to minimize the carryover effects. Upon enrollment, mares had a transrectal ultrasonographic examination performed every other day until signs of estrus (follicle > 30 mm, uterine edema, and cervical softening) were noted; mares displayed signs of estrus after 1 to 10 days from enrollment. Then, follicular growth was monitored daily, and a dose of 1.8 mg deslorelin acetate (SucroMate™, Thorn BioScience, LLC, Louisville, KY, USA) was given intramuscularly to hasten the onset of ovulation. At the induction of ovulation, samples for uterine culture and cytology were collected. After 24 h from ovulation induction, mares had a uterine body insemination with infusion with either donkey or horse semen, or donkey or horse seminal plasma, or saline. No post-breeding treatments were performed other than intramuscular administration of 20 units of oxytocin injected intramuscularly, every six hours for two days after ovulation. A transrectal ultrasonographic examination was performed daily to detect ovulation up to 48 h post insemination/infusion. Mares failing to ovulate within this time range had cycles discarded and re-enrolled.

The uterine inflammatory response was assessed via uterine edema, intrauterine fluid accumulation, measuring inflammatory cytokines, and counting uterine PMNs at 0, 6, 24, and 48 h post-infusion of semen, seminal plasma, or saline. Eight days post-ovulation, embryo flushing was performed in the donkey or horse semen, and the outcome was recorded (**Figure 3.1)**.

Semen collection and uterine infusions

Semen from a mature donkey jack and horse stallion was used throughout the entire experiment. Before each insemination or infusion, semen was collected off a tease mare using a Missouri artificial vagina (16 in length; Nasco, Fort Atkinson, WI, USA) coupled with a filter and a plastic bag (Whirl-Pak, Madison, WI, USA). Gel-free semen fraction was assessed for volume and concentration using a scale and a Nucleocounter SP100 (Chemometec, Lillerød, Denmark) following manufacturer instructions. Motility parameters were assessed with a computer-assisted sperm analyzer (CASA) using default settings recommended for horse sperm (Spermvision, Minitube of America, Verona, WI, USA). The CASA preset values were static cell area 14–80 μm^2; straightness threshold for progressive motility 50%; average path velocity threshold for static cell < 9.5 μm/s; cell intensity 10^6; and light-emitting diode illumination intensity 1,800–2,550. An aliquot from each sample was diluted to 50 million sperm/mL with a commercially available semen extender (INRA 96, IMV Technologies USA, Brooklyn Park, MN, USA) incubated for 10 min at 37 °C before each evaluation. A small aliquot (10 μL) of extended semen was placed on a pre-heated slide with a coverslip for the assessments. The sperm parameters were used for descriptive purposes and compared across species.

Inseminations for donkey and horse semen groups consisted of a dose of raw semen with 2 billion total sperm/estrous cycle. The volume of infusion was 9.5 ± 0.5 mL for donkey semen, and 13.9 ± 1.1 mL for horse semen. The semen was deposited in the uterine body through a standard pipette for horse artificial insemination. For the donkey and horse seminal plasma cycles, the semen was processed through serial centrifugations (600 × g 10 min, then 1,000 × g 20 min) until no sperm was observed under the microscope. Each infusion with seminal plasma was carried out with 20 mL of volume; seminal plasma was deposited in the uterine body through a standard

pipette for horse artificial insemination. Samples from the seminal plasma and the sperm pellet obtained after the first centrifugation were frozen in liquid nitrogen and stored at -80 °C until further analysis.

Eight days post-ovulation, a non-surgical embryo flush was performed in the mares enrolled in donkey and horse semen groups. The mare's tail was wrapped, and the perineal area cleaned. A bullet-tip catheter 28 Fr was inserted through the cervix, and 4 L of Ringer's Lactate solution was used to recover the embryo from the uterus. The fluid was retrieved in a dish coupled with a filter and inspected under the stereomicroscope to determine the presence or absence of the embryo. The embryo recovery and morphometry were calculated and accounted for in a parallel study assessing embryo morphometry (Podico and Canisso, 2024). After embryo flushing, mares received 5 mg dinoprost tromethamine intramuscularly (Lutalyse, Zoetis, Parsippany-Troy Hills, NJ, USA). Estrous cycles assigned to other groups received a mock embryo flushing 8 d post-ovulation.

Uterine inflammatory response

Markers for uterine inflammatory response (uterine edema, intrauterine fluid accumulation, pro- and anti-inflammatory cytokines, PMNs counts) were assessed at 0, 6, 24, and 48 h post-infusion. Uterine edema and intrauterine fluid accumulation were assessed via transrectal ultrasonography. Uterine edema was scored from 0 to 3. A score of 0 was given when no edema was visible in the endometrial folds. Scores 1, 2, and 3 were given when a very small, moderate, and large amount of edema was observed, respectively. Intrauterine fluid accumulation was measured at the base of one of the uterine horns with the built-in caliper of the ultrasound machine and then categorized from 0 to 3. A score of 0 was given when no fluid was visible; score

1 was given when the fluid column was less than 1 cm high; score 2 was given when the fluid column was between 1 and 2 cm high; score 3 was given when the fluid column was greater than 2 cm high.

Samples for uterine cytology were collected *via* disposable double-guarded cytobrush (Jorgensen Labs, Loveland, CO, USA) introduced manually through the vagina and cervix into the uterus. The slides were prepared by smear and stained with Romanowski stain (Diff-Quick, Siemens Healthcare Diagnostic Inc., Deerfield, IL, USA). Each sample was examined under a bright-field microscope at ×400 magnification. The same investigator counted the number of PMNs in ten HPF randomly selected in each sample. The average number of PMNs was recorded and compared across groups.

The inflammatory cytokines were assessed on samples of uterine fluid collected via low-volume uterine lavage. Briefly, 100 mL of Ringer's lactate solution was infused into the uterus and then recovered with the aid of the ultrasound and oxytocin (20 units, intravenously). The first 50 mL of the fluid recovered were discarded as this fluid was mainly contained in the catheter used in the sampling method. The rest of the fluid was collected in a 50 mL conical tube and processed immediately; the fluid was centrifuged at 400 ×*g* for 20 min at 5 °C. The supernatant recovered was centrifuged again at 1,000 ×*g* for 20 min at 5 °C to remove cellular debris. After the second centrifugation, the supernatant was saved and stored at −80 °C for further analyses. Uterine fluid samples from a randomly selected subset of mares (n = 16) were analyzed using an equine-specific multiple sandwich immunoassay based on flowmetric Milliplex Map® technology per the workflow previously published (Milliplex Map®, Millipore Sigma, Burlington, MA, USA). The concentration (pg/mL) of IL1α, IL1β, IL4, IL6, CXCL8, and IL10 in the uterine fluid obtained by low-volume uterine lavage was measured. Each sample of uterine fluid was measured undiluted,

and calibration curves for these plates were prepared in assay buffer, as previously described (Skogstrand, 2012). Additionally, samples of interleukins were measured undiluted, and standards were prepared with the serum matrix added to all standards and quality controls, following the manufacturer's guidelines, and as previously described (Fedorka et al., 2018).

Serum progesterone concentration

A blood sample was collected 6, 24, 48, 72, and 96 h post-infusion and 8 d post-ovulation in a 10 mL collection tube with no anticoagulant. The samples were kept at room temperature for 30 min and then centrifuged at 1,000 ×*g* for 20 min; serum was harvested and stored at -20 °C until analyses. Serum progesterone concentrations were measured using an automated system based on solid-phase, competitive chemiluminescence enzyme immunoassay (Immulite 2000 XPi Platform, Siemens Medical Solutions Inc., Malvern, PA, USA). Reported cross-reactivities were as follows: androstenedione 0.076%, corticosterone 0.417 %, cortisol 0.003%, DHEA sulfate 0.01%, 11-deoxycorticosterone 1.82%, 17α-hydroxyprogesterone 0.444%, medroxyprogesterone 0.029%, pregnenolone 0.047%, testosterone 0.119%. The intra-assay and inter-assay coefficient of variation were 6.12% and 5.08% for progesterone, respectively. The calibrated sensitivity of the progesterone assay was 0.01 ng/mL, and the detection limits were 0.2 to 40 ng/mL. For statistical analyses, samples with values below the lower detection limit were deemed 0 ng/mL. There were no samples above the upper limit of the detection for the assay.

Metabolomics and proteomics of sperm and seminal plasma

A subset of samples (n = 9) of sperm and seminal plasma of donkeys and horses was randomly selected; three pooled samples were formed by mixing three of the aliquots for each

sample type. The pooled samples were submitted for metabolomics and proteomics at the Roy J. Carver Biotechnology Center, University of Illinois at Urbana-Champaign, IL, USA.

The metabolomics samples were analyzed with the Q-Exactive MS system (Thermo Scientific, Bremen, Germany). The acquisition of data was based on previously published protocols (Elolimy et al., 2019). Data were acquired with the software Xcalibur 4.1.31.9. The HPLC system (Dionex Ultimate 3000, Thermo Scientific, Bremen, Germany) had a degasser, an autosampler, and a binary pump. The LC separation was performed on a Phenomenex Kinetex C18 column (4.6 x 100 mm, 2.6 µm) with mobile phase A (0.1% formic acid aqueous solution) and a mobile phase B (0.1% formic acid in acetonitrile, v/v). The flow rate was 0.25 mL/min. The linear gradient was as follows: 0-3 min, 100% A; 20-30 min, 0% A; 31-61 min, 100% A. The autosampler was set to 15 °C, and the injection volume was 20 µL. Mass spectra were acquired under both positive (sheath gas flow rate: 45; aux gas flow rate: 11; sweep gas flow rate: 2; spray voltage: 3.5 kV; capillary temp: 250 °C; Aux gas heater temp: 415 °C) and negative electrospray ionization (sheath gas flow rate: 45; aux gas flow rate: 11; sweep gas flow rate: 2; spray voltage: -2.5 kV; capillary temp: 250 °C; Aux gas heater temp: 415 °C). The full scan mass spectrum resolution was set to 70,000, with a scan range of m/z 67 ~ m/z 1,000, and the automatic gain control (AGC) target was set to 1e6 with a maximum injection time of 200 ms. The 4-chloro-DL-phenyalalnone was spiked into the sample as an internal standard. LC-MS data were analyzed with Thermo Compound Discoverer software v2.1 SP1 for chromatographic alignment and compound/feature identification/quantitation. The following settings were used in select spectra: minimum precursor mass (65 Da) and maximum precursor mass (5,000 Da); in align retention time: maximum shift (1 min) and mass tolerance (5 ppm), intensity tolerance (30%), S/N (3), and minimum peak intensity (1,000,000).

For proteomics, 3 mol guanidinium hydrochloride was added to the samples. Reduction and alkylation were performed with the addition of 10 mmol TCEP (Tris(carboxyethyl)phosphine hydrochloride) and 40 mmol CAA (2-chloroacetamide). The pH of the solutions was adjusted to be over 7 using TEAB (triethylammonium bicarbonate), if necessary. Samples were heated at 95 °C for 10 min in the dark and then cooled to 4 °C. Samples were precipitated using chloroform/methanol and air-dried. The protein levels were quantitated using the bicinchoninic acid method (Pierce™ BCA Protein Assay Kits, Thermo Scientific, Waltham, MA, USA). The samples were digested with trypsin and desalted using StageTips before drying in a vacuum centrifuge. The digested peptides were analyzed on a Thermo Scientific Fusion Tribrid mass spectrometer interfaced with a Dionex nano UPLC. A 50 cm NanoLC microPAC (Thermo Scientific) column ran at a 0.3 microliters/minute flow rate. The gradient comprised acetonitrile (B) and water (A) with 0.1% formic acid. Initial conditions were 2% B to 10% B in 3 min, followed by an increase to 36% B in 42 min, followed by an increase to 50% B in 6 min. The Fusion was set to the DDA Universal method, with a precursor range of 300 to 2000. The instrument type was ESI-TRAP.

The raw LC-MS/MS data were processed using Mascot Distiller software (Matrix Science Inc., Boston, MA, USA) to obtain the relative quantification of each protein in the mixture, determined by the exponentially modified protein abundance index (emPAI) and searched using the Mascot Database search engine v2.8.2 (Matrix Science, Inc., Boston, MA, USA) against UniprotKB proteome database (https://www.uniprot.org/) *Equus asinus asinus* (UP000694387) and *Equus caballus* (UP000002281). The fixed modification was carboxyamidomethylation, variable modification was acetyl (Protein N-term) and Oxidation (M). Peptide mass tolerance was

10 ppm, and fragment mass tolerance was 0.3 Da with 3 missed cleavages using trypsin as the protease.

Statistical analyses

Statistical analyses were performed using R v1.4.1717 and Prism v9.5.1. Normal distribution was assessed with histograms and the Shapiro-Wilk test. Data not normally distributed were transformed or assessed with non-parametric tests. Tukey-HSD was performed as a post-hoc test when a significant effect was found. Motility parameters and embryo recovery were compared with one-way ANOVA across species (donkey *vs.* horse). The number of PMNs in the uterus was compared using a linear mixed model where the group and the time were accounted for as a fixed effect and the mare as a random effect. Uterine edema and intrauterine fluid were compared using a Kruskal-Wallis rank sum test. Progesterone concentrations were compared using a linear mixed model considering the group, the time, and the number of ovulations as fixed effects and the order of the cycle and the mare as a random effect. A statistically significant difference was defined as $P < 0.05$. Data were presented as mean ± standard error of the mean (SEM).

The proteomics and metabolomics data were analyzed using an open-access software, Metaboanalyst v5.0 (Xia et al., 2009). Only proteins present in two out of three pooled samples were used for further analyses. To compare the proteomic results of donkey and horse, each protein identified in the donkey samples was paired with the ortholog protein found in the *E. caballus* database using the BLAST function and then used in the analyses. Only compounds putatively annotated based on the level 1 identification of the Metabolomics Standard Initiative were used (Chaleckis et al., 2019). Both proteomic and metabolomic data were normalized by

sum, log-transformed, and then auto-scaled before the analyses. The proteomic and metabolomic profiles of sperm and seminal plasma were compared across species with a principal component analysis (PCA) and a partial least-squares discriminant analysis (PLS-DA); proteins with VIP scores $\alpha > 1.5$ were considered relevant. Volcano plots were also constructed using fold change threshold 2.0, and $P < 0.05$. Heatmaps were created to show clustering using the normalized and autoscaled data of the top 25 proteins and compounds; the heatmaps were constructed using Euclidean distance measure and Ward as a clustering method. The PANTHER database was used to classify the proteins and their genes based on the molecular function and biological process and class of proteins (gene ontology classification).

RESULTS

Cycles, semen parameters, and embryo recovery

One hundred and four cycles were completed in this study. Of the twenty-two mares, nineteen underwent five estrous cycles each. Three mares only completed three estrous cycles before entering Winter anestrus; the cycles with the infusion of either seminal plasma were not performed.

The donkey had 23 semen samples collected, while the horse had 31 (**Table 3.1**). Volume, concentration, total sperm per ejaculate, and motility were higher in semen collected from the donkey compared to the horse (**Table 3.1**) ($P < 0.05$). A total of 26 embryos were recovered during this study. There was no difference between the groups for the number of positive flushes in donkey and horse semen (13 *vs.* 12) ($P > 0.05$).

Uterine inflammatory response

The uterine edema and the amount of intrauterine fluid were influenced by the time post-infusion ($P < 0.01$) but not by the group or their interaction ($P > 0.05$) (**Figure 3.2 A-B**). The presence of PMNs on cytology was affected by the time from the infusion ($P = 0.001$) but not by the group ($P = 0.86$); however, the analysis revealed an effect from the interaction between time and group ($P < 0.001$) (**Figure 3.2 C**). There was a transitory increase of PMNs in the uterus at 6 h post-infusion; the number of PMNs decreased to the level present before infusion by 24 h post-infusion ($P < 0.05$). At 6 h post-infusion, the inflammatory response due to the donkey semen tended to be lower than the one due to horse semen ($P = 0.074$). At 6 h post-infusion, the number of PMNs present in the uterus was lower in donkey seminal plasma compared to horse semen ($P < 0.001$) and horse seminal plasma ($P = 0.03$) and similar to the infusion with saline ($P > 0.05$) (**Figure 3.2 C**)**.**

The IL4 concentration was not affected by time, group, or their interactions ($P < 0.05$) (**Figure 3.3 A**). The concentration of IL10 was influenced by time ($P = 0.03$) but not by the group or their interactions ($P > 0.05$). The peak of IL10 was recorded 6 h post-infusion ($P < 0.05$) (**Figure 3.3 B**). CXCL8 was affected by both the time and the interaction between time and group ($P < 0.05$) but not the group ($P > 0.05$) (**Figure 3.3 C**). The concentration of CXCL8 peaked at 6 h post-infusion and was different from the other time points assessed in all groups ($P < 0.05$) except Control and horse seminal plasma ($P > 0.05$) (**Figure 3.3 C**). At 6 h post-infusion, the species did not result in different CXCL8 concentrations nor the seminal plasma or the semen; however, saline had lower CXCL8 concentration than the other groups ($P < 0.05$). The IL6 concentration was affected by the time and interaction between time and the group but not by the group (**Figure 3.3 D**). At 6 h post-AI, horse semen had higher IL6 concentration than control and groups with

seminal plasma ($P < 0.05$), and it tended to be higher than donkey semen ($P = 0.06$) (**Figure 3.3 D**). Time, group, and their interactions had an effect on the IL1α concentration ($P < 0.05$); the control group revealed a greater concentration of IL1α than donkey semen ($P < 0.05$) (**Figure 3.3 E**). There was an effect from the time post-infusion and from the interaction between time and group ($P < 0.05$) on the IL1β concentration in the uterine fluid, whereas the group did not show an effect on IL1β concentration ($P > 0.05$) (**Figure 3.3 F**). At 6 h post-infusion, the presence of sperm in both groups (i.e., donkey and horse) caused an increase in IL1β compared to the seminal plasma or saline ($P < 0.05$). Concentrations of IL1β concentrations with donkey semen were twice the ones reached after breeding with horse semen ($P < 0.05$) and almost three times the ones reached after infusion with donkey seminal plasma and saline ($P < 0.001$) **(Figure 3.3 F)**.

Serum progesterone concentrations

Both the group, time, and the number of ovulations affected the progesterone concentration ($P < 0.05$), but there were no interactions between the variables ($P > 0.05$) (**Figure 3.4)**. The progesterone concentration increased starting at 96 h post-infusion ($P < 0.05$) **(Figure 3.4)**. Estrous cycles with double ovulations ($n = 9$) had greater progesterone concentration ($P < 0.05$). The estrous cycles assigned to donkey semen had a similar progesterone concentration to horse semen and seminal plasma ($P > 0.05$) and greater progesterone concentration than donkey seminal plasma- and Control-assigned cycles ($P < 0.05$). After 8 d post-ovulation, horse semen had greater progesterone concentrations than donkey seminal plasma ($P = 0.046$), tended to have greater concentration than control-assigned cycles ($P = 0.084$), and had similar concentration to horse seminal plasma ($P = 0.7$).

Proteomics of donkey and horse sperm and seminal plasma

A total of 356, 257, 116, and 427 proteins were identified in donkey and horse sperm and seminal plasma, respectively; 69 proteins were found in common between donkey and horse sperm, and 17 proteins were found in common between donkey and horse seminal plasma. The PCAs sum (axis X = PC1 and axis Y = PC2) was 85.1% for sperm proteins and 87.1% for seminal plasma proteins (**Figure 3.5**). According to the PLS-DA, the results of the donkey and horse seminal plasma proteins were classified by the VIP score ($\alpha > 1.5$), and only one protein (A0A3Q2L4I6, alpha-mannosidase) was considered relevant in Component 2, no other proteins had VIP score with $\alpha > 1.5$ in components 1 to 5. No proteins had a high VIP score ($\alpha > 1.5$) in the sperm proteins identified in either the donkey or the horse. The volcano plot of the sperm proteins showed that there were 71 proteins present in higher abundance in donkey compared to horse semen, including F6XLB1 (lactoferrin, fold change 5.9, P = 0.03) and Q29482 (clusterin, fold change 6.3, P = 0.003) **(Figure 3.6 A).** Proteins with the highest and lowest abundance were described in **Table 3.2**. The volcano plot of the seminal plasma proteins showed that 22 proteins were present in higher abundance in donkey compared to horse seminal plasma **(Figure 3.6 B)**. The proteins in higher abundance in donkey seminal plasma also included F6XLB1 (lactoferrin, fold change 15.2, P = 0.008) and Q29482 (clusterin, fold change 5.5, P = 0.009), in addition to A0A5F5PRC5 (serotransferrin, fold change 7.7, P = 0.006), F6TIR2 (lipocalin-2, fold change 11.6, P = 0.006), and O97921 (prostaglandin H2 D-isomerase, fold change 5.4, P = 0.01) **(Table 3.2).** The heatmaps created with the top 25 most represented proteins in both donkey and horse sperm and seminal plasma depict the marked distinction between the two species and type of sample **(Figure 3.7).**

In the donkey sperm, the highest-scored genome ontology (GO) terms for molecular function were catalytic activity 31.5% (G0:0003824), binding 25.1% (GO:0005488), and transporter activity 3.8% (GO:0005215); 104 (44.3%) proteins had no PANTHER category assigned. For biological process, the highest scored GO terms were cellular process 43.0% (GO:0009987), metabolic process 26.0% (GO:0032501), and biological regulation 9.8% (GO:0065007); 111 (47.2%) proteins were not classified by any PANTHER category. The classes of protein with the highest GO score were metabolite interconversion enzymes 31.1% (PC00262) (oxidoreductase, hydrolase, transferase), cytoskeletal proteins 8.5% (PC00085), chaperones 7.7% (PC00072), protein modifying enzymes 5.1% (PC00260), and protein-binding activity modulators 4.3% (PC00095).

In the donkey seminal plasma, the highest-scored genome ontology (GO) terms for molecular function were catalytic activity 29.2% (GO:0003824), binding 26.4% (GO:0005488), and structural molecule activity 4.2% (GO:0005198); 29 (40.3%) proteins had no PANTHER category. For biological process, the highest scored GO terms were cellular process 37.5% (GO:0009987), metabolic process 26.4% (GO:0008152), response to stimulus 18.1% (GO:0050896), and biological regulation 17.4% (GO:0065007); 30 (41.7%) proteins were not classified by any PANTHER category. The classes of proteins with the highest GO score were metabolite conversion enzyme 23.6% (PC00262), protein modifying enzyme 11.1% (PC00260), chaperone 8.3% (PC00072), and defense/immunity proteins 6.9% (PC00090).

The gene ontology analyses of horse sperm revealed that the highest-scored genome ontology (GO) terms for molecular function were catalytic activity 23.2% (GO:0003824), binding 23.2% (GO:0005488), and transporter activity 3.0% (GO:0005215); 80 (47.6%) proteins had no PANTHER category. For biological process, the highest scored GO terms were cellular process

39.3% (GO:0009987), metabolic process 27.4% (GO:0032501), and biological regulation 8.9% (GO:0065007); 34 (50.7%) proteins were not classified by any PANTHER category. The classes of protein with the highest GO score were metabolite interconversion enzyme 19.6% (PC00262), protein modifying enzyme 8.9% (PC00260), cytoskeletal proteins 8.3% (PC00085), and protein binding activity modulators 4.2% (PC00095).

In the horse seminal plasma proteins, the highest-scored genome ontology (GO) terms for molecular function were binding 25% (GO:0005488), catalytic activity 18.2% (GO:0003824), molecular function regulator 9.1% (GO:0098772), and structural molecule activity 6.8% (GO:0005198); 20 (45.5%) proteins had no PANTHER category. For biological process, the highest scored GO terms were cellular process 31.8% (GO:0009987), biological regulation 20.5% (GO:0065007), metabolic process 18.2% (GO:0032501), and response to stimulus 15.9% (GO:0050896); 18 (40.9%) proteins were not classified by any PANTHER category. The classes of protein with the highest GO score were protein-binding activity modulator 13.6% (PC00095), metabolite interconversion enzyme 13.6% (PC00262), and defense protein 11.4% (PC00090).

Metabolomics of donkey and horse sperm and seminal plasma

The multivariate principal component analysis (PCA) revealed that the metabolites identified in donkey and horse sperm and seminal plasma presented a distinct separation **(Figure 3.5).** The PCAs sum (axis X=PC1 and axis Y = PC2) was 75.1% for sperm and 80.5% for seminal plasma metabolites **(Figure 3.5)**.

The volcano plots showed 55 metabolites with greater and 56 with fewer abundance in donkey compared to horse sperm **(Figure 3.8 A)**, whereas there were 130 metabolites with greater abundance and 71 fewer abundance in donkey compared to horse seminal plasma. **(Figure 3.8 B).**

Components of the inflammatory cascade were found in greater quantity in donkey than in horse seminal plasma, including DL-glutamine (fold change 36.6, P = 0.025), 3-hydroxydodecanoylcarnitine (fold change 29.9, P = 0.002), leukotriene E_4 (fold change 25.3, P = 0.005), lauric acid (fold change 21.4, P = 0.0005), prostaglandin F_1 (fold change 19.1, P = 0.0007), carnitine (fold change 18.22, P = 0.002), epinephrin (fold change 17.8, P = 0.006), prostaglandin E_3 (fold change 14.8, P = 0.006), thromboxane B_2 (fold change 14.1, P = 0.006), prostaglandin F_2 (fold change 10.5, P = 0.002), and prostaglandin E_1 (fold change 6.94, P = 0.001) **(Figure 3.8 B)**. The heatmaps showed the distinct composition of the top 25 most represented metabolites in the two species and the homogeneous composition of the three pooled samples used in the study **(Figure 3.9)**.

DISCUSSION

This study is the first to characterize the complex interplay of the mare endometrium, sperm, and seminal plasma during inter-species breeding. Clinical experiences suggested that breeding with donkey semen triggers a weaker post-breeding endometritis than horse semen when used in mares; thus, problem mares would have better fertility when bred to donkeys than horses. Indeed, our study demonstrated the presence of abundant anti-inflammatory molecules in donkey sperm and seminal plasma.

Despite the phenotypical and karyotypical differences, the inter-species breeding between donkey and horse is successful and results in satisfactory embryo recovery. Although the embryo recovery of the present study was slightly lower than the conception rate reported by other studies (Canisso et al. 2011, Carluccio et al., 2020,Segabinazzi et al., 2021), the mares enrolled included various ages, statuses, and susceptibilities to persistent breeding-induced endometritis. It is

possible that the results could have been different if multimodal post-breeding treatments like uterine lavages, or anti-inflammatory medications were used. However, it was outside the scope of this study to assess the various post-breeding therapeutic modalities currently used in equine practice (Canisso et al. 2016, Canisso et al. 2020).

As expected, PMNs peaked after 6 h from the breeding, confirming previous knowledge that sperm plays a major role in post-breeding endometritis (Kotilainen et al., 1994). The recruitment of PMNs in the uterine lumen starts with the activation of Toll-like receptors on sentinel endometrial cells by sperm and bacteria contained in the semen. The interaction with those receptors causes the activation of the Nκ-kβ intranuclear receptors that upregulates the expression and translation of genes involved in the production of pro- and anti-inflammatory cytokines (Canisso et al., 2020). Studies have elucidated this mechanism in the mare during intra-species breeding, but the effect of donkey sperm on horse endometrial cells has not been well studied. Clinical markers of persistent breeding-induced endometritis include an excessive uterine edema pre- and post-ovulation and intrauterine fluid accumulation (Canisso et al., 2016, Canisso et al. 2020). Noteworthy, there was no difference between the types of semen; it remains to be determined if the results would have been different if a population of mares susceptible to persistent breeding-induced endometritis had been used.

Our results suggested that donkey seminal plasma has anti-inflammatory properties with a decreased number of PMNs after 6 h from the infusion. The chemoattraction of PMNs is mainly mediated by the action of CXCL8 (Zerbe et al., 2003, Fumuso et al., 2007,). The concentrations of CXCL8 did not vary across assigned to either semen or seminal plasma; this could be due to the fact that only a subset of mares was used for this assay, it is possible that if a greater number of samples were analyzed, differences could have been detected. It is also possible that the anti-

inflammatory action of donkey semen and seminal plasma is mediated by one or more cytokines that were not assessed in the present study.

Seminal plasma has a species-specific composition, but literature is scant in donkeys. While it is known that the donkey seminal plasma has a greater amount of protein, glucose, lipids, and cholesterol than the horse, little is known about the anti-inflammatory molecules contained in it. Further evidence of its anti-inflammatory properties was provided by other studies that demonstrated that adding seminal plasma did not improve the quality of frozen-thawed semen (Sabatini et al., 2014) but decreased PMNs recruitment after artificial insemination and improved the fertility of jennies after artificial insemination (Rota et al., 2012). Our study confirmed that donkey sperm and seminal plasma have a great abundance of anti-inflammatory molecules.

Lactoferrin is a protein contained in stallion seminal plasma, endometrium, epididymis, and breast milk; different reports described its antimicrobial and anti-inflammatory properties and more recently, a study demonstrated that the uterine infusion of human recombinant lactoferrin after breeding in susceptible mares was able to mitigate the post-breeding inflammation characterized by a reduction in the number of PMNs, the expression of pro-inflammatory cytokine IFNγ, and an increase in the expression of IL1RN (Inagaki et al., 2002, Kolm et al., 2007, Li et al., 2015, Luo et al., 2015, Fedorka et al., 2018,). Noteworthy, lactoferrin purified from horse seminal plasma has a molecular mass of 80 kDA; the mass fits in the range (30 to 100 kDa) that was given for proteins considered responsible of the immunomodulatory role of donkey seminal plasma in another recent *in vitro* study (Inagaki et al., 2002, Miró et al., 2021). Due to the complexity of the composition of seminal plasma, it remains to be determined if lactoferrin is the main contributor of its immunomodulatory properties, and further studies, both *in vitro* and *in vivo*, are warranted.

Different types of prostaglandins were also found with higher abundance in donkey seminal plasma. Although their classical role is to induce inflammation, their anti-inflammatory properties have also been described (Scher and Pillinger, 2009). In particular, PGE_2 and PGE_3 were characterized as potent immunomodulators with anti-inflammatory properties (Cui et al., 2021, Martínez-Colón and Moore, 2018). Prostaglandin E_3, which herein was found to be 14 times in greater quantity in donkey seminal plasma than horse seminal plasma, seemed to modulate the polarization of macrophages during prostate cancer (Cui et al., 2021). The role of PGE_3 in seminal plasma has not been elucidated in any domestic species.

Another molecule well represented in the donkey seminal plasma was epinephrine. Epinephrine's role in the uterus is mostly described in the bovine species, where a high dose of epinephrine (10mL, 1:1000) is routinely given at the moment of dystocia to interrupt uterine contractions. Interestingly, it has also been reported that epinephrine given at the moment of AI seemed to increase the conception rate in cows (Fitzpatrick, 1957, Hays et al., 1958). The effect of epinephrine on uterine motility has not been investigated in the mare.

The number of proteins identified in donkey and stallion sperm and seminal plasma was lower than in recent proteomic studies on stallion semen and seminal plasma (Griffin et al., 2020, Griffin et al. 2022); differences in analytical steps like the protein digestion and extraction, the type of mass spectrometry used (UPLC *vs.* UHPLC), separation steps like FAIMS, or the bioinformatics could have may have played a major role. Regardless of the smaller array of proteins identified in this study, the analyses performed allowed the identification of significant differences between the two species and paved the way for further studies focused on one of the most common diseases of the horse, endometritis.

Furthermore, we recognize that choices made in the experimental design (i.e., breeding dose, timing of AI, post-breeding treatments) might have influenced the results. The standardized dose of 2 billion total sperm was selected after being extensively used by the authors both in a research and clinical setting to achieve a satisfactory conception rate and inflammatory response for the screening for susceptibility to persistent breeding-induced endometritis (Segabinazzi et al., 2021). No semen extender was used to breed the mares as we intended to prevent interference with the interaction of semen and the uterine epithelium. In normalizing the breeding within cycles and species, we could have introduced another variable, which was the highly variable volume and concentration across species and ejaculates. We could have normalized by volume, but then we would have favored the donkey, which is a species producing ejaculates with higher concentrations as noted herein and previously (Canisso et al. 2019). Regardless of the design, different assumptions could have been made and design limitations occurred. We believe that the design carried out here caused the least human interference with artificial insemination. It remains to be determined if other designs would have produced different results; regardless, this is the first in-depth characterization of the post-breeding inflammatory response in mares inseminated with semen from donkeys and horses, and to analyze the effects and interactions of seminal plasma of either species with the endometrium.

In conclusion, the use of donkey semen induces a similar post-breeding endometritis in mares with similar embryo recovery to horse semen. Donkey seminal plasma results in a lower post-infusion inflammatory response than other combinations in the immediate post-breeding.

Funding

The study was supported by the Department of Veterinary Clinical Medicine, College of Veterinary Medicine of the University of Illinois at Urbana-Champaign, IL, USA.

FIGURES AND TABLES

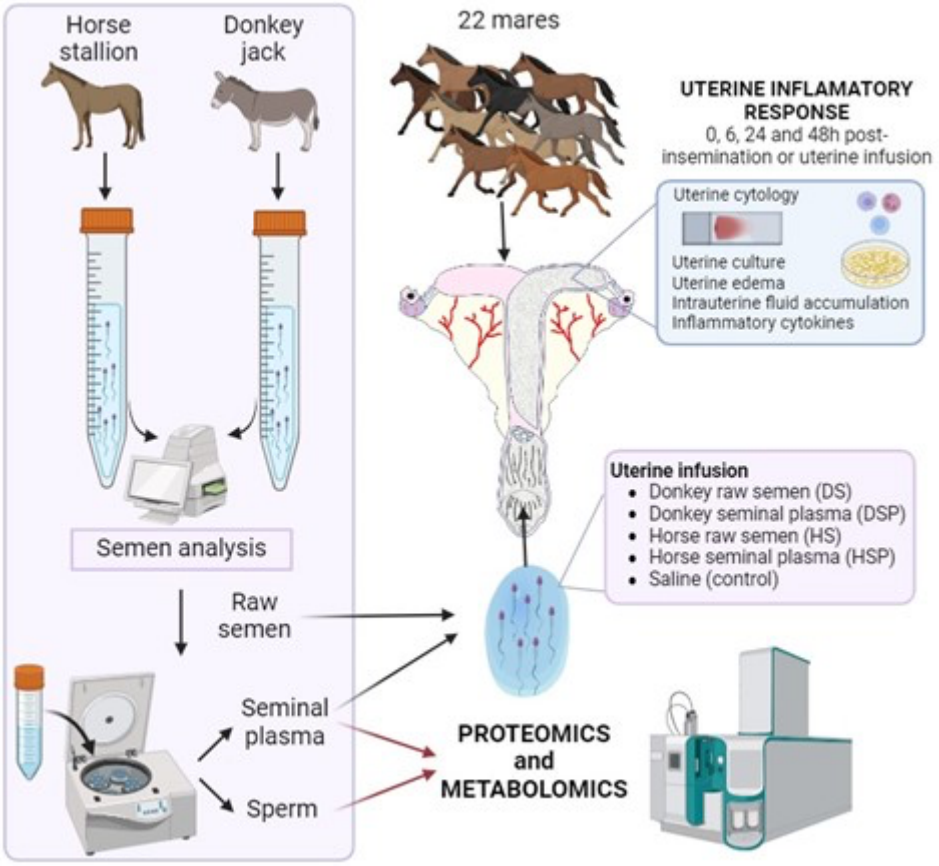

Figure 3.1. Experimental design

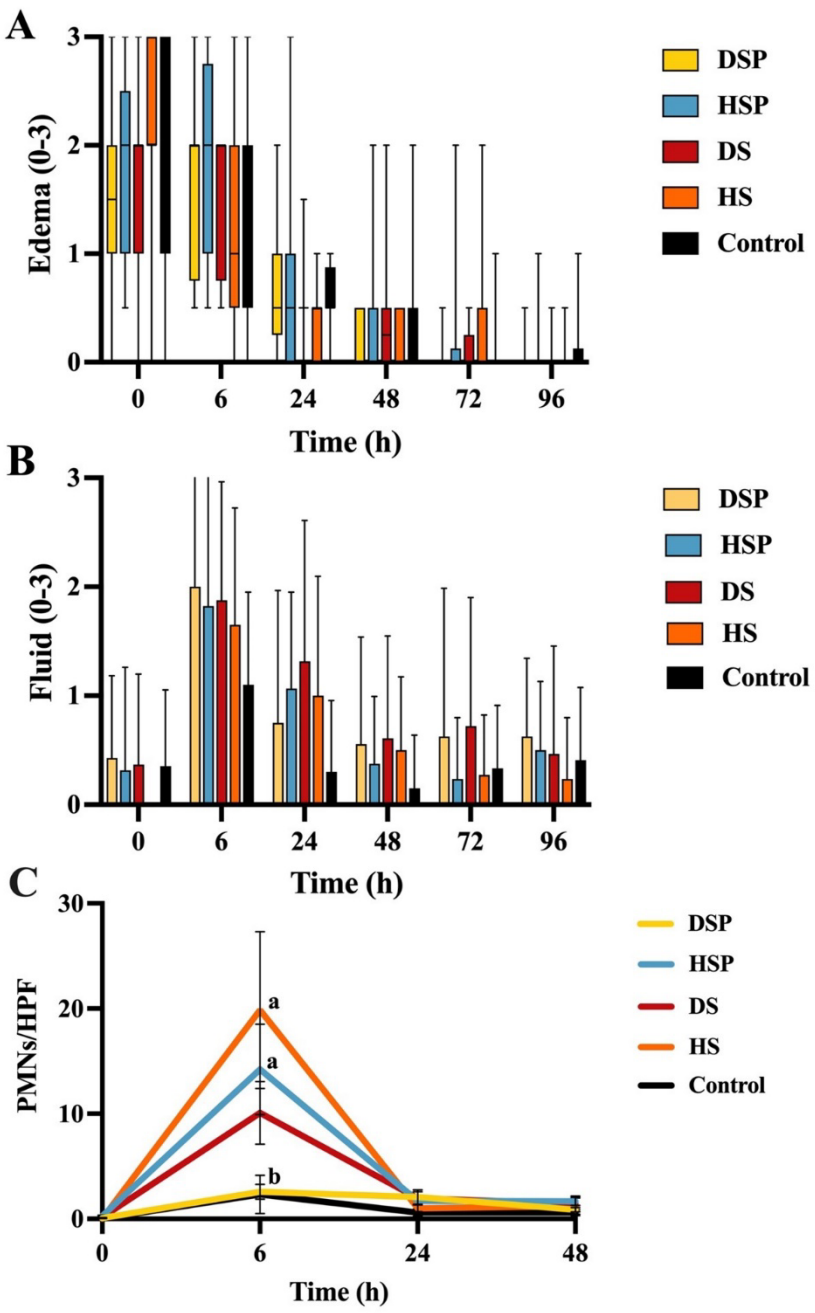

Figure 3.2 (A-C). Endometrial inflammatory response of mares after uterine infusion of donkey and horse semen (DS, HS) and seminal plasma (DSP, HSP) and saline (Control). The number of polymorphonucleate cells (PMNs) was assessed before (0 h) and 6, 24, and 48 h after the infusion; the endometrial edema and intraluminal fluid were assessed *via* transrectal ultrasound before (0 h), and 6, 24, 48, 72, and 96 h after the infusion. (^{ab}) Different superscripts denote a difference between groups ($P < 0.05$).

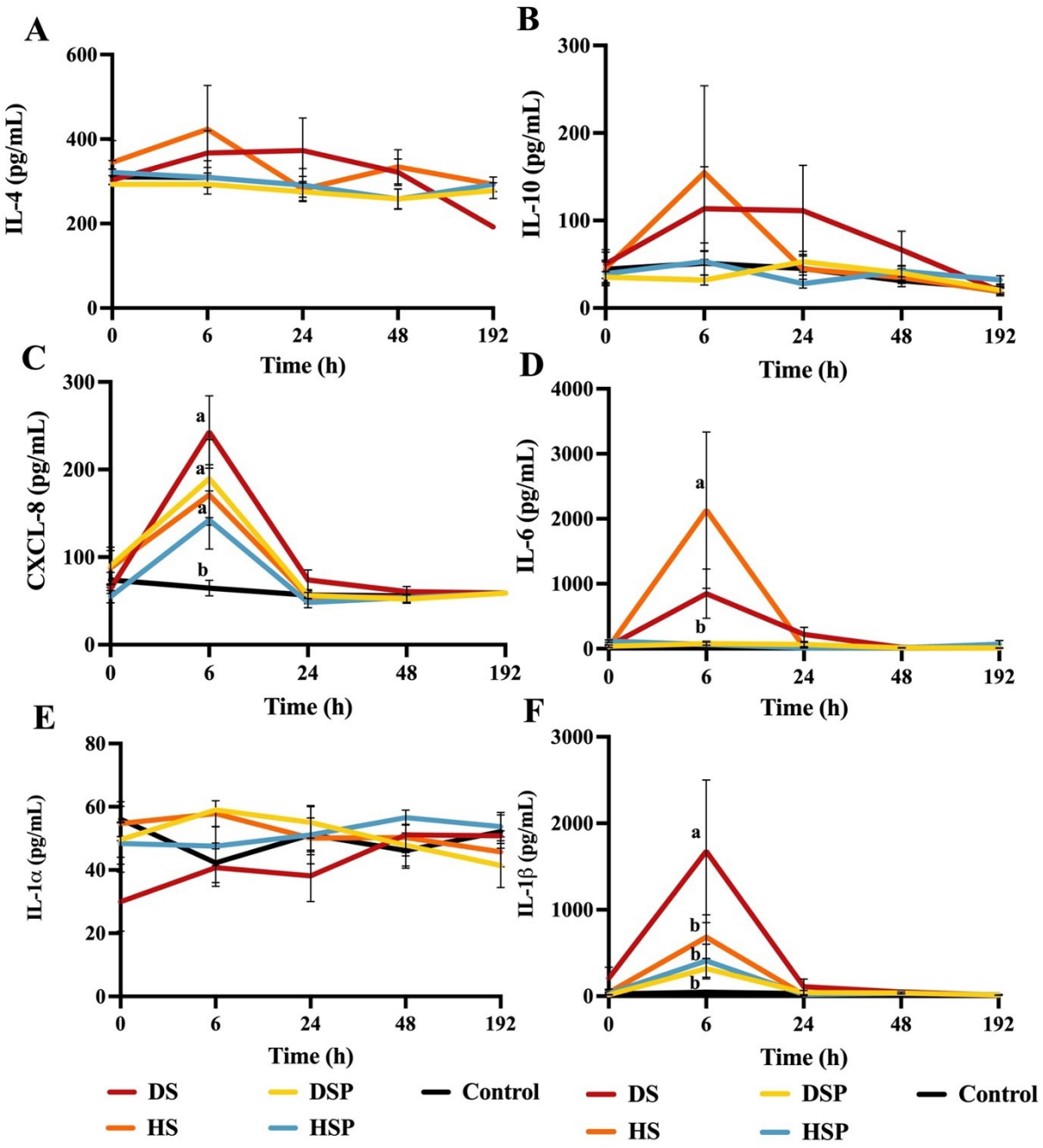

Figure 3.3 (A-F). Endometrial inflammatory response of mares after uterine infusion of donkey and horse semen (DS, HS) and seminal plasma (DSP, HSP) and saline (Control). The concentration (pg/mL) of pro- and anti-inflammatory cytokines (IL1α, IL1β, IL4, IL6, CXCL8, IL10) was assessed before (0 h), 6, 24, 48 h after the infusion, and 8 d (192 h) after the ovulation. (^{ab}) Different superscripts denote differences between groups ($P < 0.05$).

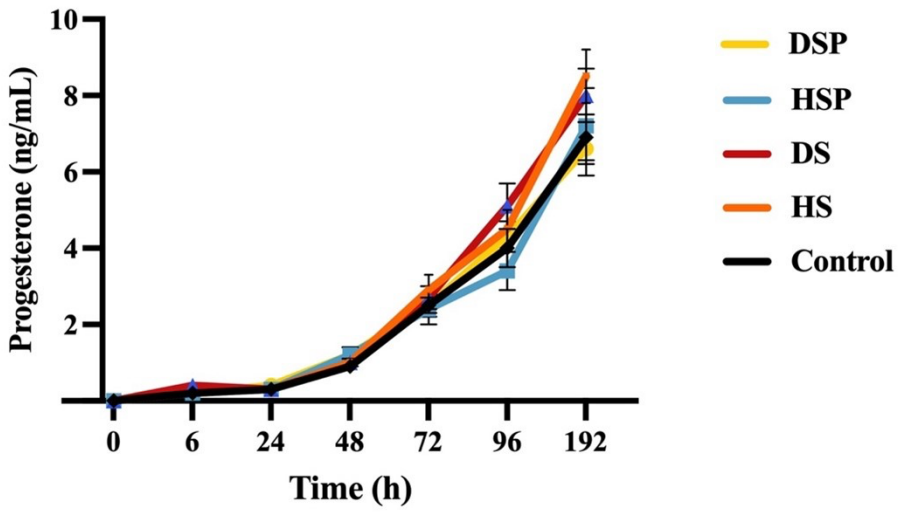

Figure 3.4. Progesterone concentration (ng/mL) was assessed in mares before (0 h) and after 6, 24, 48, 72, and 96 h from the uterine infusion of donkey and horse semen (DS, HS) and seminal plasma (DSP, HSP) and saline (Control) and 192 h (8 d) post-ovulation.

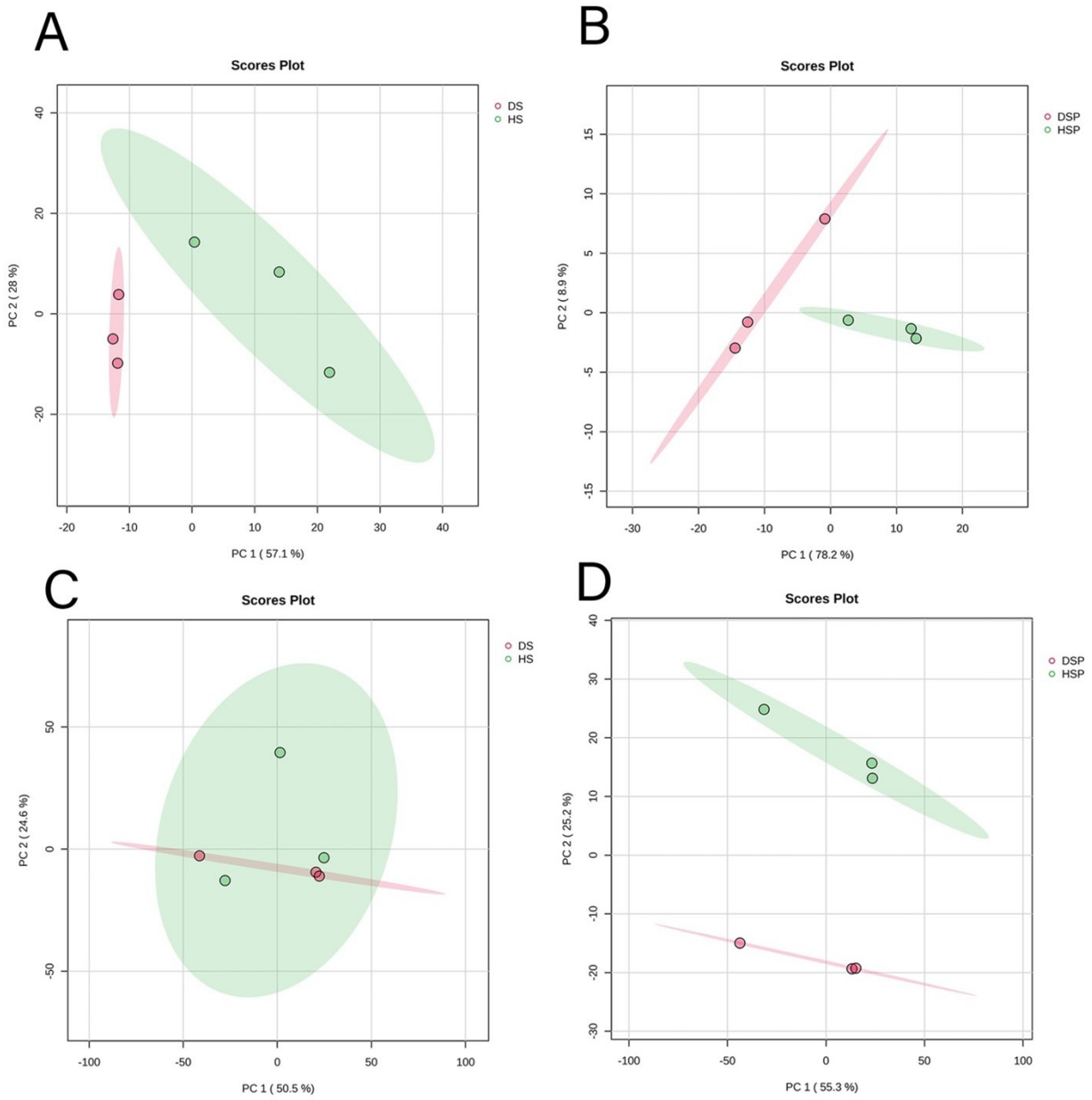

Figure 3.5 (A-D). Principal component analysis (PCA) with relative scores for donkey and horse sperm (DS, HS) and seminal plasma (DSP, HSP) proteins (A, B) and metabolites (C, D).

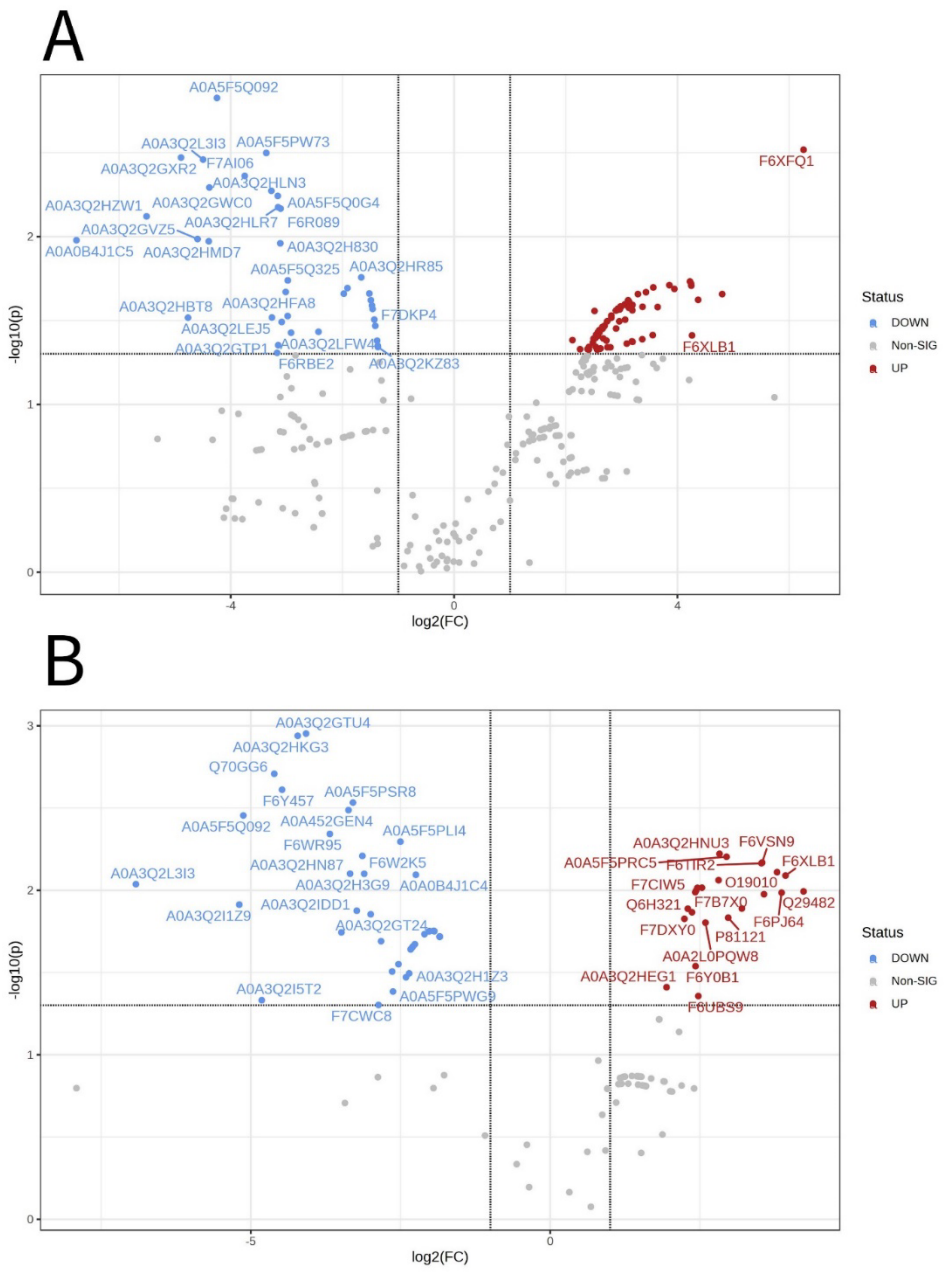

Figure 3.6 (A, B). Volcano plots of proteins identified in donkey and horse sperm (A) and seminal plasma (B). Proteins with higher abundance in donkey sperm or seminal plasma compared to the horse were depicted in red, whereas proteins and metabolites with lower abundance in donkey sperm or seminal plasma compared to the horse were depicted in blue.

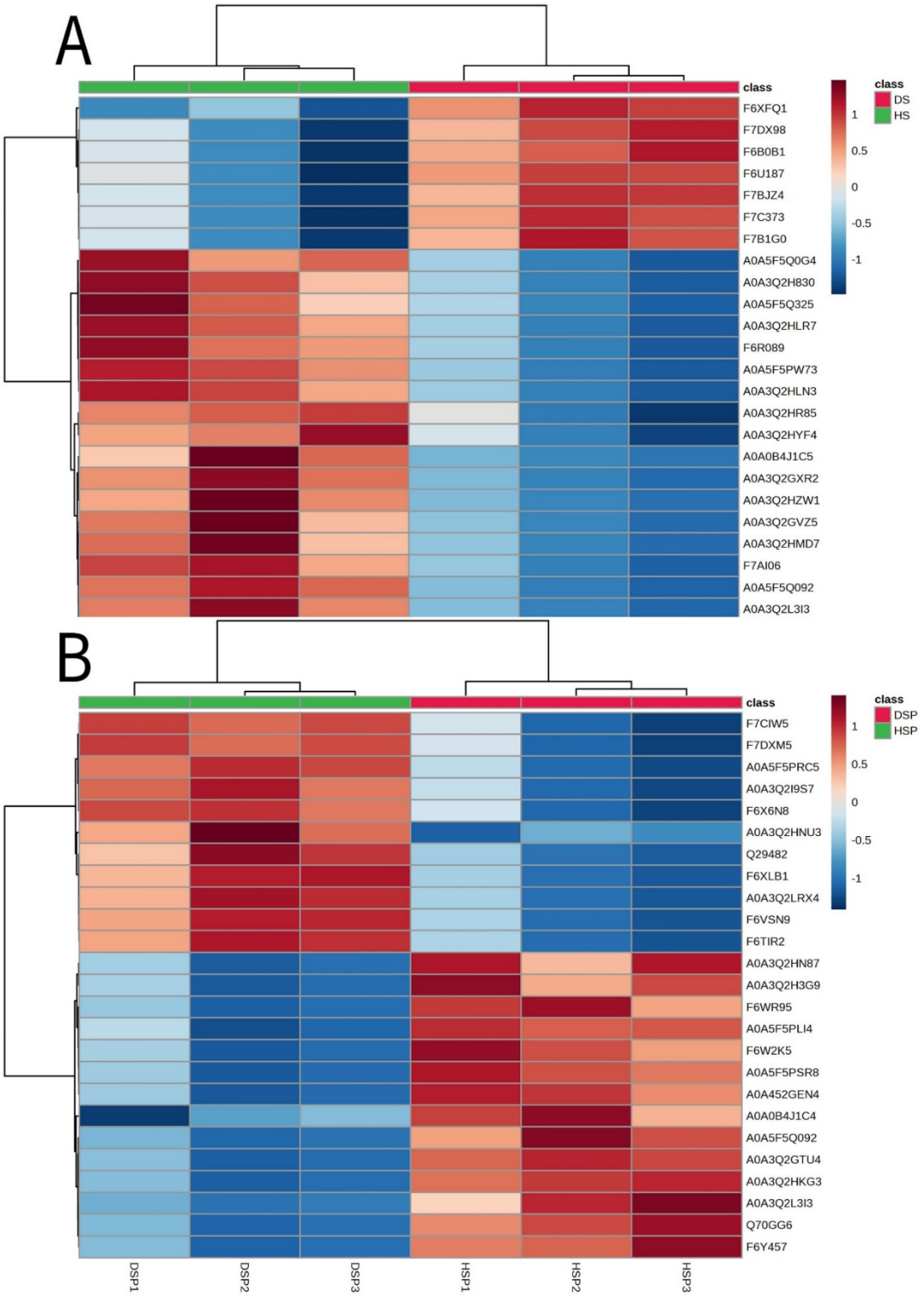

Figure 3.7 (A, B). Heatmaps of sperm (A) and seminal plasma (B) proteins from donkey (DS, DSP) and horse (HS, HSP).

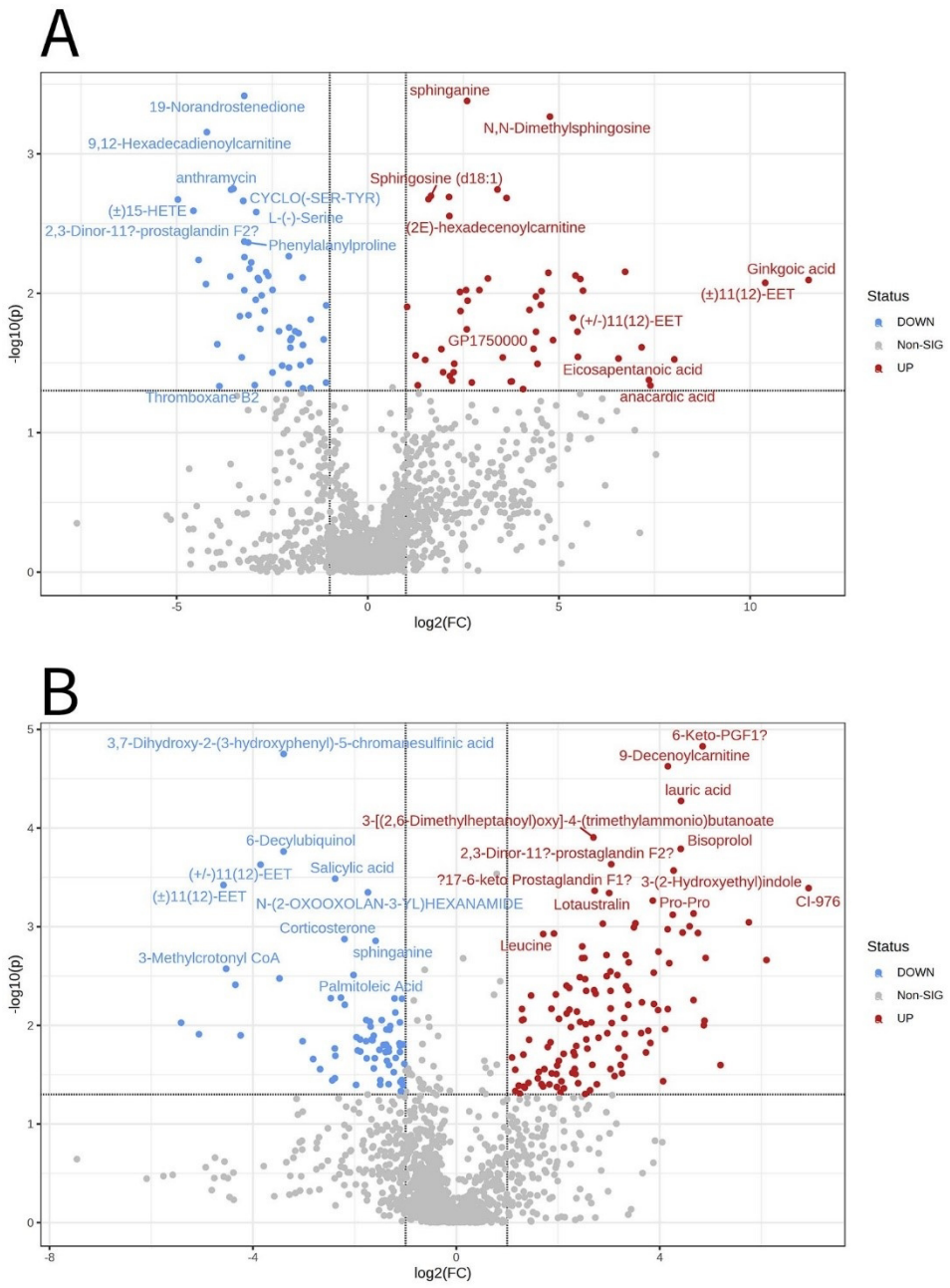

Figure 3.8 (A, B). Volcano plots of metabolites identified in donkey and horse sperm (A) and seminal plasma (B). Metabolites with higher abundance in donkey sperm or seminal plasma compared to the horse were depicted in red, whereas metabolites with lower abundance in donkey sperm or seminal plasma compared to the horse were depicted in blue.

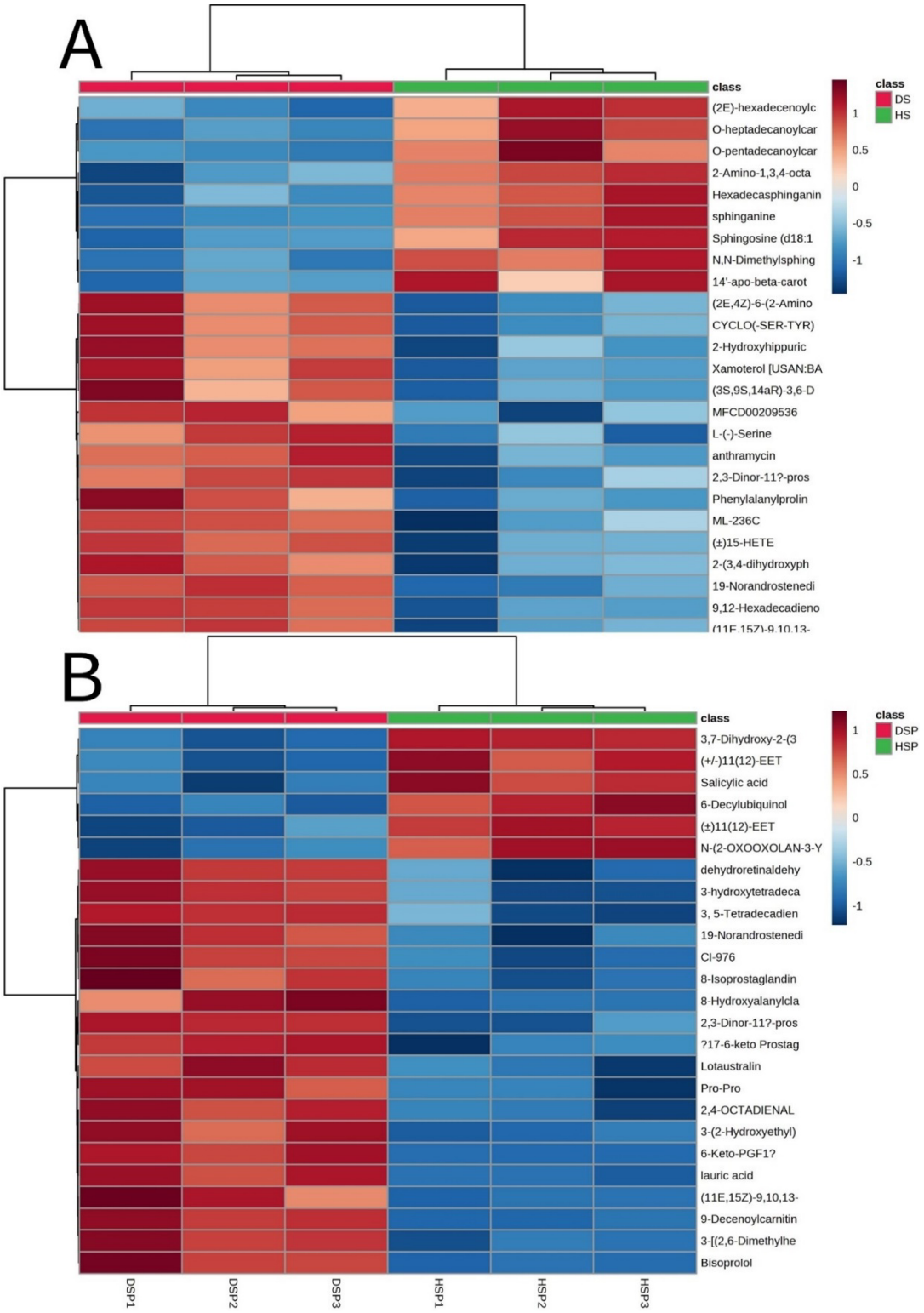

Figure 3.9 (A, B). Heatmaps of sperm (A) and seminal plasma (B) metabolites from donkey (DS, DSP) and horse (HS, HSP).

Mule embryos share identical morphological features to horse embryos[2]

ABSTRACT

This study aimed to compare the morphometry of equine and mule embryos. The study's hypothesis was that the micronuclei and nuclear fragmentation indexes are higher in mule embryos than in horse embryos. Twenty-two mares were randomly assigned in a crossover design to receive semen from both species; thirteen horse and thirteen mule embryos were obtained. All mares were bred by a fertile small standard jack or a fertile Quarter Horse stallion. Embryos were recovered eight days post-ovulation and classified according to the stage of development and quality with a score from 1 (excellent) to 4 (degenerate). Embryos were stained with Hoechst33342, and images were acquired with a fluorescence microscope. Nuclei were categorized as compact, mitotic, or fragmented. The fragmented and mitotic indices were calculated based on their proportion over the total amount of nuclei counted. Embryo size and nuclear morphometry were assessed. The number of positive flushes in cycles bred with donkey or stallion semen did not differ when compared per cycle or per ovulation (13 *vs.* 12) ($P > 0.05$). One set of twins was recovered from a mare that had a double ovulation. Mule and horse embryos were both recovered from eight mares. There was no difference in size between mule and horse embryos ($P > 0.05$). The mule embryos scored between grade 1 (n = 8) and grade 2 (n = 5); similarly, the horse embryos scored between grade 1 (n = 7) and grade 2 (n = 6). The evaluation of the nuclear morphometry revealed that horse and mule embryos have a similar number of compact nuclei per sector (148.7 ± 6.8 nuclei/sector in mule embryos *vs.* 156.5 ± 8.5 nuclei/sector in horse embryos) ($P > 0.05$); however,

[2] This work has been previously published and is modified for inclusion here with the permission of the authors: Giorgia Podico, Igor F. Canisso. Mule embryos share identical morphological features to horse embryos. *Theriogenology* 2024;**216**:196-202

the number of mitotic nuclei tended to be higher in mule embryos (5.2 ± 0.82) than in horse embryos (3.3 ± 0.3). The fragmented nuclei index was similar between mule (0.25 ± 0.1 %) and horse (0.22 ± 0.1 %) embryos (P = 0.4); the mitotic nuclei index was higher in mule embryos (3.2 ± 0.4 %) than in horse embryos (2.2 ± 0.2 %) (P = 0.02). In conclusion, this is the first study to compare the embryo morphology of mares bred with donkeys and horses. Equid embryos share similar nuclear ultrastructure features, except that mule embryos have a higher mitotic index.

Keywords: hybrid; nuclear morphometry; mitotic nucleus; embryo recovery

INTRODUCTION

Following the domestication of African wild asses (*Equus asinus africanus*) 5,000 years ago, these animals and their hybrids (*Equus mulus mulus*) with horses (*Equus ferus caballus*) played an essential role in human development and expansion of early societies from Mesopotamia to the Old Continent, and east to west expansion of the United States and Brazil (Rossel et al., 2008, Canisso et al., 2010). During these early days, domesticated donkey jacks were used to mate with horse mares to produce hybrids to be used as packing animals and to work agricultural, mining, and war fields (Canisso et al., 2019, Mclean et al., 2019). Nowadays, donkeys and their hybrids with horses are still used as packing animals in Northern Africa. In Europe, donkeys are used for milk production with hypoallergic properties. In China, the species is bred on large farms to produce *ejiao*, a collagen product extracted from the hid skin part of the traditional Chinese medicine (Magalhaes et al., 2021, Camillo et al., 2018). In the American continent, hybrids are prized leisure animals and are also massively used to herd cattle in large beef farms in Brazil (Canisso et al., 2010a, Canisso et al., 2010b, Canisso et al., 2009).

The interspecies breeding of a horse and a donkey produces a mule when the sire is a donkey, or a hinny, when the sire is a horse. The prevalence of one or the other hybrid depends on the availability of the dam species, i.e., in rural areas, the access to mares is more limited, and hinnies are more common in certain areas, but overall mules predominate (Mclean et al., 2019). Based on the latest demographic study carried out by the USDA in 2015, the number of mules and donkeys in the US has increased while the number of horses and ponies has decreased (USDA 2016). Noteworthy, using donkey semen to breed mares often results in a higher pregnancy rate than its use on jennies; anecdotally, problem mares have better fertility when bred to a donkey rather than a horse (Rota et al., 2012, De Oliveira et al., 2016, Gobato et al., 2022, Segabinazzi et al., 2021, Vidament et al., 2009, Oliveira et al., 2014). While embryo flush and transfer procedures are routinely performed in the horse industry, for mule production, most of the mares are bred and then carry their pregnancy until term; however, there is a growing demand for the production of mule embryos and also their transfers, and there is scant data regarding the early stages of embryo development and the morphological evaluation of mule embryos. The importance of embryo evaluation has been well established in other domestic species, and quality has been linked with the pregnancy rates in cows and mares (Bó and Mapletoft 2013, Cuervo-Arango et al., 2018, Ducheyne et al., 2019). Grading systems used in bovine and equine consider the development stage and the morphological characteristics of key components like the zona pellucida, the blastomeres, and the capsule (Bó and Mapletoft 2013, McKinnon and Squires 1988, McCue et al., 2009, Van Soom et al., 2003, Morris et al., 2022). Recently, advanced microscopy techniques have been used to perform in-depth ultrastructure embryo evaluation and infer its relation to the successful embryo transfer (Ducheyne et al., 2019, Rubessa 2020, Umair et al., 2023). A recent study out of the Netherlands reported that *in-vitro* produced embryos have a higher presence of

nuclear abnormalities compared to *in-vivo* produced embryos and that they could affect the likelihood of a pregnancy (Ducheyne et al., 2019). These nuclear features could be useful to assess hybrid embryos such as mule embryos.

The objectives of this study were to assess the embryo quality of mule and horse embryos and to determine if nuclear abnormalities are higher in mule embryos compared to horse embryos. While sharing other morphological features, we hypothesized that mule embryos had higher nuclear abnormalities than horse embryos.

MATERIALS AND METHODS

The present study was performed from September to December 2020 at the University of Illinois at Urbana-Champaign, IL, USA. The Institutional Animal Care Unit Committee (protocol #19141) approved all procedures carried out in the present experiment.

Animals and breeding management

Twenty-two light-breed mares (14 ± 5 years-old, range 4 – 23 years-old) were enrolled in the study; the study was performed from September to December 2020. Mares were housed on pasture at the Veterinary Teaching Hospital at the University of Illinois at Urbana-Champaign, IL, USA. Mares were fed with a free choice of mixed hay and supplemented once daily with 1.5 kg of grain (Strategy, Purina Animal Nutrition, L.L.C., St. Louis, MO, USA), each dressed in 50 mL of a commercially available multivitamin and fatty acid product (Broodmare Plus, Botupharma USA, Phoenix, AZ, USA).

Mares were randomly bred with horse and donkey semen in a crossover design with one washout cycle in between. Upon enrollment, mares had a transrectal ultrasonographic examination performed every other day until signs of estrus (follicle > 30 mm, uterine edema, and cervical

softening) were noted. Then, follicular growth was monitored daily, and a single dose of deslorelin acetate (1.8 mg, intramuscularly; SucroMate™, Thorn BioScience, LLC, Louisville, KY, USA) was given to hasten the ovulation. At the induction of ovulation, samples for uterine culture and cytology were collected; each mare had negative uterine cytology (i.e., less than 1 inflammatory cell/high-power field) (hpf) and no bacterial growth after 24-48 h of aerobic incubation to be enrolled in the study. Conversely, mares with signs of endometritis were treated as appropriate and had an additional washout cycle (Canisso et al., 2016, Canisso et al., 2020).

One donkey jack and one horse stallion were used throughout the entire experiment. Before each insemination, semen was collected off a tease mare using a Missouri artificial vagina (16 in length; Nasco, Fort Atkinson, WI, USA) coupled with a filter and a plastic bag (Whirl-Pak, Madison, WI, USA). An artificial insemination was performed with ~2 billion total sperm of raw semen after 24 h from ovulation induction. No post-breeding treatments were performed other than oxytocin administration (20 units, intramuscularly, every six hours for two days after ovulation). A transrectal ultrasonographic examination was performed every two hours from 34 h post-induction to the time of ovulation. Mares failing to ovulate within 48 h from the ovulation induction agent had cycles discarded and re-enrolled. The interval between the artificial insemination and the ovulation was compared among groups and the outcomes of the embryo flush. The relation between the interval between the artificial insemination and the ovulation and the embryo size and age of the mare was assessed.

Embryo recovery and evaluations

A non-surgical embryo flush was performed in the mares from 192 to 220 h from the ovulation. Mares younger than 13 years-old had the flush performed between 192 and 200 h,

whereas older mares (> 13 years-old) had it between 200 and 220 h. Each mare had the tail wrapped and the perineal area cleaned for the procedure. A bullet-tip catheter 28 Fr was inserted through the cervix, and 4 L of Ringer's Lactate solution was used to recover the embryo from the uterus. The fluid was retrieved in a dish coupled with a filter and inspected under the dissection microscope to determine the presence or absence of the embryo. After embryo flushing, mares received a dose of dinoprost tromethamine (5 mg im) (Lutalyse, Zoetis, Parsippany-Troy Hills, NJ, USA).

Stereomicroscopy evaluation

Embryos were immediately evaluated under a stereomicroscope after their recovery. The evaluation was based on previous *in-vivo* horse embryo grading systems; a single clinician used the same morphological grading for horse and mule embryos (McKinnon and Squires 1988, McCue et al., 2009). The grading system considered the degree of compactness, color, presence of extruded cells, and the degree of cell granulation and cytoplasmic fragmentation. Each embryo was scored from 1 to 4. Score 1 (excellent quality) was given when no abnormalities were observed; Score 2 (good quality) was given when minor abnormalities like a thin zona pellucida, few extruded blastomeres, and light irregularities in shape, size, or color were noted; Score 3 (poor quality) was given when moderate imperfections were found, like a considerable amount of extruded cells and the collapse of the blastocoele and trophoblast; Score 4 (degenerate embryo) was given when major abnormalities like rupture of the zona pellucida, a high amount of extruded cells or a complete collapse of the blastocoele, were found (McKinnon and Squires 1988, McCue et al., 2009). After the evaluation, the embryos were fixed in buffered formalin 2% at room temperature for 30 min and then stored at 4 °C until the immunostaining.

Fluorescence microscopy evaluation

The immunostaining was based on a published protocol for horse embryos [18]. The fixed embryos were washed three times for 5 min each in PBS and PVP (3 mg/mL) (Cat. P0930, Sigma-Aldrich, St. Louis, MO, USA) and then left in PBS with 0.1 % w/v saponin (Cat. 47036, Sigma-Aldrich, St. Louis, MO, USA) to permeabilize for 30 min at room temperature. Next, each embryo was incubated for 30 min in a 1:500 dilution of Hoechst33342 (Cat. 14533, Sigma-Aldrich, St. Louis, MO, USA) in PBS-PVP; Hoechst33342 is a fluorescent dye that binds to the nuclear DNA. Each embryo was then mounted in 10-20 µL droplets of antifade mounting medium (Cat. H-1900, Vectashield®, Vector laboratories, Newark, CA, USA) and then sealed with nail polish using spacer wells 0.12 mm deep (S24735, Sigma-Aldrich, St. Louis, MO, USA); two spacers were utilized for larger embryos to not compromise their integrity.

The immunostained embryos were scanned using a Keyence BZ-X800 (Keyence Corporation of America, Itasca, IL, USA), inverted widefield fluorescence microscope, coupled with a ×60 objective; Hoechst33342 was excited using a 405 nm laser. Eight sectors of the embryo were randomly selected and scanned with planes at 1 µm of distance. Images were then analyzed using ImageJ v1.54a (https://imagej.net/Fiji/Downloads); a grid tool was used over each image to not count each nucleus more than once. Nuclei were manually counted and evaluated for morphology in each embryo (**Figure 3.10**). Nuclei were categorized as 1) Compact nuclei – interphase nuclei with strong Hoechst33342 signal; 2) Mitotic nuclei – nuclei in prophase, metaphase, or anaphase of mitosis; 3) Fragmented nuclei (Ducheyne et al., 2019, Tremoleda et al., 2003). The absolute number of nuclei in each category was compared across the species, and the following indexes were calculated: fragmented nuclei index = fragmented nuclei/total nuclei *100; mitotic nuclei index = nuclei in mitosis /total nuclei *100 [18].

Statistical analyses

Statistical analyses were conducted using GraphPad Prism v10.0.2. The relation between embryo size, age of the mare, and the interval between ovulation and embryo flush was assessed by a mixed linear regression, and the relation between embryo size and interval from artificial insemination and ovulation by a Pearson correlation. A linear mixed model was used to evaluate the effect of the outcome of the embryo flush, the type of semen used, the number of ovulations; the cycle order, and the mare were considered random effects. Results were reported as mean ± SEM; differences with $P < 0.05$ were considered statistically significant.

RESULTS

Embryo recovery and evaluations

Forty-four embryo flushings were performed, and a total of 26 embryos were recovered; one set of twins was recovered from a mare bred with horse semen and a double ovulation. The number of positive flushes in cycles bred with donkey or stallion semen did not differ when compared per cycle or per ovulation (13/22 *vs.* 12/22) ($P > 0.05$), respectively **(Figure 3.11).**

The size of mule and horse embryos did not differ (915.5 ± 288 µm *vs.* 575.8 ± 69.6 µm, respectively) ($P > 0.05$). The mule embryos were all scored between grade 1 (n = 9) and grade 2 (n = 4) **(Table 3.12);** similarly, the horse embryos were scored between grade 1 (n = 6) and grade 2 (n = 7) **(Table 3.13)**. The most common abnormalities found in both mule and horse embryos were abnormal shape and extruded cells **(Figure 3.12).**

One embryo was recovered in both the donkey- and horse-semen cycles in eight mares out of twenty-two (**Figure 3.13**). When only those mares were considered, the embryo size and quality seemed more homogeneous; the mule embryos were 664.3 ± 79.8 µm and mainly classified as

grade1 (n = 5), and the horse embryos were 592.1 ± 39.1 μm and mostly classified grade 1 (n = 4).

The interval between the artificial insemination and the ovulation did not differ in mares bred with donkey or horse semen and did not affect the outcome of the embryo flush (P > 0.05). The interval was 15 ± 1.3 h and 13.2 ± 0.6 h in mares bred with donkey semen with a negative and a positive embryo flush, respectively; the interval was 16.3 ± 3.2 h and 14.8 ± 0.9 h in mares bred with horse semen with a negative and a positive embryo flush, respectively (P > 0.05). There was no correlation between the interval between artificial insemination to ovulation and the embryo size in the horse (r = -0.5559; P = 0.1202) and in the mule (r = 0.1932; P = 0.5693) embryos. The multiple linear regression revealed that the mule embryo size was not affected by the interval from ovulation to embryo flush (P = 0.6594), the age of the mare (P = 0.4107), or their interaction (P = 0.4102). Conversely, the multiple linear regression revealed that the horse embryo size was not affected by the interval between ovulation and embryo flush (P = 0.0869), the age of the mare (P = 0.2801) or their interaction (P = 0.2509).

The evaluation of the nuclear morphometry revealed that horse and mule embryos have a similar number of compact nuclei per sector (148.7 ± 6.8 nuclei/sector in mule embryos *vs.* 156.5 ± 8.5 nuclei/sector in horse embryos) (P > 0.05); however, the number of mitotic nuclei per sector tended to be higher in mule embryos (5.2 ± 0.82 mitotic nuclei) than in horse embryos (3.3 ± 0.3 mitotic nuclei) (P = 0.0827). The fragmented nuclei index was similar between mule (0.25 ± 0.1 %) and horse (0.22 ± 0.1%) embryos (P = 0.3818); the mitotic nuclei index was higher in mule embryos (3.2 ± 0.4 %) than in horse embryos (2.2 ± 0.2 %) (P = 0.0209) (**Figure 3.14**).

DISCUSSION

This study was set forth to assess and compare mule embryos with horse embryos using a standard grading system established for horses and as well an advanced confocal microscopy. Our findings demonstrated that horse and mule embryos have identical morphological features ~8d post-ovulation, thus, the well-established horse embryo grading system can also be used to assess mule *in vivo* produced embryos (McKinnon and Squires 1988, McCue et al., 2009).

The embryo recoveries were not different between mare cycles bred to either the donkey or the horse. The embryo recovery in the interspecies breeding concurred with the high pregnancy rates reported by other studies (De Oliveira et al., 2016, Carluccio et al., 2020, Paolucci et al., 2012). Out of the twenty-two mares, eight of them produced embryos when bred with either male, five yielded embryos when bred with donkey semen, and four with horse semen. Although the mule embryos in the present study showed excellent morphology, their fertility potential was not assessed as it was not the objective of the present study. Previous studies from another research group demonstrated that mule embryos similar to this study have excellent potential and all the three mule embryos transferred into a mule recipient resulted in pregnancy (Fanelli et al., 2022).

The morphological evaluation of an embryo is a critical step of the embryo flushing and transfer procedures; the stage of development and presence of abnormalities have been linked to a decreased pregnancy rate in both the mare and the cow (Ducheyne et al., 2019, McCue et al., 2009, Umair et al., 2023). A retrospective study on almost 500 horse embryo flushes reported that 80% of the embryos were of excellent quality and only 2% were of poor quality; remarkably, no degenerated embryo was found; our study mirrored those results with both embryo species (McCue et al., 2010). The absence of degenerated embryos seemed to be linked to the active mechanism required by the uterotubal junction to open at day 5.6-6 post-ovulation, where only viable embryos

can produce PGE_2 that is necessary (Weber et al., 1991). This mechanism has not been demonstrated in the presence of mule embryos; however, the absence of degenerated embryos seemed to support a similar mechanism.

The size of both mule and horse embryos in this study was not affected by the age of the mare or the time between ovulation and embryo flush; this finding was different from another study that reported that horse embryo size was influenced by the age of the mare but not by the type of semen or interval to ovulation (Cuervo-Arango et al., 2018). This lack of difference could mean that there is no true difference, or that the study's sample size did not allow for a statistical differentiation of sizes. Noteworthy, in mares that gave an embryo both when bred with donkey and horse semen, the month of the year seemed to play a role. Embryos harvested at the beginning of the study (September-October) were larger than embryos harvested at the end (November-December). It is possible that if embryos were harvested in the spring and summer, the sizes could have been different.

The index accounting for the number of mitotic nuclei indicated that mule embryos had a higher mitotic index than horse embryos. The presence of a high mitotic index is common in cancer cells, aneuploid cells, and polyploid cells (Sazonova et al., 2021, Denisenko et al., 2016). Surprisingly, in neoplastic tissues, the mitotic index is also tightly correlated to the apoptotic index; apoptosis seems to drive the ability of cancer to metastasize to other organs and tissue (Lipponen 1999, Lipponen et al., 1994, Harris et al., 1983). No apoptosis marker was used in this study; thus, we were not able to determine if mitosis and apoptosis are related in the mule embryos and if they could be a leading cause of the higher reported rate of pregnancy loss in mule pregnancies (Boeta and Zarco 2005, Boeta and Zarco 2010, Harris and Thompson 2000, Korsmeyer 1992). In human *in vitro* produced embryos, nuclear abnormalities during mitosis have been studied with stainings

of the centromeres and revealed that they were linked to aneuploidy, one of the most common causes of developmental arrest and miscarriage. Although the difference in the number of chromosomes of the horse and donkey could make aneuploidy the putative reason for nuclear abnormalities in mule embryos, our study did not include differential staining or microscopic techniques that were able to prove its presence; therefore, the impact on mule embryo development remains to be determined. Furthermore, the higher number of mitotic nuclei could explain the apparent larger size of mule embryos compared to horse embryos.

In conclusion, mule embryo shares morphometric features with horse embryos that allow the use of the standard horse embryo grading evaluation system. To the authors's knowledge, this was the first study investigating the morphology of mule embryos; further studies are needed to elucidate the meaning of the high rate of mitotic cells and its impact on embryo development and developmental competency.

FIGURES AND TABLES

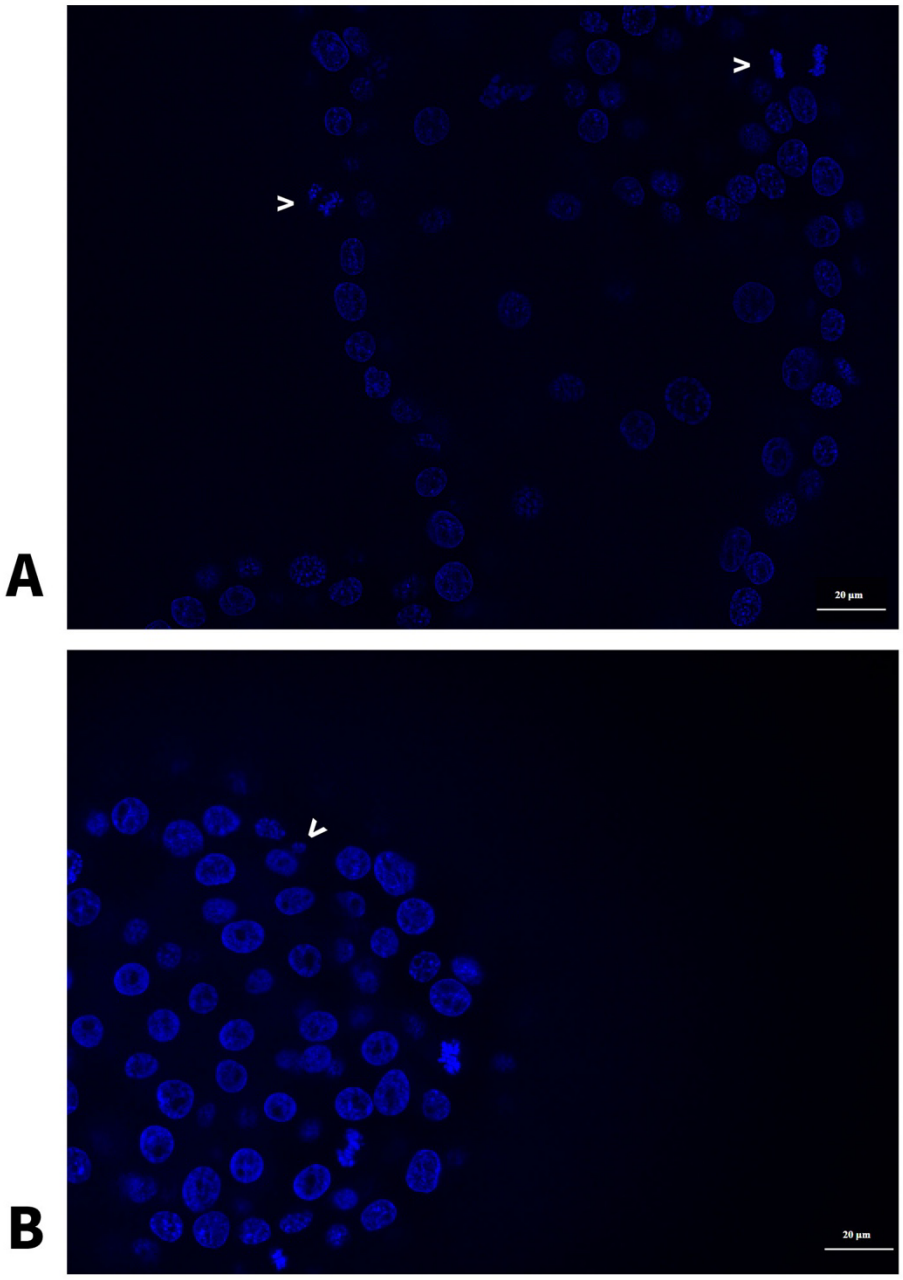

Figure 3.10. Representative fluorescent image of a horse embryo harvested after ~8d post-ovulation with two mitotic (A) and one fragmented nuclei (B).

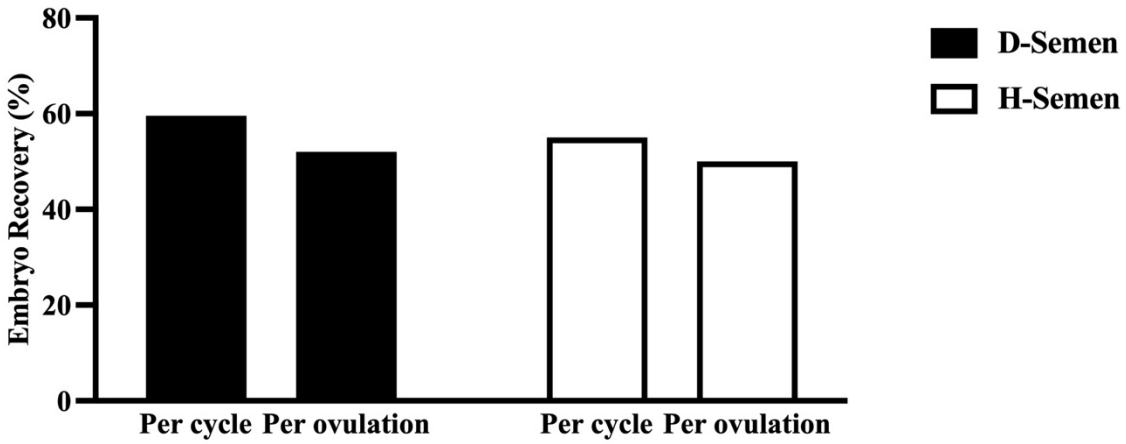

Figure 3.11. Embryo recovery of mares (n = 22) inseminated with donkey semen (DS; n = 25 ovulations among 22 cycles) and horse semen (HS; n = 24 ovulations among 22 cycles). There were no differences in embryonic recovery ($P > 0.05$).

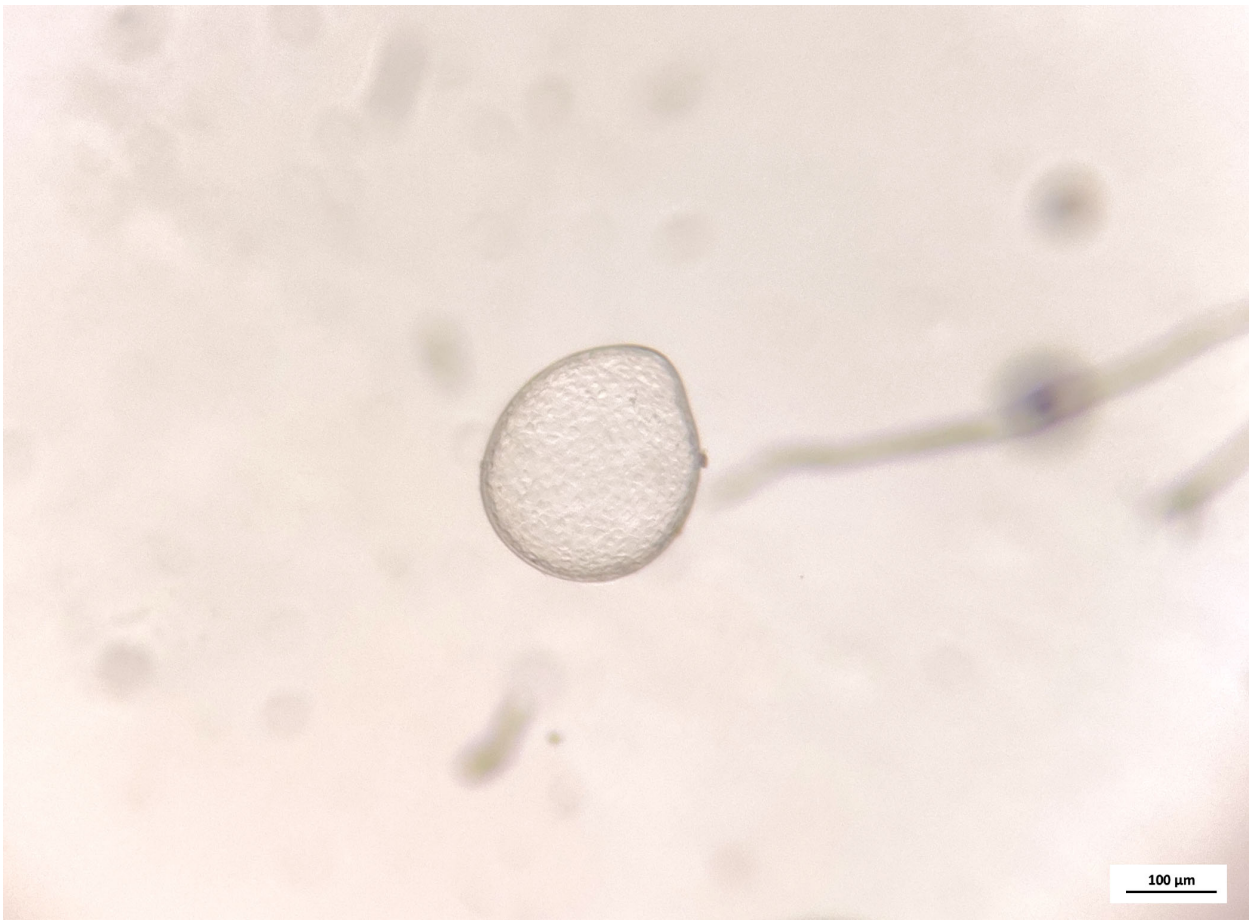

Figure 3.12. Mule embryo harvested from a mare ~8d post-ovulation. The embryo showed abnormal morphology with a piriform shape (Grade 2).

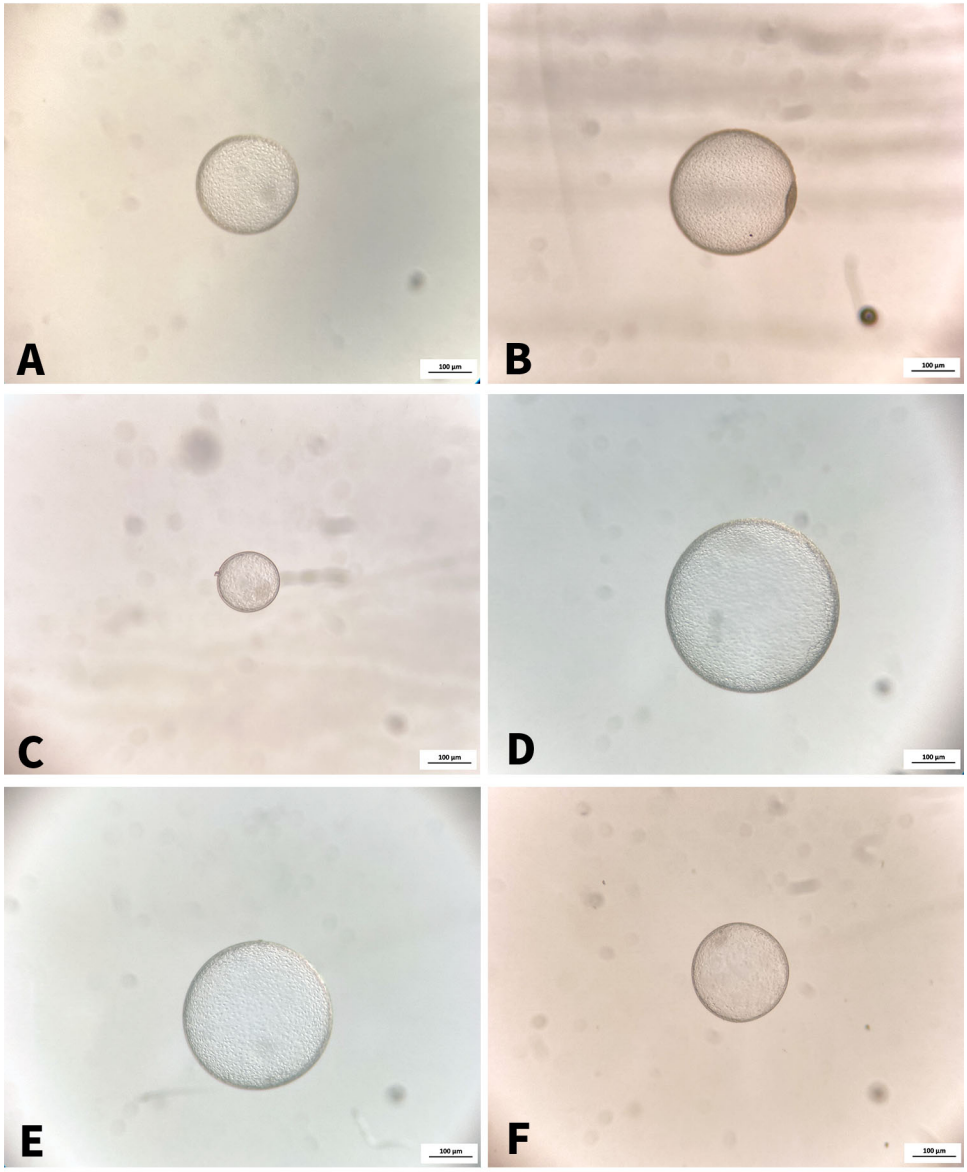

Figure 3.13. Pairs of mule (A, C, E) and horse (B, D, F) embryos from three of the eight mares that conceived on both cycles. Interestingly, despite the embryo genotype, two of the three mares showed a marked difference in embryo size when the flush occurred in early Fall, September (D, E), or Winter, November-December (C, F). Embryos were collected after ~8d post-ovulation.

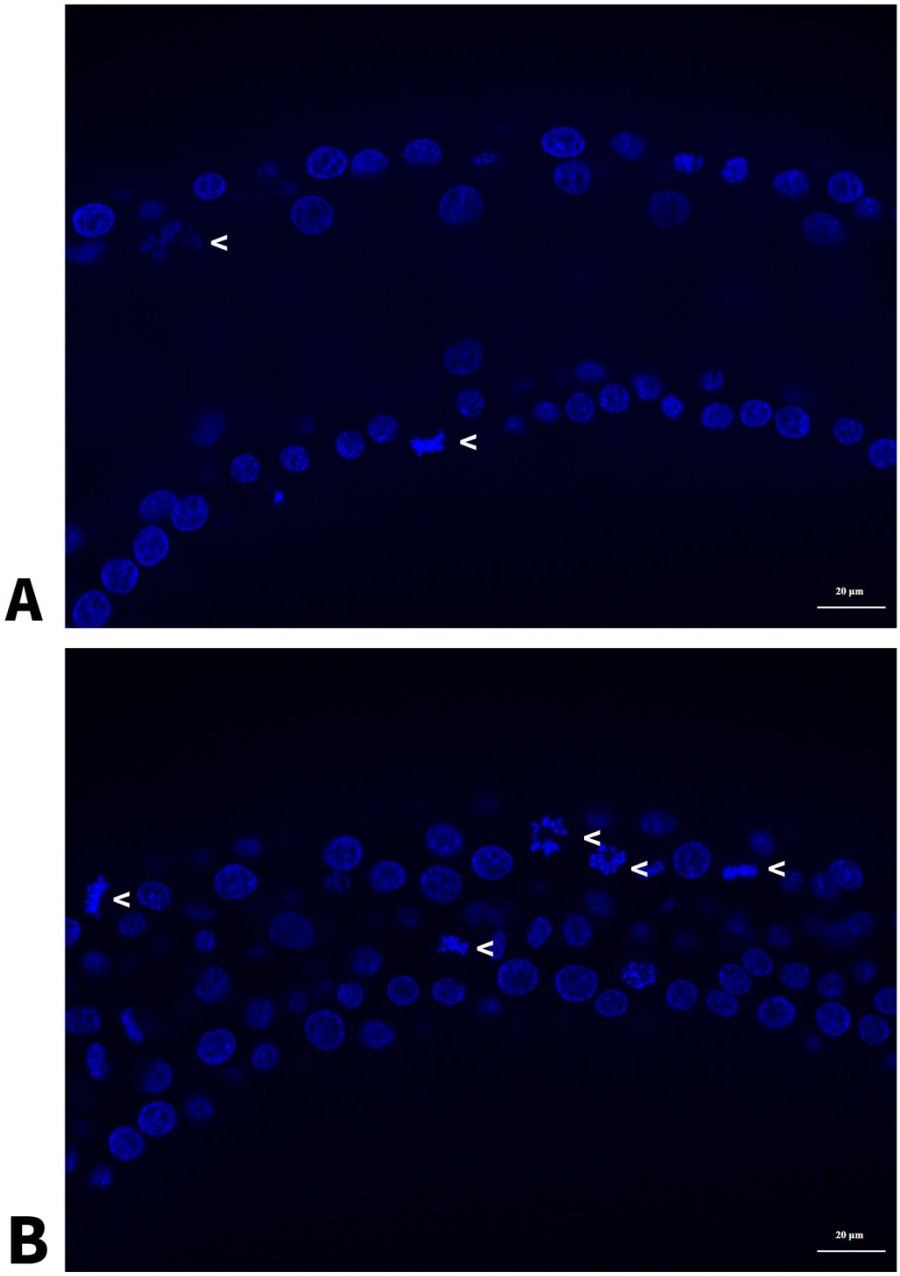

Figure 3.14. Representative images of a horse (A) and a mule (B) embryo with low and high presence of mitotic nuclei, respectively. Embryos were collected after ~8d post-ovulation.

Table 3.1 Semen parameters of donkey jack and horse stallion used in the study.

Semen characteristics	Donkey (n= 23)	Horse (n= 31)	P-value
Gel-free volume (mL)	115 ± 6.8	73 ± 3.9	0.0001
Sperm concentration ($x10^6$ sperm/mL)	222.7 ± 14	160.7 ± 11	0.0015
Total sperm ($x10^9$)	19.5 ± 2.5	8.0 ± 1.1	0.0002
Total motility (%)	87.2 ± 1.6	82.6 ± 1.1	0.0274
Progressive motility (%)	80.2 ± 2.0	72.4 ± 1.4	0.0023
VAP (mm/s)	87.7 ±3.0	77.6 ± 1.8	0.0035
VSL (mm/s)	67.3 ± 3.3	55.6 ± 1.6	0.0012
VCL (mm/s)	149.0 ± 4.5	140.7 ± 3.6	0.1553

Abbreviations: VAP, average path velocity; **VSL**, straight-line velocity; **VCL**, curvilinear velocity.

Table 3.2 Proteins identified in donkey sperm and seminal plasma compared to the horse

More abundant proteins in donkey sperm				
Accession ID	**Name**	**Function**	**Fold change**	**P-value**
F6XFQ1	**L-lactate dehydrogenase**	Catalytic activity	76.5	0.003
O19010	**Cysteine-rich secretory protein 3**	Spermatogenesis, cell adhesion	27.8	0.022
F6RR95	**High mobility group box 4**	DNA binding	20.7	0.023
F6XLB1	**Lactoferrin**	Immune system	19.7	0.038
F7B1G0	NA		19.0	0.019
Less abundant proteins in donkey sperm				
A0A0B4J1C5	**Ubiquitin-ribosomal protein eL40 fusion protein**	Protein synthesis	0.009	0.01
A0A3Q2HZW1	**Heat shock protein family A (Hsp70) member 2**	ATP binding, chaperone, heat shock protein	0.02	0.007
A0A3Q2GXR2	**T-complex protein 1 subunit beta**	Chaperone	0.03	0.003
A0A3Q2HBT8	NA		0.03	0.033
A0A3Q2GVZ5	**Jacalin-type lectin domain-containing protein**	Binding	0.04	0.01
More abundant proteins in donkey seminal plasma				
Q29482	**Clusterin**	Extracellular chaperone	18.8	0.01
F6XLB1	**Lactoferrin**	Immune system	15.2	0.008
F6PJ64	**Lipocalin**	Transport	14.5	0.01
A0A3Q2LRX4	**CUB domain-containing protein**	Fertilization	13.8	0.007
O19010	**Cysteine-rich secretory protein 3**	Spermatogenesis, cell adhesion	11.9	0.01
Less abundant proteins in donkey seminal plasma				
A0A3Q2L3I3	**Clusterin**	Extracellular chaperone	0.008	0.009
A0A3Q2I1Z9	**Calmodulin 2**	Binding	0.027	0.01
A0A5F5Q092	**Cysteine-rich secretory protein 2**	Fertilization, cell adhesion	0.028	0.003
A0A3Q2I5T2	**Cystatin C**	Protease inhibitor	0.035	0.04
Q70GG6	**Binder of sperm 1**	Sperm capacitation, binding	0.040	0.001

Table 3.3 Morphologic features and quality of mule embryos (n = 13) harvested ~8d post-ovulation during the Fall-Winter in the Northern hemisphere.

Embryo	Size	Stage	Grade	Quality
1	1175 µm	Expanded blastocyst	2	Regular trophoblast, few extruded cells
2	812 µm	Expanded blastocyst	1	Regular trophoblast, no extruded cells
3	297 µm	Expanded blastocyst	1	Regular trophoblast, no extruded cells
4	169 µm	Expanded blastocyst	2	Regular trophoblast, extruded cells
5	1275 µm	Expanded blastocyst	1	Regular trophoblast, no extruded cells
6	3700 µm	Expanded blastocyst	1	Regular trophoblast, no extruded cells
7	205 µm	Expanded blastocyst	2	Irregular trophoblast, extruded cells
8	589 µm	Expanded blastocyst	1	Regular trophoblast, no extruded cells
9	310 µm	Expanded blastocyst	1	Regular trophoblast, rare extruded cells
10	353 µm	Expanded blastocyst	2	Irregular shape (pyriform), no extruded cells, regular trophoblast
11	1673 µm	Expanded blastocyst	1	Regular trophoblast, no extruded cells, very thick zona pellucida
12	428 µm	Expanded blastocyst	1	Regular trophoblast, no extruded cells
13	NA	Expanded blastocyst	1	Regular trophoblast, no extruded cells

Table 3.4 Morphologic features and quality of horse embryos (n = 13) harvested ~8d post-ovulation during the Fall-Winter in the Northern hemisphere.

Embryo	Size	Stage	Grade	Quality
1	637 µm	Expanded blastocyst	2	Irregular trophoblast, no extruded cells
2	497 µm	Expanded blastocyst	1	Regular trophoblast, no extruded cells
3	653 µm	Expanded blastocyst	2	Irregular trophoblast, extruded cells
4	323 µm	Expanded blastocyst	2	Irregular trophoblast, extruded cells
5	905 µm	Expanded blastocyst	1	Regular trophoblast, no extruded cells
6	337 µm	Expanded blastocyst	1	Regular trophoblast, no extruded cells
7	286 µm	Blastocyst	2	Irregular shape, irregular trophoblast, extruded cells, thick zona pellucida
8	1115 µm	Expanded blastocyst	2	Regular trophoblast, no extruded cells
9	812 µm	Expanded blastocyst	2	Irregular trophoblast, no extruded cells
10	515 µm	Expanded blastocyst	1	Regular trophoblast, no extruded cells
11 Twin	381 µm	Expanded blastocyst	1	Regular trophoblast, no extruded cells
12 Twin	854 µm	Expanded blastocyst	1	Regular trophoblast, no extruded cells
13	320 µm	Expanded blastocyst	2	Irregular trophoblast, extruded cells, thick zona pellucida

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CHAPTER 4: The uterine microbiome of mares during inter- and intraspecies breeding[3]

ABSTRACT

An extra-species model (donkey-to- and horse-to-mare) was designed to determine the effects of the male seminal microbiome on the mare uterine microbiome. The semen and uterine microbiomes were analyzed among seventy-five estrous cycles of mares (n=15) inseminated with a single fertile donkey or horse raw semen or infused with saline, donkey, or horse seminal plasma in a crossover design. After filtering and agglomeration, sequencing the full 16S gene resulted in the assignment of 99 taxa. Alpha diversity differed between donkey and horse semen, whereas beta diversity was similar across species. The most prevalent phyla in mare-cycles infused with donkey semen were Proteobacteria, Actinobacteria, and Firmicutes; similarly, with the horse semen, Proteobacteria, Actinobacteria, and Firmicutes were the most abundant. The beta diversity of the uterus differed across the mare-cycles based on the type of infusion and on the presence of an embryo in both mare-cycles infused with donkey and horse semen. In conclusion, the semen microbiome varied between the two equids used in the study, and more importantly, the semen microbiome affected the mare uterine microbiome and suggested the presence of a temporary combined male and female microbiome.

Keywords: Equine hybrid; mule embryos; donkey semen microbiome;

[3] This work has been submitted for publication to the journal Scientific Report at the moment of the dissertation's depostit and it is modified for inclusion here with the permission of the authors. Giorgia Podico, Negin Valizadegan, Christopher J. Fields, Igor F. Canisso. The uterine microbiome shifts of mares during intra- and interspecies breeding.

INTRODUCTION

The direct communication of the uterus with the caudal/lower reproductive tract (i.e., vagina, vestibule) and with the outside contributes to the dynamicity of the uterus. It is considered one of the main routes for the establishment of uterine conditions such as endometritis, metritis, or placentitis (Romero et al., 2019, Lapinsky, 2013, Canisso et al., 2020). However, it is reasonable to assume that bacterial transplantation from the vagina, the male reproductive tract, or the outside also occurs during physiological uterine functions like breeding and parturition. The uterine microbiome has been described in the mare for more than a decade using 16S gene sequencing, confirming the presence of bacteria that remained undetected by classic culture-based microbiology techniques (Rock et al., 2011). However, it was not until recently that a study detailed the intricate panorama of bacteria colonizing the uterus of healthy mares (Holyoak et al., 2022). The composition of the mare uterine microbiome includes *Lactobacillus*, *Escherichia/Shigella*, *Streptococcus*, *Blautia, Staphylococcus*, *Klebsiella*, *Acinetobacter*, and *Peptoanaerobacter* (Holyoak et al., 2022).

Mating is thought to affect the uterine microbiome *via* the inherent semen microbiome and external contamination; the concept of "complementary semino-vaginal microbiota" was developed to depict the interplay between the female and the male reproductive microbiomes (Altmäe et al., 2019, Mändar et al., 2015). Noteworthy, in humans, the semen is deposited vaginally during mating and intrauterine in horses; these physiological differences could be of interest to understanding the local interaction (Altmäe et al., 2019, Mändar et al., 2015). In the horse industry, most mares are artificially inseminated, which consists of semen being collected from a stallion and deposited intrauterine with or without the addition of extenders. In domestic animals, the semen microbiome fingerprint has been investigated in a few species, including the

stud dog, the bull, and the stallion (Koziol et al., 2022, Quiñones-Pérez et al., 2024, Banchi et al., 2024, Mocé et al., 2022). Still, the interactions with the female uterine or vaginal microbiome have never been elucidated.

Interspecies breeding is surprisingly successful in equids; the seven extant species of the genus *Equus* are all able to cross producing a viable offspring (Benirschke et al., 1967, Benirschke et al., 1962, King et al., 1967). The mule, product of the mating of a male donkey and a female horse, is the most common equid hybrid. The mule played an essential role in human history from the second century when its size, power, and stamina were exploited in the fields and at war (Podico et al., 2024, Canisso et al., 2019). It has been commonly believed that the above-referenced crossing is even more fertile than intraspecies breeding, and this has not been critically assessed beyond the semen and seminal proteome and metabolome composition and post-breeding uterine inflammatory response (Podico et al., 2024). It is reasonable to suggest that donkey jacks have a distinct semen microbiome compared to horse stallions and horse mares, thus serving as an excellent research model to study the dialog between semen and uterine microbiome around breeding and concerning the breeding outcome. It is possible that equid interspecies breeding results in a unique microbiome dialog; however, this hypothesis has not been investigated. In women, recent studies demonstrated that the microbiome composition deeply affects the uterus during physiological and pathological conditions. A microbiome with high alpha diversity (species richness within samples) is associated with pathological conditions (Khan et al., 2016, Fang et al., 2016, Diaz-Martínez et al., 2021). Women with chronic endometritis and endometrial polyps have a high abundance of Firmicutes; women with endometriosis had uterine samples with a prevalence of *Streptococcaceae* and *Moraxellaceae* (Khan et al., 2016, Fang et al., 2016, Diaz-Martínez et al., 2021). Furthermore, repeated failures during in vitro fertilization seemed

connected to the presence of vaginal dysbiosis (Hyman et al., 2012). A high abundance of bacteria of the genus *Lactobacillus* seems necessary for normal reproductive functions in women (Moreno et al., 2022, Moreno et al., 2016). In veterinary medicine, the functional role of the uterine microbiome and its interplay with the uterus has not been thoroughly investigated. Researchers from Japan have confirmed that a uterine microbiome with a high abundance of *Lactobacillus spp*. is associated with a lower number of inflammatory cells in the uterus of postpartum dairy cows (Wu et al., 2021). In the horse, preliminary data showed that subfertile mares have higher bacterial abundance than fertile mares[25]; there are no studies investigating the association between uterine microbiome composition and embryo recovery.

This study was set forth to characterize the changes in the mare uterine microbiome after interacting with the male seminal microbiome using the inter-species breeding model. We hypothesized that the semen and seminal plasma microbiomes from either species contribute to the changes in the mare uterine microbiome after breeding. Thus, they initiate changes in the richness and diversity of the microbiome of the mare uterus with potential trickle-down effects on the mare fertility.

MATERIALS AND METHODS

The animal work was conducted at the College of Veterinary Medicine, University of Illinois Urbana-Champaign, from August to December 2020; all the experimental procedures were reviewed and approved by the Institutional Animal Care and Use Committee of the University of Illinois at Urbana-Champaign (Protocol #19141). This study is reported here in accordance with ARRIVE guidelines.

Animals, experimental design, and sample collection

The experimental design was described in a concurrent study that investigated the uterine inflammatory response to interspecies breeding (Podico et al., 2024). The animals enrolled in the study included fifteen light-breed mares (12.2 ± 0.2 yrs old) housed on pasture at the Veterinary Teaching Hospital of the University of Illinois, a single fertile horse stallion (11 years old), and a fertile standard donkey jack (6 years old), housed in single stalls at the Veterinary Teaching Hospital. The animals were fed daily a free choice of mixed hay and 2.5 lb of grain (Senior, Purina) topped with 50 mL of multivitamin supplement (Broodmare Plus, mares, and Stallion Plus, stallion and jack; Botupharma USA, Phoeniz, AZ).

Mares were infused with semen and seminal plasma from the donkey and the horse and saline 24 h post-induction of ovulation (i.e., 18-24 h before ovulation) in a crossover design. A uterine fluid sample was collected 24 h before the infusion (0 h), then 6 h, 24 h after the infusion, and then again 8 d after the ovulation; a sample of the donkey and horse semen and seminal plasma used for each infusion was also stored for further microbiome analyses.

The mares were monitored with serial ultrasonographic examinations until a periovulatory follicle was detected (≥35 mm diameter, in the presence of uterine edema and cervix softening). A dose of GnRH agonist (deslorelin acetate 1.8 mg, Sucromate, Dechra Veterinary Products, Overland Park, KS) was given intramuscularly 24 h before infusion (0 h) as described elsewhere[51]. Right at the administration of GnRH agonist, an aliquot of the uterine fluid was used to assess the presence of uterine inflammation and infections using classic aerobic culture and uterine cytology; only mares with the absence of any signs of uterine inflammation and infection (i.e., no inflammatory cells in the cytology smear, no bacterial growth in the aerobic culture) were used at every cycle.

The semen was collected right before the infusion using an artificial vagina Missouri coupled with a filter and a plastic bag off the back of an estrous mare. Each infusion of raw semen consisted of 2 billion total sperm in a volume of 9.5 ± 0.5 mL for the donkey and 13.9 ± 1.1 mL for the horse. To obtain seminal plasma, the raw semen was processed through serial centrifugations (1,000 ×g, 20 min) until no sperm was observed under the microscope; each infusion of seminal plasma consisted of 20 mL of volume. For the control group, 20 mL of saline was used. Each infusion was performed using a sterile standard plastic pipette for equine artificial insemination, with deposition into the mare's uterine body. The uterine culture, cytology, and microbiome analysis samples were collected via low-volume uterine lavage. Briefly, the mare was restrained in a palpation stock, and the perineal area was aseptically prepared. Next, 100 mL of Ringer's Lactate solution were infused in the uterus using two catheter tip syringes connected with a 28 Fr silicon catheter and then distributed through the uterine lumen, recovered with the aid of an ultrasound and oxytocin (20 UI, intravenously). The first 50 mL of the fluid recovered was discarded as it was contained in the catheter used, and one aliquot of the remaining fluid was stored in sterile cryotubes at -80 °C until further processing. At 8 d post-ovulation, the fluid was initially recovered in a dish coupled with a search grid, allowing the search of the embryo; when an embryo was not present in the first 100 mL of fluid passed through the uterus, one to two more liters of Ringer's Lactate solution were used to distend the uterus and then passed through a dish coupled with a filter. The cycle infusion and distension were performed for three rounds. The dish was then examined under a stereomicroscope and the presence or absence of an embryo was recorded and used for the analyses. Mare-cycles infused with seminal plasma and saline had a mock embryo flushing performed after the standard sampling as described for the mare-cycles infused with semen.

DNA purification

Uterine fluid, semen, and seminal plasma were kept at -80 °C until the bacterial DNA extractions. One aliquot of each specimen was thawed at room temperature and then processed following the manufacturer's recommendations (QIAamp DNA microbiome Catalog #51704, Qiagen, Germantown, MD) and manufacturer's handbook (QIAamp DNA microbiome handbook 05/2014). The first step entailed the lysis of the host cells; each aliquot of uterine fluid, semen, or seminal plasma (1 mL) was mixed with 500 mL of buffer AHL, incubated on a shaker (500 rpm) for 30 min at room temperature and then centrifuged at 10,000 ×g for 20 min. The supernatant was discarded, and the pellet containing the bacteria and host cells was mixed with a nuclease (benzonase 2.5 mL) and 190 µL of buffer RDD and then incubated at 37 °C for 30 min at 600 rpm; this step degraded the nucleic acids derived from the host cells. The nucleases were then removed by exposure to the proteinase K contained in the kit (20 mg/mL) during an incubation of 30 min at 56 °C at 600 rpm. Next, the bacterial and nuclear cell walls were chemically and mechanically disrupted using 200 µL of buffer ATL and homogenizing tubes containing beads (10 min at 30 Hz; Tissuelyzer II). Proteins that may denature DNA were eliminated by incubating the supernatant with proteinase K (20 mg/mL) at 56 °C at 600 rpm for 30 min. Next, the bacterial nucleic acids were precipitated by mixing them with APL2 buffer, incubating it at 70 °C for 10 min, and mixing it with 200 µL ethanol. A silica-based membrane column was used to bind the bacterial DNA through a brief centrifugation (6,000 ×g for 1 min); two washing steps were then performed using two separate buffers (AW1 500 µL and AW2 500 µL, respectively) followed by centrifugation (6,000 ×g for 1 min, 20,000 ×g for 1 min, respectively). The bacterial DNA was eluted in 50 µL of buffer AVE after incubation at room temperature for 5 min. The eluted DNA was quantitatively analyzed using a fluorometer (Qubit) following the manufacturer's recommendations (Qubit

dsDNA Quantification Assay Kit High Sensitivity Catalog #Q32851, Invitrogen, Carlsbad, CA) and then stored at -80 °C until the PCR amplification and sequencing.

PCR amplification and DNA sequencing

An aliquot of bacterial DNA was submitted to the Roy J. Carver Biotechnology Center of the University of Illinois at Urbana-Champaign for PCR amplification and sequencing; 333 samples were submitted for sequencing. The full-length 16 S gene was amplified through PCR using the forward primer AGRGTTYGATYMTGGCTCAG and the reverse primer RGYTACCTTGTTACGACTT; both primers had a 5' barcode and Kinnex linker extension. The PCR was run for 35 cycles using the Kapa Hotstart Ready Mix (Kapa Biosystems, Wilmington, MA); the products were measured with SpectraMax Quant AccuClear Nano kit (Molecular Devices LLC, San Jose, CA) and run on an automated parallel capillary electrophoresis (Agilent Fragment analyzer, Santa Clara, CA) for quality control. Samples were pooled according to concentration and cleaned twice with 0.6% volume of magnetic beads. Before library production, the cleaned pool was checked by electrophoresis (Agilent Fragment Analyzer, Santa Clara, CA).

Full-length 16S Kinnex libraries and sequencing were constructed in the PacBio Revio at the Roy J. Carver Biotechnology Center, University of Illinois at Urbana-Champaign. The full-length 16 S amplicons were generated with the barcoded Full-Length Kinnex 16 S primers from PacBio and with the 2x Roche KAPA HiFi Hot Start Ready Mix following the manufacturer protocol from PacBio (Rhoads et al., 2015). The amplicons were concatenated 12-fold with the PacBio Kinnex Concatenation Kit and Kinnex PCR 12-Fold Kit to produce an 18 kb library. The concatenated library was quantitated with Qubit and the run on a Fragment Analyzer (Agilent Fragment Analyzer, Santa Clara, CA) to confirm the presence of DNA fragments of the expected

size, and it was sequenced on the Pacbio Revio instrument with a 30h movie time. Circular Consensus analysis (CCS), de-concatenation, and demultiplexing were done using SMRTlink 13.1 with RQ ≥ 0.999.

Microbiome data analysis

The codes were run using R version 4.4.0. Sequence data were processed using the TADA workflow, which utilizes DADA2 for identifying and quantifying ASVs in samples (Callahan et al., 2018, Ras et al., 2021). Taxonomic assignment was performed using DADA2's implementation of the RDP classifier using the Silva database (release 138.1). Multiple sequence alignment was performed using DECIPHER with a maximum likelihood-based phylogenetic tree (midpoint-rooted) generated using fasttree (Wright, 2015, Price et al., 2010). Microbiome data were filtered to eliminate artifacts (from the host or other sources), contaminants, low-count taxa, non-bacterial taxa, eukaryotic sequences, and organelle 16 S sequences (i.e., mitochondria, chloroplast). Further, the proportion of ASVs that remained unassigned was determined at each rank; ASVs that were not classified up to the phylum level were removed.

The alpha diversity (within sample species diversity) was characterized by using indexes for its richness (Observed ASVs, Chao1, Faith's PD) and its evenness (Shannon, Simpson). Data was checked for normality with the Shapiro-Wilk test, and a non-parametric test (Wilcoxon rank-sum test) was used for each index to assess the microbial diversity within samples of donkey and horse semen and seminal plasma, and uterine fluids. A linear mixed model was also run to include fixed and random (i.e., individual animal) effects, followed by Tukey HSD as a post-hoc test.

Before proceeding with the beta diversity analyses, we filtered the data to exclude taxa with low features and unassigned taxa; then, we selected a prevalence threshold of 0.02%. Next,

we performed a taxonomic agglomeration per rank, setting the family level to start the agglomeration of the unassigned taxa. Beta diversity (between samples diversity) indexes were calculated based on the Bray-Curtis metric and then plotted using a canonical correspondence analysis (CCA). A PERMANOVA analysis was conducted on beta diversity values.

The contribution of the semen and seminal plasma to the mare core uterine microbiome was evaluated by comparing the core microbiome present in at least 10% of the samples in each group. Briefly, the core microbiome of the mare-cycles infused with saline was subtracted from the one from the mare-cycles infused with donkey and horse semen and seminal plasma, and then each group pair was compared to detect the taxa that were in common.

RESULTS

Sequencing data and filtering

After the QIIME2 taxonomy assignments, 26,099 taxa across seven ranks and 333 samples were found. During the initial filtering, five samples were excluded because they had less than 1,000 reads; one sample was obtained from donkey seminal plasma, one from horse semen, two from mare uterine fluid, and one from a control obtained from water; next, we found eight taxa with 0 counts that were removed. The percentage of amplicon sequence variants (ASVs) that remained unassigned was 3.30% at the domain level, 3.10% (723 taxa) at the phylum level, 3.45% at the class level, 3.87% at the order level, 15.02% at the family level, 34.58% at the genus level, and 70.33% at the species level. The non-bacterial taxa included Archaea (116), Eukaryota (15), mitochondria (2455), chloroplast (433), and unclassified taxa (87). After filtering, 22,364 taxa and 328 samples were included in the subsequent analyses and data plots. The range of total counts per taxon was between 0 and 3,032,358, and the range of total counts per sample was between

1,247 and 266,517; only 18 samples had less than 5,000 counts per sample. After the prevalence filtering, we obtained 1,576 taxa among 328 samples, and after the tip agglomeration, we obtained 99 taxa among 328 samples, which were used for the beta diversity analyses.

Microbiome composition

Fourteen phyla were identified across the samples **(Figure 4.1 a)**. The Proteobacteria phylum was the most prevalent; the lowest abundance was observed in the uterine fluid collected after 6 h from the mare-cycles infused with donkey semen (42.8%), the highest abundance was observed in the uterine fluid collected 8 d from the mare-cycles infused with horse seminal plasma (77.8%). Other prevalent phyla included Actinobacteriota, Firmicutes, Bacteroidota, and Verrucomicrobiota. Twenty classes were identified across the samples **(Figure 4.1 b)**. The most prevalent classes were Gammaproteobacteria, Actinobacteria, Alphaproteobacteria, Bacilli, and Bacteroidia. The class of Gammaproteobacteria was the most prevalent one; the lowest abundance was observed in the uterine fluid collected 6 h from the mare-cycles infused with horse seminal plasma (20.1%), the highest abundance was observed in the uterine fluid collected 6 h from the mare-cycles infused with horse semen (68.7%).

The five most abundant orders of bacteria included Pseudomonadales, Burkholderiales, Propionibacteriales, Rhizobiales, and Lactobacillales **(Figure 4.1 c)**. Interestingly, Lactobacillales were more abundant in mare-cycles infused with saline and donkey semen; the lowest abundance was in the uterine fluid collected 8 d from the mare-cycles infused with donkey seminal plasma (2.3%), and the highest abundance was in the uterine fluid collected 6 h from the mare-cycles infused with donkey semen (28.3%).

The five most abundant families of bacteria were *Pseudomonadaceae*, *Propionibacteriaceae*, *Rhizobiaceae*, *Streptococcaceae*, and *Comamonadaceae* **(Figure 4.1 d)**. *Pseudomanadaceae* had the lowest abundance in the uterine fluid collected 6 h from the mare-cycles infused with horse seminal plasma (7.6%), whereas the highest abundance was found in the uterine fluid collected 6 h from the mare-cycles infused with horse semen (38.9%).

Alpha diversity

Alpha diversity measures the observed richness (number of taxa) or evenness (relative abundance of each taxon) of an average sample; alpha diversity is expressed by different indexes, including Observed ASVs, Chao1, Shannon, Simpson, and Faith's PD. The Wilcoxon rank-sum test was used to compare groups for all the indexes; group comparisons were made between semen and seminal plasma from the donkey and the horse, between the time points and the treatment groups. There was no difference in the species richness (Observed ASVs, Chao1) and evenness (Shannon, Simpson, Faith's PD) between the donkey seminal plasma and horse seminal plasma ($P > 0.05$) and between the donkey semen and donkey seminal plasma ($P > 0.05$) **(Figure 4.2 a)**. There was a difference in species richness (Observed ASVs, Chao1) and evenness (Shannon, Simpson, Faith's PD) between the horse semen and horse seminal plasma ($P < 0.05$) **(Figure 4.2 a)**. The Faith's PD showed a difference in the species richness between the horse semen and the donkey semen ($P = 0.048$); Faith's PD is a measure of biodiversity based on phylogeny **(Figure 4.2 a**); the other alpha diversity indexes (Observed ASVs, Chao1, Shannon, Simpson) showed no differences ($P > 0.05$). The groups were defined by the type of uterine infusion was performed during each mare-cycles; seventy-five estrous cycles of fifteen mares were assigned to be infused with donkey semen (DS), donkey seminal plasma (DSP), horse semen (HS), horse seminal plasma

(HSP), or saline (C) in a crossover design. There was no difference in species richness (Observed ASVs, Chao1) and evenness (Shannon, Simpson, Faith's PD) between mare-cycles infused with horse semen and horse seminal plasma ($P > 0.05$), and saline and donkey seminal plasma ($P > 0.05$) **(Figure 4.2 b)**. There was a difference in the species richness (Observed ASVs, Chao1) and evenness (Shannon) between mare-cycles infused with donkey semen and donkey seminal plasma ($P < 0.05$). The species richness (Observed ASVs, Chao1) was different in mare-cycles infused with donkey semen and horse semen compared to the infusion of saline ($P < 0.05$**) (Figure 4.2 b)**. The alpha diversity (Observed ASVs, Chao1, Faith's PD) of mare-cycles infused with donkey seminal plasma was similar to mare-cycles infused with saline ($P > 0.05$**) (Figure 4.2 b)**, whereas the alpha diversity (Shannon, Simpson) of mare-cycles infused with horse seminal plasma was different from mare-cycles infused with saline ($P < 0.05$). The alpha diversity was evaluated over time, before (0 h) and after (6 h, 24 h, 8 d) the uterine infusions. The non-parametric test showed that there was a transitory difference in the species richness (Observed ASVs and Chao1) at 6 h from the infusion ($P < 0.05$), but no changes in the alpha diversity were detected after 6 h from the infusion ($P > 0.05$) **(Figure 4.2 c)**. A Linear Mixed-Effect model was also conducted to ascertain the role of fixed and random effects in the alpha diversity **(Table 4.1)**. No significance was found after Tukey's HSD multiple comparisons**.** The outcome of the embryo flush did not affect any alpha diversity index based on the T-test and the Linear Mixed-Effect Model ($P > 0.05$) **(Figure 4.2 d, table 4.2)**.

Beta diversity

Beta diversity measures the similarity or dissimilarity between sample groups, and it is calculated by measuring the distances between microbiomes of two groups based on microbial features; the current study used the Bray-Curtis dissimilarity metric.

The filtered prevalence of the 20 most abundant families of bacteria was used to build a heatmap. Although a pattern cannot be established, there were similarities in the bacterial abundance in the semen and seminal plasma across species, especially regarding more abundant families of bacteria like Gammaproteobacteria and Actinobacteria **(Figure 4.3 a)**.

The beta diversity plots showed the presence of three distinct clusters: one formed by the samples obtained from semen and seminal plasma from either species, one formed by most of the bacteria present in the uterine fluid of mare-cycles infused with donkey and horse seminal plasma, and one formed by the bacteria present in the uterine fluid of mare-cycles infused with semen of either species or saline **(Figure 4.3 b-c)**. In mare-cycles infused with donkey or horse semen, the bacterial composition of the uterine microbiome differed in mare-cycles that had or did not have an embryo recovered 8 d post-ovulation ($P = 0.02$) **(Figure 4.3 d)**.

The PERMANOVA confirmed that the type of infusion performed during the mare-cycles had an effect on the clustering (R-squared 0.1687; F-value 2.7984; P-value < 0.001) **(Figure 4.4)** and that the beta diversity of the uterine fluid after 8 d post-ovulation and infusion with semen was different based on the embryo outcome in both mare-cycles infused with donkey semen and horse semen (R-squared 0.06929; F-value 2.0102; P-value = 0.02).

Contribution of the semen and seminal plasma to the mare core uterine microbiome

Our analyses showed that the semen and seminal plasma microbiome have several Families of bacteria in common; 24 were found in common between the donkey semen and the uterine microbiome of mare-cycles infused with donkey semen **(Figure 4.5 a)**, nine were found in common between the horse semen and the uterine microbiome of mare-cycles infused with horse semen **(Figure 4.5 b)**, 14 were found in common between the donkey seminal plasma and the uterine microbiome of mare-cycles infused with donkey seminal plasma **(Figure 4.5 c)**, and 15 were found in common between the horse seminal plasma and the uterine microbiome of mare-cycles infused with horse seminal plasma **(Figure 4.5 d)**. The abundance of each shared family was higher in the semen and seminal plasma than in the uterine fluid **(Figure 4.5 a-d)**.

DISCUSSION

Interspecies breeding was used as a research model to study the intricate relationship between the mare uterine and the seminal microbiome. Equids are known to cross species and to yield fertile mating; thus, the breeding of donkey ♂ to horse ♀ and horse ♂ to horse ♀ in a crossover sequential design allowed us to investigate the effects of males on the mare uterine microbiome. The exciting results revealed a temporal contribution of semen and seminal plasma in the mare uterine microbiome, and another fascinating finding was that the mares with a positive embryo recovery had a distinct uterine microbiome from those failing to recover an embryo. This latter finding may suggest that uterine microbiome plays a role in embryonic survival from arrival in the uterus ~6-6.5 d post-ovulation to 8 d post-ovulation. The physiological and clinical significance of this finding remains to be determined with future studies, but this is certainly an interesting foundation concept that needs to be explored further. Traditionally, the presence of

bacteria in the mare uterus was exclusively associated with pathological statuses, i.e., endometritis, metritis, and placentitis (Canisso et al., 2016, Canisso et al., 2020).

Several studies have shown the functional roles of the uterine microbiome in humans (Hyman et al., 2012, Giudice et al., Vitale 2021); however, this field of research is still unexplored in veterinary medicine. The limited number of biological of 75 estrous cycles, technical replicates, and one anatomical site of the present study may have affected the results and urged a cautious interpretation of them. Regardless, we believe that the novel information contained herein will support the development of a new field for veterinary medicine that potentially will originate strategies to modulate the uterine microbiome to decrease diseases and promote embryo survival upon arrival in the uterus.

The uterine microbiome composition identified in the present study mirrors the ones recently described in healthy mares in other studies where Proteobacteria, Actinobacteria, Firmicutes, and Bacteroidota are the most prevalent phyla (Heil et al., 2024, Holyoak et al., 2022). A similar composition was also described in some women studies (Chen et al., 2017, Verstraelen et al., 2016, Wang et al., 2021, Winters et al., 2019); but different than other human studies, where Firmicutes was the most abundant phylum (Baker et al., 2018, Mitchell et al., 2015, Moore et al., 2000). A preliminary study detected significant differences in the uterine microbiome of mares with endometritis (Virendra et al., 2024). It is well documented in women that the uterine microbiome composition is strongly associated with reproductive success. Subfertile women showed a lower abundance of *Lactobacillus* than fertile women in one study (Wee et al., 2018). Shifts in the uterine microbiome composition occur after hormonal changes due to the menstrual or estrous cycle, inflammatory processes, or changes in geographical location. Additionally, the direct connection with the vagina makes the vaginal bacterial translocation a likely source of the

changes in the uterine microbiome in women, and it could also explain the different uterine composition of portions closer or farther from the vagina in women. In mares, the vagina and the uterus are in direct contact; but a transverse fold separates the vagina from the vestibule vagina and the outside; it is possible that the transverse fold functions as a barrier and modulate the microenvironments and minor shifts in the reproductive tract when compared with women. Cattle shows a different microbiome in uterine horns *vs.* in the uterine body and it is likely a reflection of the anatomy of the cow uterus characterized by long uterine horns and a short uterine body (Murga Valderrama et al., 2023). A recent study showed similarities in the samples collected from the uterus and the cervix, suggesting that a discrepant pattern may not be present in the mare (Heil et al., 2018).

The present study used a low-volume uterine lavage to collect the samples for the microbiome analyses; this type of sampling does not allow for differentiation from different sites of the mare uterus; however, it has been recently shown that this technique enables a more sensitive detection of low-abundant taxa compared to the endometrial swab and biopsy (Heil et al., 2018). This is interesting because our design allowed us to explore abundant and less abundant bacteria, probably enhancing our ability to detect differences even with a small sample size of seventy-five estrous cycles.

The present study compared the microbiome of the semen and seminal plasma of two species for the first time; surprisingly, the bacterial richness and diversity were similar between the donkey and the horse in both the seminal plasma and the semen. Albeit only one male of either species was used, it is possible if more animals were used, we could have found more discrepancies. In addition, as we did not study at the species level, the microbiome may be conserved at the genus level but variable at the species level; thus, additional studies should be

carried out addressing these suggestions. In men, it has been demonstrated that the seminal microbiome is the result of the microbial contribution of the different portions of the reproductive tract, i.e., epididymis, vas deferens, urethra, accessory sex glands (Altmäe et al., 2019, Hou et al., 2013); in the domestic species, the literature is scant, and no study has been conducted on the seminal microbiome of donkeys. Noteworthy, it has been demonstrated in rams and men that the seminal microbiome is linked to the gut microbiome and that the gut-testes microbiome axis is also present in domestic animal species (Magill et al., 2023, Wang et al., 2023). Donkey and horse males have similar anatomy and physiology of the reproductive and digestive tracts, which may explain the similar bacterial composition.

Our study revealed that the mare uterine microbiome is significantly affected by the type of fluid the uterus is exposed to; the uterine microbiome of mares infused with seminal plasma and semen from either species had a different microbial profile from the one of the uterus infused with saline; additionally, the microbiome was different from the microbial profile given by the semen and seminal plasma alone. It remains to be determined if the response we observed in the uterine microbiome could have been different using a different method of breeding (natural mating *vs.* artificial insemination) and a larger number of males. Those findings confirm the presence of a "complementary semino-vaginal microbiota" in a domestic species for the first time. This concept was proposed almost a decade ago in human medicine; one of the possible origins of the seminal microbiome was thought to be the transfer from vaginal bacteria to the male reproductive tract during copulation (Koedooder et al., 2019, Mändar et al., 2015). Although further studies challenged this hypothesis, other researchers revealed that the seminal microbiome and the vaginal microbiome of couples share a significant amount of taxa[7].

The origin and composition of the seminal microbiome in the horse are not well studied; a recent work revealed that the four more abundant phyla in horse semen were Firmicutes, Bacteroidetes, Actinobacteria, and Proteobacteria (Quiñones-Pérez et al., 2022); noteworthy, those were also the most abundant phyla found in the uterine fluid of the mares included in the present and other studies (Heil et al., 2018, Heil et al., 2019, Heil et al., 2024, Holyoak et al., 2022, Jones, 2017, Krekeler et al., 2023, Schnobrich et al., 2017, Schnobrich et al., 2018). After interacting with seminal plasma, the uterine microbiome profile differed from the one given by the semen of either species. It is well known that sperm and bacteria elicit an immediate response in the uterine innate immune system; the results of a concurrent study assessing the uterine inflammatory response to intra- and interspecies breeding confirmed that the endometrium responds differently to each male species and type of exposure (semen *vs.* seminal plasma) (Podico et al., 2024). The interaction between male and female reproductive tract microbiome proved to have snowball effects on the endometrium receptivity and immune response to the embryo, either presence or survival, suggesting that the "temporary combined male and female microbiome" plays an essential role in fertilization and embryo development (Koedooder et al., 2019, Robertson et al., 2016, Schoenmakers et al., 2019). In the horse, the impact of the microbiome on uterine functions has not been demonstrated, and the present study certainly provides an early seed to be explored further.

In conclusion, the mare uterine microbiome changes after interacting with the seminal microbiome. Our results showed that a different microbial profile was identified in the presence of an embryo in the uterus; more research is warranted to seek the specific effects of the bacteria involved in those changes. This study may also represent an important step toward developing

alternative treatments to modulate the uterine microbiome and control the ever-growing antimicrobial resistance.

Author contributions

G.P. and I.F.C. contributed to the study design, and collection and processing of the samples. I.F.C. provided the funding. N.V. and C.J.F. performed the bioinformatic analyses. G.P. wrote the first draft of the manuscript. All authors contributed to the manuscript revisions and read and approved the submitted version.

Data availability statement

The datasets generated during the present study are available at the Sequence Read Archive (SRA) of NCBI under the accession number PRJNA1186186.

Competing interest statement

The authors declare no competing interests.

FIGURES AND TABLES

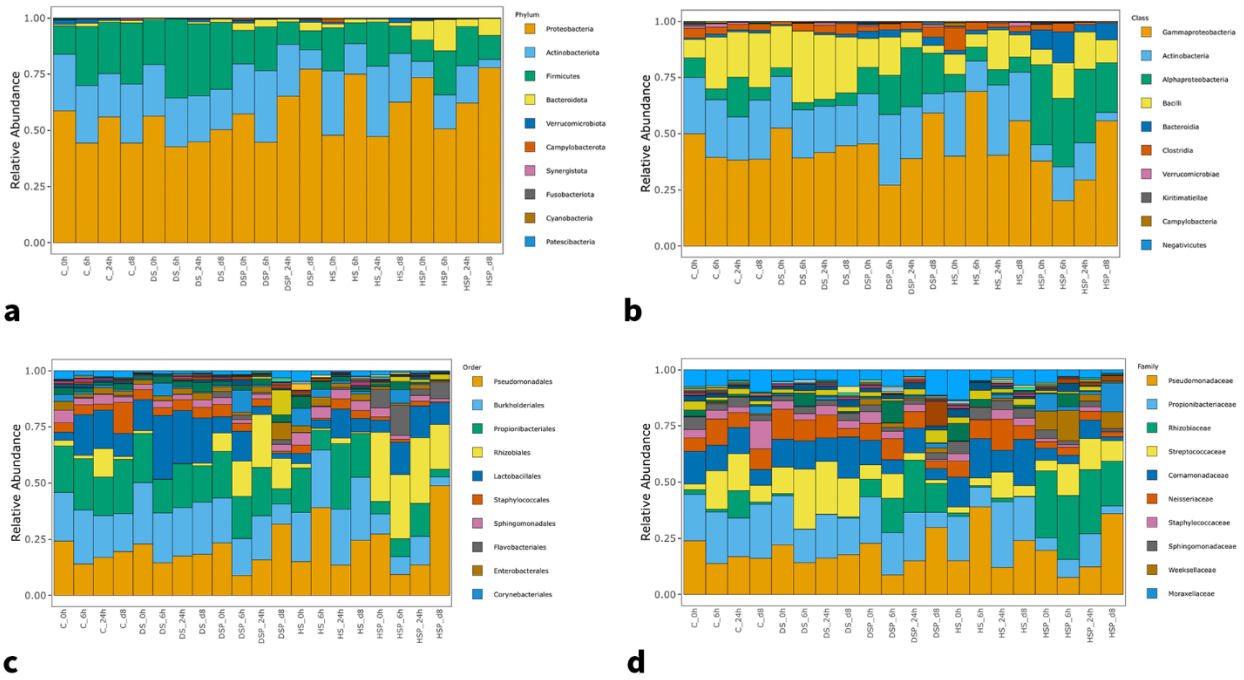

Figure 4.1 a-d. The ten most abundant **(a)** phyla, **(b)** classes, **(c)** orders, and **(d)** families of the uterine microbiome of seventy-five mare-cycles of fifteen mare infused with saline (C), donkey semen (DS), donkey seminal plasma (DSP), horse semen (HS), and horse seminal plasma (HSP). A sample of uterine fluid was collected at the moment of induction of ovulation (0 h), then the uterine infusion was performed ~24 h later. Additional samples of uterine fluids were collected 6 h and 24 h from the infusion and 8 d post-ovulation.

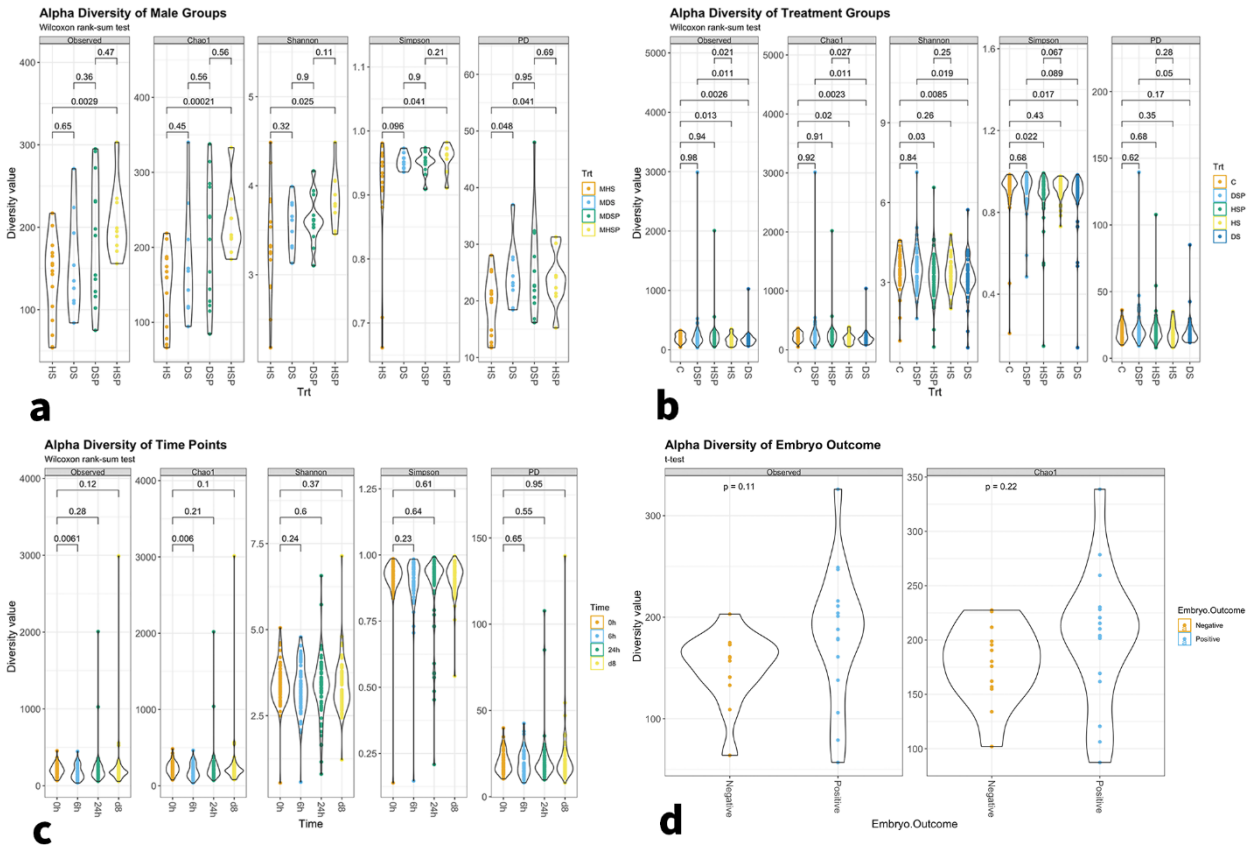

Figure 4.2 a-d. Alpha diversity indexes (Observed ASVs, Chao1, Shannon, Simpson, and Faith's PD) of the microbiome present in **(a)** donkey semen (MDS) and seminal plasma (MDSP) and horse semen (MHS) and seminal plasma (MHSP); **(b)** the uterine fluid of mare-cycles infused with donkey semen (DS), donkey seminal plasma (DSP), horse semen (HS), horse seminal plasma (HSP), and saline (C); **(c)** uterine fluid collected 24 h before the infusion, at the moment of induction of ovulation (0 h), and 6 h and 24 h from the infusion and 8 d post-ovulation; **(d)** the uterine fluid of mare-cycles from whom an embryo was collected after being bred with donkey or horse semen.

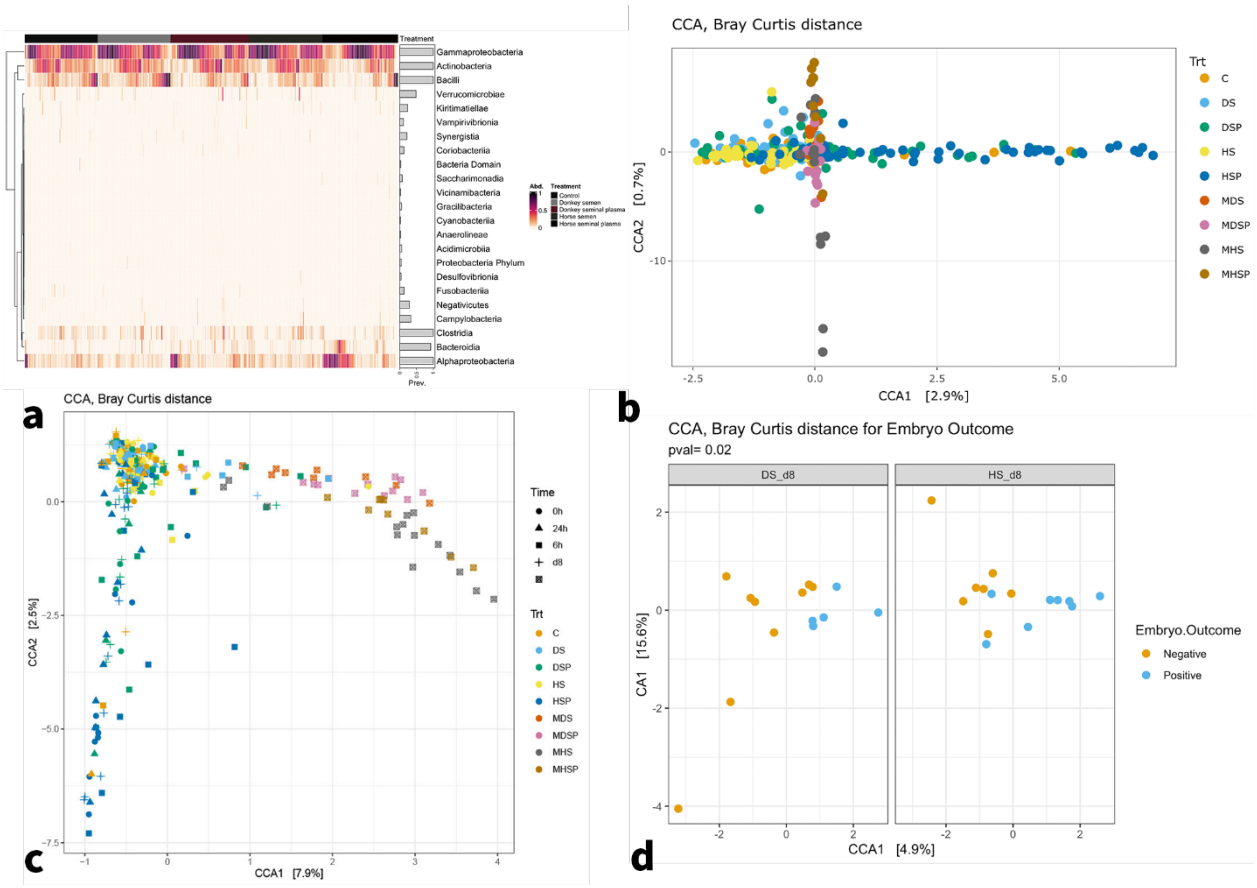

Figure 4.3 a-d. Beta diversity of the uterine microbiome of **(a-c)** seventy-five mare-cycles infused with saline (C), donkey semen (DS), donkey seminal plasma (DSP), horse semen (HS), and horse seminal plasma (HSP); samples of uterine fluid were collected 24 h before (0h) and after 6 h, 24 h from the infusion and 8 d post-ovulation. **(d)** After 8 d post-ovulation, mares infused with DS and HS had an embryo flush performed, and the embryo recovery was included in the analyses (positive, embryo present; negative, embryo not present).

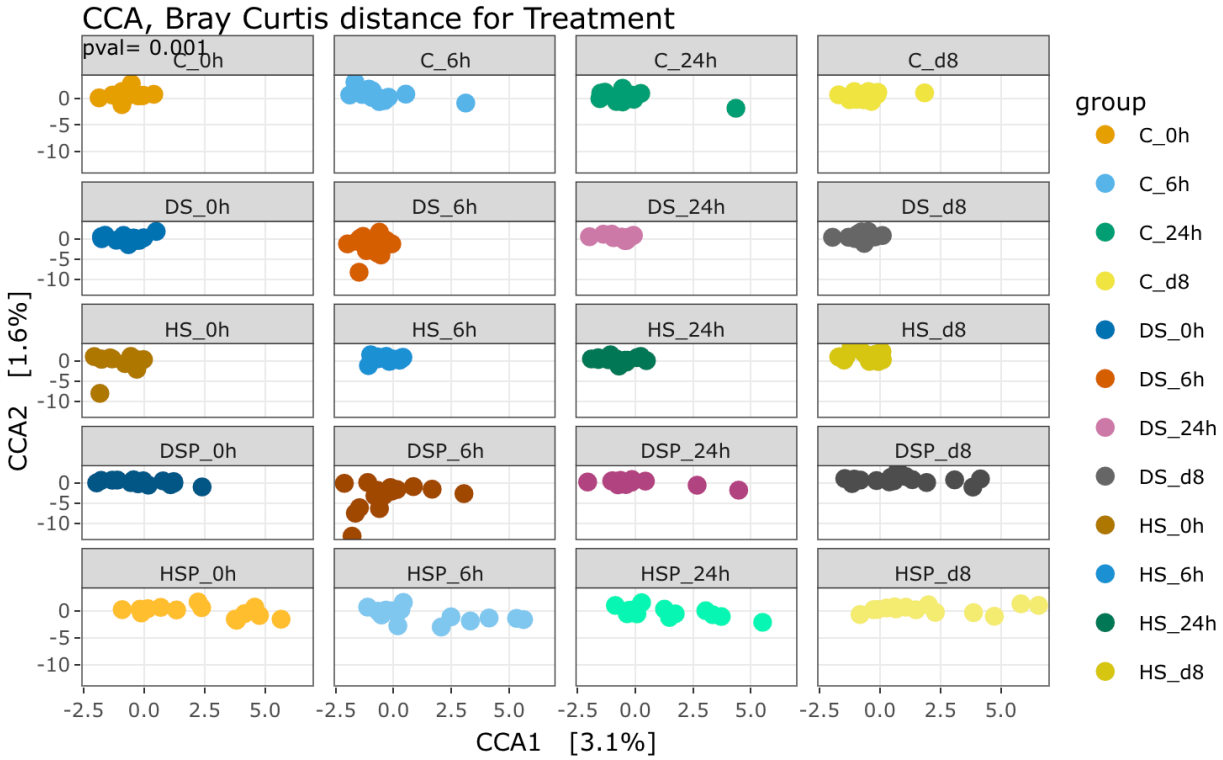

Figure 4.4. The type of uterine infusion (C, saline; DS, donkey semen; HS, horse semen; DSP, donkey seminal plasma; HSP, horse seminal plasma) affected the biodiversity of the mare-cycles uterine microbiome over time (P = 0.001). A sample of uterine fluid was collected at the moment of induction of ovulation (0 h), then the uterine infusion was performed ~24 h later. Additional samples of uterine fluids were collected 6 h and 24 h from the infusion and 8 d post-ovulation.

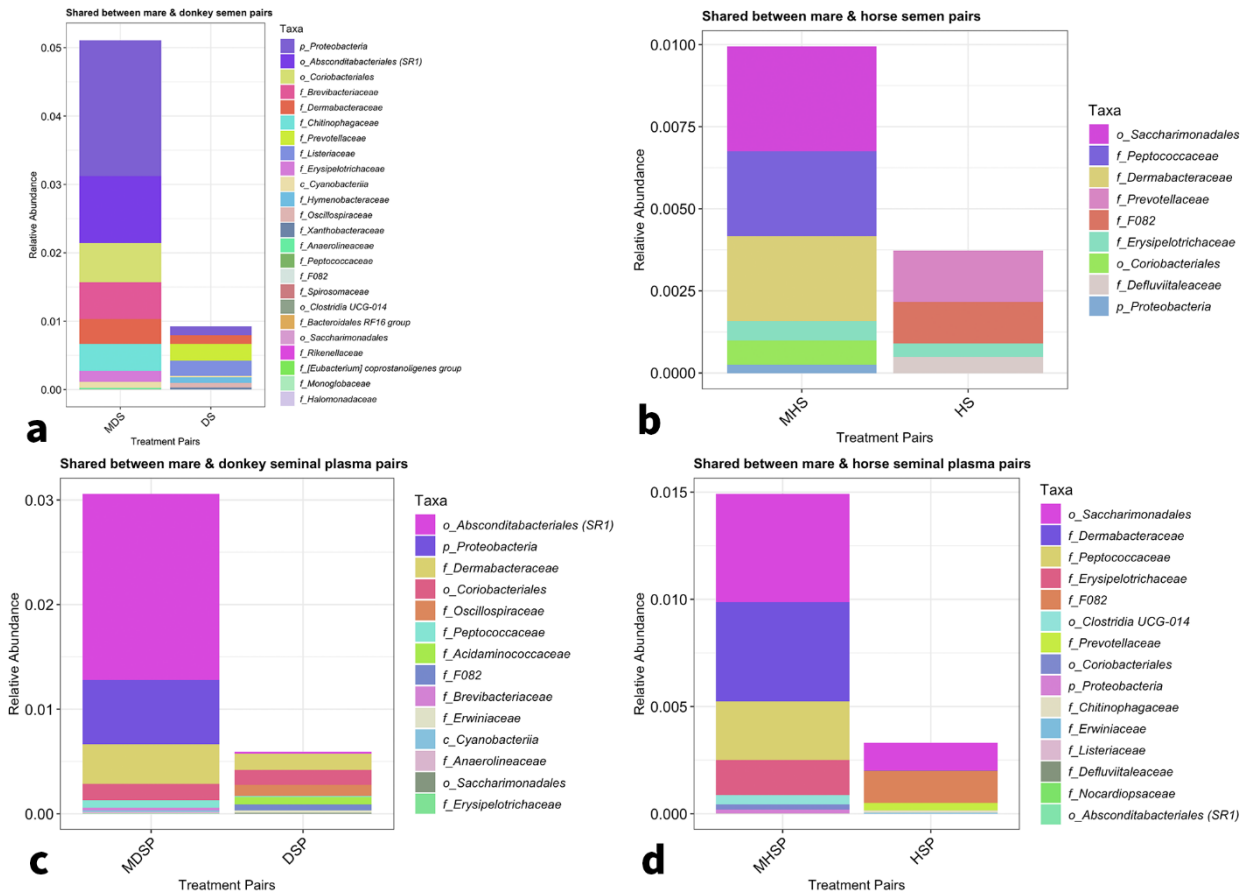

Figure 4.5 a-d. Shared families of bacteria that characterize the mare uterine microbiome (Right column) and the male seminal microbiome (Left column). Seventy-five estrous cycles of fifteen mares infused with donkey semen (MDS), donkey seminal plasma (MDSP), horse semen (MHS), and horse seminal plasma (MHSP).

Table 4.1. Alpha diversity indexes of the uterine fluid of seventy-five estrous cycles of fifteen mares infused with donkey and horse semen and seminal plasma or saline.

Alpha Diversity Index	Factor	Chi-square	DF	P-value
Observed ASVs	Treatment	0.8147	4	0.9364697
	Time	0.1065	3	0.9910436
	Treatment × Time	11.0546	12	0.5242495
Chao1	Treatment	0.7738	4	0.94192
	Time	0.1068	3	0.99102
	Treatment × Time	11.1791	12	0.51363
Shannon	Treatment	3.3149	4	0.50658
	Time	5.1285	3	0.16262
	Treatment × Time	23.4476	12	0.02416
Simpson	Treatment	5.6204	4	0.22935
	Time	6.4226	3	0.09277
	Treatment × Time	14.0279	12	0.29893
Faith's PD	Treatment	1.3849	4	0.8468
	Time	1.5936	3	0.6609
	Treatment × Time	16.7831	12	0.1579

Table 4.2. Linear mixed-effect model of the alpha diversity indexes of the uterine samples classified based on the outcome of the embryo flush (positive *vs.* negative).

Alpha diversity index	Chi-square	DF	P-value
Observed ASVs	2.4312	1	0.1189
Chao1	1.4467	1	0.2291
Shannon	0.011	1	0.9166
Simpson	1.653	1	0.1985
Faith's PD	0.1415	1	0.7068

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CHAPTER 5: Conclusions and future directions

The objectives of this dissertation were to study the horse's uterine inflammatory response, particularly in response to exposure to sperm from another equid and changes in the core uterine microbiome after the challenge with sperm and seminal plasma. Overall, this work demonstrated that donkey seminal plasma exerts an anti-inflammatory action on the mare endometrium and that the uterine microbiome shifts after the interaction with the male seminal microbiome. The studies also showed for the first time in horses that uterine microbiome beta diversity is linked to the presence or absence of an embryo in the uterus.

Donkey seminal plasma had an anti-inflammatory property compared to horse semen and seminal plasma as determined by fewer neutrophils in uterine cytology in Chapter 3. Interestingly, the metabolomic analyses revealed that PGE1, PGE3, and lactoferrin were significantly more abundant in donkey sperm and seminal plasma; the three molecules seemed to have immunomodulation roles in the uterus in other species and the mare. Further studies are necessary to identify and confirm the molecules responsible for the anti-inflammatory action of the donkey seminal plasma. Additionally, Chapter 3 showed that the embryo recovery of mares bred to a donkey, or a horse is similar despite the morphological and karyotypic differences between the two species. Chapter 3 also provided the opportunity to apply the horse embryo morphology grading system to mule embryos as they share the same morphological features.

The study contained in Chapter 4 explored the interactions between the uterine microbiome and the seminal microbiome. Overall, the results revealed that the mare uterine microbiome is significantly affected by the type of fluid (semen *vs.* seminal plasma *vs.* saline) the uterus is exposed to and by the presence of an embryo. Those findings confirm the presence of a "complementary semino-vaginal microbiota" in a domestic species for the first time and suggest

that the “complementary semino-vaginal microbiota” could play a role in essential physiological functions. Further studies should be aimed at determining how to modulate the uterine microbiome to prevent or treat dysbiosis and pathological conditions.