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NEW INSIGHTS INTO THE DIAGNOSIS AND TREATMENT OF ENDOMETRITIS IN THE MARE

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*a Nonna Edda,
tutto questo è grazie a te*

Table of contents

List of abbreviations	7
List of figures	7
List of tables	9
List of supplementary materials	9
CHAPTER 1: Introduction	10
1 Endometritis	11
1.1 Persistent breeding-induced endometritis	11
1.1.1 Mechanical pathway	11
1.1.2 Immunological pathway	12
1.2 Infectious endometritis	13
1.2.1 Microorganisms involved	13
1.2.2 Biofilm infections	15
1.3 Diagnosis	16
1.3.1 Ultrasound	17
1.3.2 Cytology	18
1.3.3 Microbial culture	20
1.3.4 Biopsy	21
1.4 Treatment	23
1.4.1 Correction of anatomical defects	23
1.4.2 Uterine lavage	23
1.4.3 Ecbolics	23
1.4.4 Immunomodulatory agents	24
1.4.5 Antibiotics	24
1.4.6 Alternative non-antibiotic treatments	26

1.4.6.1 N-Acetylcysteine	26
1.4.6.2 Hydrogen peroxide	26
1.4.6.3. Povidone-iodine	26
1.4.6.4 DMSO	26
1.4.6.5 Kerosene	26
1.4.6.6 Tris-EDTA	27
1.4.6.7 Ozone therapy	27
1.5 Reproductive microbiome	29
CHAPTER 2: Thesis aims	33
2 Aims of the thesis	34
CHAPTER 3: The endometrial microbiome of the mare	35
3.1 Introduction	36
3.2 Materials and Methods	37
3.2.1 Animals and experimental design	37
3.2.1.1 Experiment 1	37
3.2.1.2 Experiment 2	37
3.2.2 Bacterial culture	38
3.2.3 16S rRNA gene sequencing	39
3.2.4 Data analysis	40
3.3 Results	40
3.3.1 Experiment 1	40
3.3.1.1 Alpha and Beta diversities	44
3.3.2 Experiment 2	46
3.3.2.1 Bacterial culture	46
3.3.2.2 16S rRNA sequencing	46

3.3.2.2.1 Alpha and Beta diversities	51
3.3.3. Comparison between healthy and diseased mares	52
3.4 Discussion	54
3.5 Conclusions	59
CHAPTER 4: Ozone therapy	60
4.1 Introduction	61
4.2 Safety and effects of a commercial ozone foam preparation on endometrial environment and fertility of subfertile mares	64
4.2.1 Materials and Methods	64
4.2.1.1 Animals and clinical records	64
4.2.1.2 Treatment	64
4.2.1.3 Experimental design	65
4.2.1.4 Cultural examination	66
4.2.1.5 Cytological examination	66
4.2.1.6 Histological examination	66
4.2.1.7 Statistical analysis	67
4.2.2 Results	67
4.2.2.1 Uterine culture	67
4.2.2.2 Cytology	67
4.2.2.3 Histology	68
4.2.2.4 Effects on clinical and reproductive parameters	68
4.2.3 Discussion	69
4.2.4 Conclusions	71
4.3 <i>In vitro</i> effects of different ozone preparations on microorganisms responsible for endometritis in the mare	72
4.3.1 Materials and Methods	72

4.3.1.1 Sample collection	72
4.3.1.2 Isolation of bacterial and yeast strains	72
4.3.1.3 Experiment 1: antimicrobial activity of gaseous ozone	73
4.3.1.4 Experiment 2: antimicrobial activity of the ozonated water and oil	74
4.3.1.5 Statistical analysis	75
4.3.2 Results	75
4.3.2.1 Experiment 1	75
4.3.2.2 Experiment 2	76
4.3.3 Discussion	77
4.3.4 Conclusions	80
CHAPTER 5: Influence of IUF detection on pregnancy rate and early embryonic loss In a commercial ET program	82
5.1 Introduction	83
5.2 Materials and Methods	83
5.2.1 Donor mares and embryo flush	83
5.2.2 Recipient mares and ET	84
5.2.3 Statistical analysis	85
5.3 Results	86
5.4 Discussion	87
5.5 Conclusions	90
CHAPTER 6: General conclusions	91
References	94
Supplementary Materials	115
Research Products	118

LIST OF ABBREVIATIONS

Polymorphonuclear neutrophils (PMN)
Persistent breeding-induced endometritis (PBIE)
Prostaglandin F₂alpha (PGF₂α)
Interleukin (IL)
Extracellular polymeric substances (EPS)
Intrauterine fluid (IUF)
High-power fields (hpf)
Platelet-rich plasma (PRP)
N-acetylcysteine (NAC)
Dimethyl sulfoxide (DMSO)
Ribosomal RNA (rRNA)
Veterinary Teaching Hospital of the University of Turin (OVU)
Matrix assisted laser desorption ionization time of flight/ mass spectrometry (MALDI-TOF/MS)
Amplicon Sequence Variants (ASVs)
Antimicrobial resistance (AMR)
Tryptic soy agar (TSA)
Sabouraud dextrose agar (SDA)
Colony forming units /ml (CFU/ml)
Columbia agar with 5% sheep blood (CA-SB)
Minimum inhibitory concentrations (MIC)
Minimum bactericidal concentration (MBC)
Minimum fungicidal concentration (MFC)
Embryo transfer (ET)
Pregnancy rate (PR)
Early embryonic loss (EEL)

LIST OF FIGURES

<u>Figure 1.</u> The uterine response to introduction of sperm in resistant and susceptible mares	16
<u>Figure 2.</u> Taxonomy rarefaction curves for the endometrial samples included in Experiment 1.	41
<u>Figure 3.</u> Total number of phyla detected in estrus and diestrus.	41
<u>Figure 4.</u> Relative abundance of the 10 most abundant phyla detected in the 11 mares	42

during estrus and diestrus.

<u>Figure 5.</u> Relative abundance of the 10 most abundant phyla grouped according to cycle phase.	42
<u>Figure 6.</u> Total number of genera detected in estrus and diestrus.	43
<u>Figure 7.</u> Relative abundance of the 30 most abundant genera detected in the 11 mares during estrus and diestrus.	43
<u>Figure 8.</u> Relative abundance of the 30 most abundant genera grouped according to cycle phase.	44
<u>Figure 9.</u> Variation in endometrial microbial diversity based on the cycle phase assessed by observed richness, Shannon, Simpson, and Chao 1.	45
<u>Figure 10.</u> Two dimensions plot by Bray-Curtis based on cycle phase.	45
<u>Figure 11.</u> Taxonomy rarefaction curves for the endometrial samples included Experiment 2.	47
<u>Figure 12.</u> Total number of phyla detected before and after treatment.	47
<u>Figure 13.</u> Total number of genera detected before and after treatment.	48
<u>Figure 14.</u> Relative abundance of the 10 most abundant phyla detected before and after treatment grouped according to time point.	48
<u>Figure 15.</u> Relative abundance of the 10 most abundant phyla detected before and after treatment in each mare.	49
<u>Figure 16.</u> Relative abundance of the 40 most abundant genera detected before and after treatment grouped according to status	50
<u>Figure 17.</u> Relative abundance of the 40 most abundant genera detected before and after treatment in each mare.	50
<u>Figure 18.</u> Variation in endometrial microbial diversity based on time point assessed by observed richness, Shannon, Simpson, and Chao 1.	51
<u>Figure 19.</u> Two dimensions plot by Bray-Curtis based on time point.	52
<u>Figure 20.</u> Variation in endometrial microbial diversity based on status assessed by observed richness, Shannon and Simpson.	53
<u>Figure 21.</u> Two dimensions plot by Bray-Curtis based on status.	53
<u>Figure 22.</u> Number of PMN/hpf observed with uterine cytology in mares treated with ozone and in the control group at different time points.	68
<u>Figure 23.</u> Ozone generator (Vet Bo3x, Eco3 S.r.l, Brandizzo, Italy) coupled to a glass hermetic box containing a tested sample.	74
<u>Figure 24.</u> An example, Petri plates of <i>Pseudomonas aeruginosa</i> after 24 h of incubation. A: control untreated plate. B: plate exposed to gaseous ozone at a concentration of 40 µg/ml for 5 min.	75

LIST OF TABLES

<u>Table 1.</u> Diagnostic techniques for endometritis.	17
<u>Table 2.</u> Histopathological classification of equine endometrium according to Kenney and Doig classification.	22
<u>Table 3.</u> Results of bacterial culture before (T0) and after (T1) antibiotic treatment	46
<u>Table 4.</u> Mean of CFU/ml $\pm$ SEM of each species after exposure to oxygen-ozone gas mixture, 100% O ₂ or left untreated.	76
<u>Table 5.</u> MIC and MCB/MFC values (% v/v), presented as modal values and range of ozonated oil, ozonated water and controls (non-ozonated oil and water) on antibiotic-resistant bacteria and <i>C. albicans</i> .	77
<u>Table 6.</u> Different factors affecting pregnancy rate and early embryonic loss.	87

LIST OF SUPPLEMENTARY MATERIALS

<u>S1.</u> Relative abundances at phylum level of Experiment 1.	115
<u>S2.</u> Relative abundances of the 40 most abundant genera of Experiment 1.	116
<u>S3.</u> Relative abundances at phylum level of Experiment 2.	116
<u>S4.</u> Relative abundances of the 40 most abundant genera in Experiment 2.	117

CHAPTER 1

Introduction

1. ENDOMETRITIS

Equine endometritis is considered one of the major causes of sub-fertility in the broodmare [1,2], and it was ranked by veterinarians of North America as the third most common medical problem in adult horses [3]. It is defined as the local inflammation of the lumen of the uterus and can be the result of various causes. An acute inflammatory reaction of the endometrium, characterized by the influx of polymorphonuclear neutrophils (PMN), occurs physiologically after mating or insemination [4]. While the inflammation should resolve within 48 hours in normal mares, it persists in mares susceptible to endometritis [5]. This condition is referred to as persistent breeding-induced endometritis (PBIE) [4]. In contrast to PBIE, which is caused by sperm and other components of the ejaculate, infectious endometritis is characterized by the presence of microorganisms in the uterus [6]. Therefore, endometritis can be divided into both infectious and non-infectious causes, and yet in clinical practice, they often occur in association with another [4].

1.1 Persistent breeding-induced endometritis

In the past, endometritis was believed to be caused solely by a bacterial or fungal infection. However, this opinion was challenged when studies found that saline as well as sperm or bacteria were able to induce a neutrophil response in the uterus [4,7,8]. Nowadays, it is known that a transient inflammation is a physiological response of normal healthy mares to natural mating or insemination [7,9]. It is a local inflammatory response necessary to remove excess spermatozoa, bacteria and debris introduced into the uterus that aims to restore an environment suitable to receive the embryo, that descends into the uterus few days after the insemination [10]. This inflammation should resolve within 48 hours of breeding [2,9]. Two response pathways are mainly involved in the resolution of inflammation: the mechanical pathway and the immunological pathway. These pathways work together to prevent the establishment of PBIE [2]. If the physiological post-breeding endometritis is not solved within 48 hours, the mare is deemed susceptible to PBIE, which predisposes her to secondary uterine infections [2,11].

1.1.1 Mechanical pathway

Mechanical clearance plays a key role in the uterine response to the contamination, and deficits in myometrial contractility could be responsible for delayed uterine clearance. The myometrial contractions are stimulated by oxytocin that is released following the stimulation of cervix and vagina after mating or insemination [2,12]. Furthermore, bacterial or seminal contamination after mating trigger a massive invasion of PMNs into the uterine lumen. The PMNs phagocytize the bacteria/spermatozoa and the inflammation results in the release of prostaglandin F₂α (PGF₂α) that causes myometrial contractions. The contractions are essential for uterine clearance via the cervix into the vagina. Mares susceptible to persistent endometritis have impaired myometrial contractions resulting in insufficient uterine clearance [13]. Troedsson et al. performed a bacterial challenge on

mares resistant and susceptible to endometritis. They observed that resistant mares have a greater myometrial activity measured as frequency, duration and intensity of the uterine activity. Differences were most marked between 10 and 20 h after the intrauterine inoculation of bacteria. Therefore, they concluded that myometrial activity is an important part of the uterine defense mechanism in mares. In addition, an intrinsic contractile defect of the myometrium has been observed in mares with delayed uterine clearance, which may contribute to reduced uterine contractility following breeding [14]. Nitric oxide may also play a role in PBIE in mares since it exerts an inhibitory effect on uterine contractility and susceptible mares were shown to have higher amounts of nitric oxide in uterine secretions [15,16].

After ovulation, moreover, the cervix closes and intrauterine fluid is drained via the uterine lymphatics [2,17]. However, anatomical or degenerative defects could interfere with mechanical uterine drainage. These defects include alteration of the internal and external reproductive organs such as poor perineal conformation, vaginal stretching, an incompetent cervix, a pendulous uterus, or degenerative defects of the myometrium and/or endometrium. Injuries to the cervix can allow pathogens to enter. In addition, fibrosis of the cervix as a result of a traumatic birth or the presence of a tight fibrotic cervix, typical of old maiden mares, can lead to fluid accumulation due to impaired cervical drainage [10,18]. These defects are associated with increasing age and number of matings/foalings and increase the susceptibility of the mare to PBIE [2].

1.1.2 Immunological pathway

After the entrance of semen into the uterus, the first line of defence is represented by the innate immune response that is non-specific, rapid and transient and involves cells such as neutrophils, macrophages and dendritic cells [2,4]. On the contrary, the antibody-mediated response is less involved [4]. This innate immune response enables spermatozoa to challenge the immune system without developing anti-sperm antibodies [2,4]. Spermatozoa and bacteria have both allogenic, pathogen-recognised receptors that activate the innate immune pathway that is mediated by toll-like receptors on epithelial cells [2]. During the first 24 h after the breeding, there is a rapid increase in the pro-inflammatory cytokines, interleukin (IL)-1 β , IL-8 and interferon- γ , as well as activation of PMNs [19,20] that release lytic enzymes and phagocyte bacteria. A balance between pro- and anti-inflammatory factors is observed in resistant mares and is necessary to interrupt the inflammatory response to mating. In susceptible mares, however, endometrial expression of pro-inflammatory cytokines is inadequate and prolonged, the abundance of mRNA expression for the anti-inflammatory cytokines is reduced but that of pro-inflammatory cytokines increased [19–21]. This prolonged uterine inflammatory response culminates with excessive accumulation PMNs and intrauterine fluid accumulation in the uterus up to 96 h and beyond after breeding, impairing embryonic survival and the establishment of pregnancy [4].

1.2 Infectious endometritis

If PBIE is not managed, it determines an immense inflammation and may progress to infectious endometritis or the development of endometrial fibrosis [2] (Fig.1). It is likely that at the origin of both breeding-induced and infectious endometritis there are common failures in the physical mechanisms of uterine clearance [10]. Additionally, due to impaired innate immune response activation, microbes introduced into the uterus at the time of breeding are not eliminated efficiently, leading to potential infection [4]. Furthermore, uterine fluid accumulations provide an appropriate environment for the growth of bacteria [51]. Infection of the endometrium, however, can also occur in mares that have never been bred, but where anatomical defects together with impaired uterine clearance allow bacteria to enter the uterus. Infectious endometritis affects 25%–60% of barren [18]. It can determine conception failure, early pregnancy loss, abortion due to bacterial placentitis, the birth of septic foals and postpartum metritis [19].

1.2.1 Microorganisms involved

Many retrospective studies have been published describing the frequency of isolation of bacteria and fungi from uterine samples [22–30]. Especially in the last year two large studies were published: Mouncey et al. [29] described the results of 18,996 endometrial-swab samples performed in UK, while Kohne et al. [22] of 28,887 samples from Germany. The percentage of positive culture ranged between 25.9% and 89%. The most commonly isolated bacterium was *Streptococcus equi zooepidemicus*. However, *S. zooepidemicus* is one of several β -haemolytic Streptococci associated with infectious endometritis. Other species isolated from the endometrium included *S. dysgalactiae* subsp. *equisimilis* and *S. equi* subsp. *equi* [2,31]. These isolates belong to a genetically distinct subpopulation, that differ from respiratory or abortive strains [2,32,33]. Petersen et al. [34] demonstrated using fluorescence in situ hybridization that *Streptococcus zooepidemicus* resides deep in the endometrium of mares with chronic infections. This may account for the absence of clinical signs and the challenging diagnosis of subclinical endometritis in mares. Nielsen [35] described that an endometrial biopsy, which collects samples deep in the endometrium, is a superior diagnostic test for *S. zooepidemicus* compared to an endometrial swab, which only collects samples from the superficial layer of the endometrium [31]. Furthermore, it was observed that some strains of *S. zooepidemicus* can establish a subclinical dormant persister infection in the endometrium of the mare that tolerate 32-times of the minimal inhibitory concentration of penicillin compared to normal growing cells [34]. Persister cells had not acquired penicillin resistance but seemed to tolerate the antimicrobial due to dormancy. Therefore, endometritis can origin from a reservoir of dormant bacteria residing within the endometrium, and not only as an ascending infection [34].

The other most common isolated bacterium from uterine samples is *Escherichia coli*. Unlike *Streptococcus* spp. no uterine-specific strains of *E. coli* have been reported in the horse suggesting that it could be a contaminant [2]. It is however believed to cause endometritis in the mare [36] but the interpretation of a positive culture for *E. coli* is difficult. Some laboratories and practitioners assess mild growth of *E. coli* in combination with a lack of clinical signs for endometritis as indicative of contamination of the uterine swab, while others treat every mare with a culture result positive for *E. coli*, irrespective of the number of colonies or presence of mixed flora, with antibiotics [22]. Generally, a culture positive for *E. coli* is considered pathogenic only if growth is severe or shows hemolytic properties [22]. Furthermore, it is suggested to include cytological examination and clinical symptoms as important diagnostic tools for assessing the pathogenicity of cultured *E. coli* [22].

Pseudomonas aeruginosa is another pathogen that is able to produce biofilm and is often cultured from the uterus of mares with chronic endometritis [37,38]. Other commonly isolated bacteria are *Klebsiella Pneumoniae* and *Staphylococcus aureus* [4]. Furthermore, there are increasing reports associating *Corynebacterium uterequi* to endometritis and pregnancy complications, suggesting a potential role of *Corynebacterium* strains in the disease. Moreover, the ability to form biofilms suggests the potential for chronic infection [39,40].

Interestingly, even bacteria that are considered to be non-pathogens (*Micrococcus*, *Bacillus* and alpha-hemolytic *Streptococcus*) determined a reduction of pregnancy rate [28]. *Micrococcus* and *Bacillus* are considered skin commensals and were more commonly isolated from barren mares. However, the authors hypothesized that these bacteria were not the cause of the lower pregnancy rate but were associated with conditions that would decrease fertility. Barren and older foaling mares may have breaches in the physical barriers that protect against contamination (the vulvar seal, the vestibulovaginal fold and the cervix) making them more susceptible to infection by skin commensals and organisms that reside in the clitoris and vestibule [28].

Fungal endometritis is rarer, accounting for 1–5% of all cases of endometritis. According to Beltair et al., the most common causes of fungal endometritis are yeast (69%, primarily *Candida spp.*), mould with septated hyphae (26%, primarily *Aspergillus spp.* [18%]) and mould with non septated hyphae (5%, primarily *Mucor spp.* [4%]) [41,42].

Fungal endometritis in the mare is typically thought to occur as an opportunistic infection, with the primary reservoir being the caudal reproductive tract, including the vagina and clitoral fossa [42]. It relies on significant compromise of the normal vaginal and/or uterine defence mechanisms, or a generalized immuno-suppression. This is probably the reason why fungal endometritis is commonly associated with a history of uncorrected pneumovagina or recurrent persistent post-breeding endometritis [43]. Risk factors for infection include poor perineal conformation, compromised uterine

defenses, retained fetal membranes, abortion, trauma following dystocia and endocrine/immune disorders resulting in immunosuppression. Furthermore, another important risk factor is the repeated use of intrauterine antibiotics, probably due to a combination of a disrupted vaginal flora caused by the antimicrobial treatment and potential iatrogenic introduction of pathogens into the uterus [42].

1.2.2 Biofilm infections

In equine reproduction, many Gram-negative bacterial pathogens (e.g. *E. coli*, *P. aeruginosa* and *Klebsiella pneumoniae*) isolated from the equine uterus showed the ability to produce biofilm [36–38]. Bacterial biofilms are usually defined as aggregates of bacteria encased in extracellular polymeric substances (EPS) produced by the bacteria. They cause a significant problem in treating bacterial infections and are one of the main reasons for the persistence of infections [44]. Bacterial endometritis that is refractory to traditional antimicrobial treatment is a significant challenge in the equine breeding industry [18,38]. Bacterial pathogens commonly utilize biofilm production as a persistence strategy for survival [37]. Biofilms are resistant to clearance by the host immune response and to treatment with antimicrobial drugs. The immune system frequently fails to detect infections related to biofilm because of the protective matrix of EPS surrounding the bacterial cells [38,45]. The EPS matrix limits the ability of antibodies to target bacteria within the biofilm and interferes with the local function and mobility of white blood cells. Furthermore, it reduces the effect of antibiotics by neutralizing antimicrobial agents or limiting diffusion, protecting bacteria by forming a barrier that reduces the concentration of antibiotics reaching the bacterial colonies within the biofilm's core [44]. This creates a nidus of infection. In addition, the biofilm microenvironment decreases bacterial metabolism, resulting in a reduced replication rate of bacteria [38]. The majority of antibiotics primarily target bacteria that are undergoing rapid growth and division. Consequently, the decrease in metabolic activity that occurs determines an increase in the ability of bacteria to tolerate the effects of the antibiotic [38,44]. Biofilm infections are usually difficult to diagnose and treat and antibiotics should be associated with specific molecules that act as biofilm disruptors (DMSO, Tris EDTA, N-acetylcysteine) [37,38].

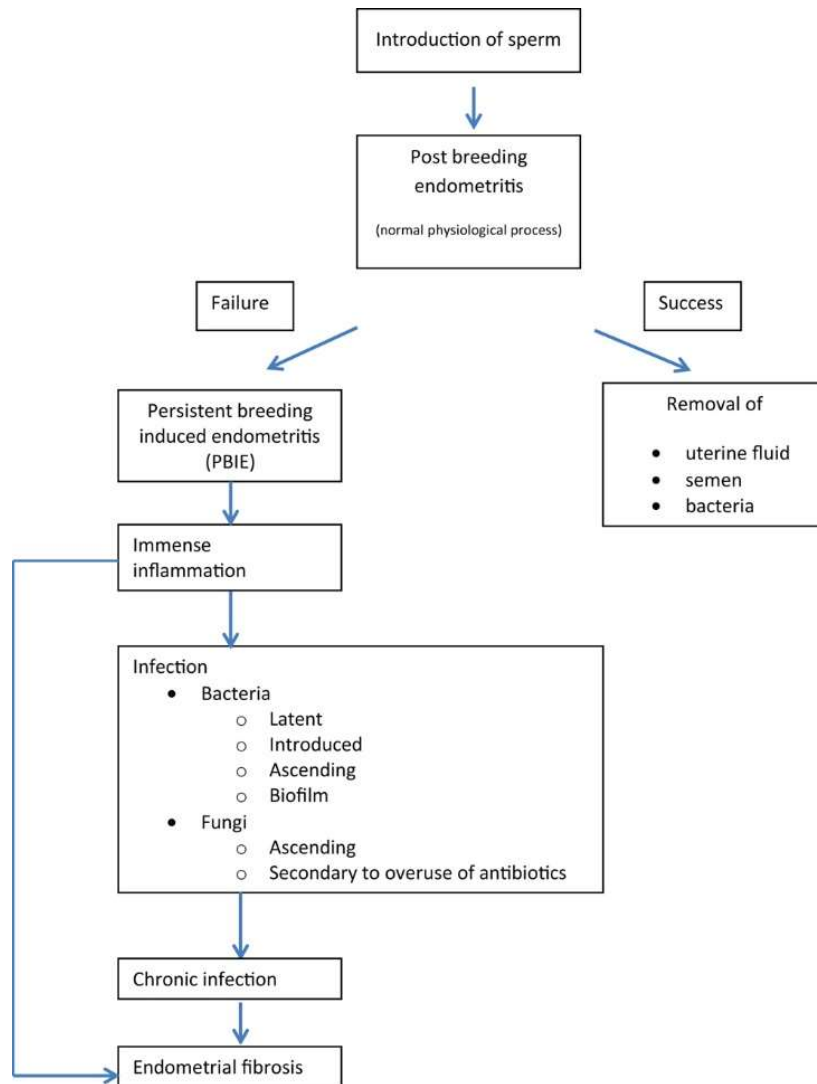


Figure 1: The uterine response to introduction of sperm in resistant and susceptible mares. From Morris et al. [2]

1.3 Diagnosis

The diagnosis of endometritis, due to its multifactorial nature [46], requires a complete clinical evaluation, with the use of ancillary diagnostic aids, coupled to a complete reproductive anamnesis (Table 1) [4,10]. The most common tools for diagnosis are ultrasonography, cytology, culture and histopathological examination [4].

The clinical signs of endometritis may be hidden, but intrauterine fluid accumulation is the hallmark sign [18]. Furthermore, vaginal discharge, short inter-estrus intervals, shortened luteal phase and reduced fertility can be detected [10].

Technique	Advantages and applications	Limitations
Ultrasound	Used as a screening tool to detect the presence, amount, and appearance of IUF, which is a common clinical sign of endometritis.	Not all the mares with endometritis have IUF accumulation. Usually, other techniques are required to confirm the diagnosis.
Double-guarded swab	Practical and easy to use in the field. It can be used for cytology and culture, although it more commonly used for culture. Low risk of contamination.	Sample only a small area of endometrium, focal infections can be missed. If used for cytology, smears are of lower quality compared to cytobrush.
Double-guarded cytobrush	Practical and easy to use in the field. It can be used for cytology and culture, although it is more commonly used for cytology. Low risk of contamination.	Sample only a small area of endometrium, focal infections can be missed. If used for cytology, smears are of higher quality compared to swab.
Low-volume lavage	It can be used for cytology and culture. All the surface of the uterus can be sampled. It is considered the most sensitive test for the diagnosis of bacterial endometritis.	More time-consuming. More difficult to perform in the field and requires one assistant. More risk of contamination from vaginal microflora if a double-guarded system is not used.
Biopsy	Considered the gold standard to diagnose endometritis. Mainly used for histological examination, but it can be used also for cytology and bacterial culture.	More invasive than cytobrush or swab to obtain samples for cytology or culture. Requires lab personnel and an experienced pathologist to perform histological evaluation.

Table 1: Diagnostic techniques for endometritis. Adapted from Canisso et al. [4].

1.3.1 Ultrasound

Detection of intraluminal uterine fluid accumulations in mares by rectal palpation of ventral dilations of the uterus was first reported by Knudsen in 1964 [47,48]. However, since the advent of ultrasound, the detection of intrauterine fluid (IUF) has been reported both in estrus and diestrus, and the awareness of the frequency of this abnormality has increased [49,50] and nowadays, ultrasonography is used as a screening tool to detect the presence, amount, and appearance of IUF, which can be suggestive of endometritis [4]. The relevance of the uterine fluid accumulation can be evaluated by examining the depth, the echo-texture and persistence of the fluid [10,51].

A small quantity of anechoic IUF is a common finding within the uterine lumen of healthy mares during estrus [26] and generally is considered not to affect pregnancy rates. Detection of small quantities of IUF during the follicular phase does not necessarily indicate inflammation, but it can also be transudate from the vessels, associated with the same mechanism that causes normal oestrous oedema; in fact, estrogens increase the permeability of the endothelium of uterine vessels, endometrial secretion and edema during estrus [47–50]. Furthermore, in many cases, the IUF detected during oestrus is sterile and does not contain inflammatory cells [48,50].

However, the presence of > 2 cm of fluid is a good indicator of a mare susceptible to endometritis [52,53] and has been associated with decreased pregnancy rates [27–29]. Fluid can accumulate because of excess production by endometrial glands, transudation from vessels, or because the clearance of fluid is impaired [54]. It has been shown that mares that accumulate fluid during estrus have more glands with a larger diameter and wider lumens than mares without IUF [55,56]. In a study, intra-uterine fluid during estrus was associated with increased numbers of neutrophils, since mares with IUF on day 2 or 3 of estrus were 1.4 times more likely to have >5 neutrophils/400· field on cytological specimens than those with no or mild inflammation [52,57]. The detection of uterine fluid was seen more frequently when β -haemolytic *Streptococcus*, *Klebsiella pneumoniae*, *Enterobacter cloacae*, or yeast were isolated compared to *Escherichia coli*, *Staphylococcus aureus* or *Pseudomonas* spp. [10,57].

Furthermore, even small accumulations of anechoic IUF during the post-ovulation period could possibly adversely affect fertility [26], and it has been shown that free fluid within the uterine lumen during diestrus should be considered abnormal because it indicates an inflammatory process and/or impaired uterine clearance and is associated with endometritis [58,59]. Adams et al. observed that IUF during diestrus was associated with the presence of an inflammatory process as indicated by a high biopsy score, reduced progesterone concentrations, and a shorter interovulatory interval. Mares with fluid collections at diestrus had also a lower pregnancy rate at Day 11 and a higher embryonic loss rate by Day 20 [59].

A high echogenicity in the uterine fluid is also associated with the presence of neutrophils and debris, thus indicative of inflammatory components [10,51]. Another aspect that can be evaluated through ultrasonography is oedema patterns. Some mares may exhibit abnormal edema patterns, such as excessive edema pre- or post-mating, or may exhibit short, thick, hyperechoic lines within the uterus, signifying either air or exudate [52]. Hyperedema has been considered a marker of uterine pathology and can be associated with fluid accumulation due to drainage deficiency [10].

1.3.2 Cytology

Endometrial cytology is a rapid and inexpensive technique to assess the presence of endometrial inflammation and is widely used a diagnostic method for endometritis [28,60]. The cytological smear

can be stained using different methods, but commercially available quick stains are nowadays usually used, e.g. Hemacolor, Diff-Quik, or equivalent [61]. The stained slide obtained is evaluated at 400× magnification over multiple high-power fields (hpf) and examined for uterine epithelial cells, white blood cells, debris, red blood cells, bacteria, yeast or other fungi and spermatozoa.

Neutrophils are the predominant white blood cells identified on uterine cytological slides and are present in the uterine lumen following breeding, uterine lavage, during the post-partum period or in cases of endometritis [51,60,62]. Other white blood cells such as macrophages, lymphocytes or eosinophils are more rarely found on equine uterine cytology preparations [60].

To assess the degree of inflammation two methods have been mainly used: the first one is to count the number of PMN/hpf, while the second one is to count the ratio of PMN/endometrial cells. The average number of PMN/hpf is determined after evaluating at least 10 fields in multiple areas of the slide. The slides are generally graded as not inflammatory (0–2 neutrophils/field), moderate inflammation (2–5 neutrophils/field), severe inflammation (>5 neutrophils/field) [28]. If the ratio PMN/endometrial cells is used, the cut off to diagnose endometritis can range between 0,5 to 5% according to different studies [51,63–65]. According to Kozdrowski et al., a ratio of 2% indicates inflammation in swab samples, but only 0.5% in cytobrush samples because cytobrushes collect more material than swabs. For cytology from a bioptic sample, Kozdrowski et al. suggested 1% of PMNs as a threshold for inflammation [61,65].

However, cytology has a relatively high rate of false negative results, and it does not provide information about the cause of the inflammation. Furthermore, mares may also yield positive uterine cytology with negative uterine culture, and vice versa. Therefore, it is recommended that both culture and cytology samples be collected to detect more accurately the presence or absence of endometritis, since the number of mares identified as positive for endometritis is significantly higher than with either technique alone [10,35,64,66,67]. It has been shown that cytology can detect twice as many mares with endometritis than endometrial culture [28,66]. Furthermore, the occurrence of PMNs in smears was significantly correlated with the occurrence of cultures of β -hemolytic streptococci [68]. Various interpretations have been made to explain the absence of a correlation between the two techniques. In particular, to justify negative cultures, it has been hypothesized that uterine swabs may miss focal infections, the presence of antimicrobial preparations in the uterus, deep-seated infection, the presence of biofilm, or non-infectious endometritis induced by an unresolved inflammation [10,25,66].

Riddle et al. [28], in a large study performed on 2123 paired cytological and microbiological samples, compared culture swab and cytology results to pregnancy rates. It was observed that pregnancy rate per cycle was significantly affected by the number of neutrophils per hpf, since mares with 0–2 neutrophils had a per cycle pregnancy rate of 60% compared to 36% for mares with 2–5 neutrophils per field and 23% for mares with >5 neutrophils per field. Isolation of bacteria on culture swab was also

associated with a decreased pregnancy rate (36%) even when cytological samples were normal (0-2 PMN/hpf) [28,52].

Also fungal agents can be often easily identified in cytology smears. Yeasts have a diameter of 5-8 μm and a distinct cell wall, which measures 100-200 nm and creates a clear 'halo' surrounding the organism. Hyphate fungi are narrower, measuring 3–5 μm in width and 8 μm in length. These individual cells are frequently connected in long branching chains. Often, the identification of a fungal organism during cytological testing may be the sole indication of fungal endometritis, as these organisms may not always grow in a culture. Mares with fungal endometritis frequently have inflammatory cells in the cytology sample, however not always [41,60].

1.3.3 Microbial culture

In a survey of 117 veterinarians practicing in Germany, the most commonly used diagnostic technique for the diagnosis of infectious endometritis was the guarded swab (61.2%). Surprisingly, cytology was performed routinely by only 1.7% of practitioners [6], in contrast to the USA where almost two-thirds of practitioners reported using this technique in conjunction with the culture [69]. Therefore, although uterine culture may yield false positive or false negative results, aerobic endometrial culture is still the most common method for diagnosing infectious endometritis [10,66]. Endometrial culture can be done via double-guarded cotton-tip swab, low-volume uterine lavage, or biopsy [4,70]. When performed with double-guarded cotton-tip swab it has lower sensitivity in comparison with endometrial culture of a biopsy or low-volume uterine lavage [4,35,71]. However, the specificity of low-volume uterine lavage is reported to be lower than the other two techniques, probably due to the higher risk of contamination [4,71]. Therefore, the use of a double-guarded system for low-volume lavage is recommended [71]. Several studies conclude that low-volume lavage is the most sensitive test for the diagnosis of bacterial endometritis [2,61,71]. In fact, while double-guarded swab has the advantage of being quick and easy to perform in the field, the sample is taken from only a small area of the endometrium and if focal endometritis is present, it could be missed [52]. A small volume uterine flush (50 to 100 mL) is more representative of the entire uterine surface; however, it is more time-consuming and difficult to perform in the field, and processing is required [70,72].

Samples for endometrial culture should always be collected before any uterine or vaginal procedure to avoid potential contamination. After collection, the sample should be processed or placed in transport media (e.g., Ames or Steward) and kept refrigerated until arrival at the laboratory [4]. In a recent publication, it was suggested that the addition of enrichment-broth culture can increase the rate of positivity for the detection of bacteria in equine uterine swabs compared to the direct plating of samples alone, increasing the sensitivity of bacteriological analysis in mares with endometritis [73].

The degree of inflammation observed with endometrial cytology varies among different bacteria [75]. Indeed, *Streptococcus equi zooepidemicus* or *Klebsiella* showed more often a positive uterine cytology, whereas *E. coli*, *S. aureus* and *Pseudomonas spp.* had fewer positive cytological results [28,57,68]. Confronting the two most frequent isolated bacteria, β -hemolytic streptococci and *E. coli*, the first one was found to be more frequently associated with the presence of PMNs in the slide, while the second one with debris [28,57,68,72].

Endometrial culture to diagnose fungal endometritis is more challenging because it requires prolonged incubation (up to 4 weeks) in a specialist medium (e.g. Sabouraud's dextrose agar (SDA)) and a level of expertise for accurate identification. It was observed that most of the samples positive for fungal growth on SDA were negative on aerobic culture, highlighting the importance of specific fungal media for the diagnosis [41,42].

1.3.4 Biopsy

Endometrial biopsy is still considered the gold standard for diagnosis of endometritis according to literature [35,66]. Nielsen considered the presence of neutrophil infiltration of the luminal epithelium and stratum compactum as the gold standard to indicate the presence of endometritis in 212 uterine biopsies while confronting different techniques to diagnose endometritis [35]. The same gold standard was adopted by Overbeck et al. [66]. However, according to a recent survey, endometrial biopsy is not used from practitioners in Germany as a standard diagnostic tool for the detection of endometritis, probably due to its invasive nature and the availability of different less invasive sampling methods [6].

To perform the biopsy, an alligator-type biopsy rod is used, and the specimens are placed in 10% buffered formalin, that is suitable for histopathological and several immunohistological investigations [74]. The junction between the uterine body and one uterine horn, that is the implantation site, is recommended as collection site [74]. Subsequently, the samples are processed and usually stained with hematoxylin and eosin, while also other fixatives and stains are currently used [75].

The current grading system for biopsies was established by Kenney in 1978 and revised in 1986 by Kenney and Doig [76]. The main parameters considered are inflammation and fibrosis of the endometrium and according to the classification, it provides an estimation of the foaling rate (Table 2).

Grade	Findings	Foaling rate
I	normal endometrium or slight, focal inflammation or fibrosis	80-90%
II a	mild, scattered inflammation; mild fibrosis	50-80%
II b	moderate, scattered inflammation; moderate fibrosis	10-50%
III	severe, irreversible changes including fibrosis and inflammation	<50%

Table 2. Histopathological classification of equine endometrium according to Kenney and Doig classification. Adapted from Snider et al. [75].

One of the most important factors evaluated is inflammation. Neutrophils are present during the acute inflammatory stages of infection; lymphocytes, plasma cells and macrophages indicate a more chronic process. Eosinophils are seen associated with fungal infections or pneumovagina. Furthermore, fibrosis is another factor evaluated [75].

A recent retrospective study performed on 726 biopsies, observed the following category distribution: 6.3% category I; 42.3% category IIA; 44.9% category IIB; 6.5% category III [77].

Since the equine uterus is a large organ, it has been questioned whether a single-sample endometrial biopsy in the mare would be representative of the entire uterus. It is generally considered that the collection of one endometrial biopsy with a minimal size of 10 × 3 × 3 mm is representative for the entire endometrium [74]. However, in a recent abstract Muderspach et al. collected 82 samples from the base of left and right horn of the same mares. It was observed that in 46% of mares, the specimens were assigned either of two adjacent Kenney and Doig categories, while in 5% of mares, the specimens varied with two categories. Therefore, they recommended to interpretate with caution results obtained from a single biopsy especially in older mares and recommended to evaluate two biopsies [78].

Furthermore, in another study, 8 experienced pathologists were asked to evaluate blindly the same set of 63 equine endometrial biopsy slides. However, agreement was slight to moderate, since 6 of 63 slides were assigned all 4 possible Kenney–Doig categories and additionally, 9 of 63 slides were categorized as a category I or III by at least 2 pathologists. Therefore, the authors concluded that the system is subject to significant observer variability and suggest that care should be taken when communicating Kenney–Doig categories to submitting clinicians with emphasis on the quality of endometrial lesions present instead of the category and associated expected foaling rate [79].

1.4 Treatment

The traditional treatment for endometritis involves uterine lavage, ecbolics, and antimicrobials [4]. However, many new therapies have been proposed and developed in the last years due to the lack of response to traditional therapy, the increasing prevalence of antimicrobial-resistant pathogens and the economic impact of endometritis [4,80].

1.4.1 Correction of anatomical defects

Anatomical defects and incompetence of one of the three barriers from microbial colonization of the uterus (vulva, the vestibule-vaginal fold and the cervix) have been associated with endometritis and placentitis. Without correction of physical defects, any treatment of endometritis would only represent a temporary solution [81]. Poor vulval conformation can be treated with a Caslick's vulvoplasty operation, while a urethral extension may be required to prevent recurrent pneumo or urovagina. Tears in the cervix can also be surgically repaired, but the prognosis is guarded [82].

1.4.2 Uterine lavage

The rationale behind uterine lavage is the physical clearance of microorganisms, debris, inflammatory cells and mediators, and dead sperm from the lumen, which can be detrimental for the sperm before breeding or the embryo after breeding [4,83]. Uterine lavage can be safely performed during the follicular phase, immediately before insemination if there is evidence of an accumulation of fluid in the uterus at the time of breeding [83,84] or after four hours after insemination [85]. When performed 0.5 or 2 hours after insemination, however, a decrease in pregnancy rate was observed, probably due to excessive removal of spermatozoa from the uterus during the lavage process or damage to oviductal spermatozoa [86]. However, in a recent report from Samper et al. [87], it was observed that performing the lavage as early as one hour after insemination (deep horn insemination technique with frozen semen) determine an increase in pregnancy rate compared to mares that were not flushed and was non different from mares flushed 4 hours later.

Uterine lavage is recommended in mares when intrauterine fluid accumulation is excessive (>2 cm depth) or highly echogenic [4]. Crystalloid solutions such as lactated Ringer's solution (LRS) and 0.9% saline are most commonly used to lavage mares' uteruses [83] and they can be supplemented with anti-septic (iodine, hydrogen peroxide) or mucolytics solutions (N-acetylcysteine, DMSO, Tris-EDTA) [4,80]. Vinegar is also used if fungal endometritis is diagnosed [42].

1.4.3 Ecbolics

Ecboic drugs are widely used in equine reproduction to increase uterine clearance. The most used ecbolics are oxytocin and PGF₂ α [2]. Oxytocin has a short half-life of 6.8 minutes [88] and the effect on

uterine contractions lasts up to one hour [89]. Furthermore, lower doses induce short methodical contractions from the tip of the horn to the body that effectively induce uterine clearance, while doses of 30 U.I. or higher result in tetanic, less effective contractions [4,90]. The use of oxytocin successfully increased pregnancy rate in mares with endometritis according to several reports [91–93]. The ecboic effect of oxytocin is higher during estrus but lasts until 48 h after ovulation. During estrus, indeed, there is higher oxytocin receptor gene expression in the myometrium compared to diestrus [2,94].

PGF2 α , and especially the analog cloprostenol, was effective in inducing uterine contractions that lasted longer than oxytocin (up to 5 hours) [2,95]. However, if given more than 18-24 hours after ovulation, it could result in impaired luteal function and decreased progesterone concentration [4,96].

1.4.4 Immunomodulatory agents

Immunomodulatory agents that influence either systemic or local immune defense systems have been investigated as a possible treatment for endometritis in the mare. Platelet-rich plasma (PRP) have been shown to significantly improve embryo recovery rates and reduce the duration and intensity of the post-breeding inflammatory response in mares susceptible to PBIE. Furthermore, it showed antimicrobial properties [97–99]. Bucca et al. [100] observed that a single dose of dexamethasone administered at the time of breeding is safe and can be used to modulate the uterine inflammatory response to breeding in susceptible mares, resulting in higher pregnancy rates. However, the use of corticosteroids should be careful because of the potential side effects of steroid administration in equids, such as laminitis and muscle wasting [80]. Upregulation of local defense mechanisms has also been explored as a means to improve fertility rates in susceptible mares [80]. Treatment with Mycobacterial cell wall extract resulted in a significant decrease in the number of mares positive for endometritis at ovulation and at 7 days post-ovulation [101]. A similar product containing *Propionibacterium acnes* used as an adjunct to conventional treatments in mares with a cytologic diagnosis of endometritis improved pregnancy and live foal rates [102,103].

1.4.5 Antibiotics

Antibiotic treatment is a necessary treatment when bacterial endometritis is diagnosed [4]. However, antibiotics were and still are administered as a routine post-covering treatment even in absence of clinical signs or antimicrobial sensitivity test [104–107], a dangerous habit that could lead to the development of antimicrobial resistance. Nowadays, routinary use of antibiotics is not acceptable anymore, and, according to guidelines of American Association of Equine Practitioners for the judicious use of antimicrobials, they should be used only when indicated through laboratory assessment, clinical experience, physical examination, and relevant history. Furthermore, the molecule should be selected according to the results of culture and antimicrobial sensitivity test [108]. Therefore, the use of

prophylactic intrauterine antibiotics aimed at treating the PMIE that may arise, although has been common-place in the past, is no longer justified in the current age of responsible antibiotic use [109].

The route of administration can be systemic or intrauterine, but there are conflicting data in the literature. As there is no clear consensus, both routes are used, which may have specific advantages and disadvantages [2,4]. In Germany, most of the practitioners administer antibiotics systemically for at least 5 days [6,23], while in other countries the preferred site is intrauterine [6,69,91,110]. Historically, intrauterine administration was the method of choice [2,110], as the effects of antibiotic treatment on the intestinal flora are low, the local concentration is higher and the amount of antibiotic required is lower than with systemic administration [2,4]. However, systemic treatment is currently also widely used because the risk of iatrogenic contamination is lower, since repeated invasions of the cervix are avoided, treatment is possible regardless of the phase of the oestrus cycle and the antibiotic is evenly distributed in the uterine mucosa [2,111]. Furthermore, the efficacy of systemic antibiotic treatment to reach minimum inhibitory concentration for common pathogens in the endometrium has been demonstrated for several antibiotic molecules [112–114]. On the contrary, it is assumed that intrauterine antibiotics lead to inconsistent tissue penetration. Presumably, deep-seeded and dormant endometrial infections, such as those caused by *Streptococcus*, may benefit from systemic administration, as intrauterine infusions may not result in deep penetration of antimicrobials [4]. To overcome this mechanism of defence, a commercial product called b-Activate (Bojesen and Petersen Biotech, Copenhagen, Denmark) has been developed to activate dormant *S. zooepidemicus*, making the infection detectable by conventional culture technique and treatable with antibiotics [115].

Furthermore, in order to treat endometritis caused by biofilm infections, antibiotics can be used in association with non-antibiotic substances (Tris-EDTA, DMSO, hydrogen peroxide) since the association enhances bacterial killing and disruption of biofilm, compared to treatment with the non-antibiotic agent or antibiotic alone [2,116].

Treatment of mares with fungal endometritis is multifaceted and includes uterine lavage and systemic and/or intrauterine infusion of antifungals. Topical antifungal treatment of the vagina and clitoris is also suggested since the vagina and clitoral fossa and sinuses can act as potential reservoir of infection [42,117]. Commonly used uterine lavage therapies include N-Acetylcysteine solution, DMSO, hydrogen peroxide, Tris-EDTA, vinegar. Furthermore, the two major classes of antifungals used are azoles (e.g. ketoconazole, fluconazole, miconazole) and polyenes (e.g. nystatin, amphotericin B), whose mechanism of action is to interfere with the cytoplasmic membrane of fungi [42]. After the treatment for fungal endometritis, it is common to witness secondary bacterial endometritis. Therefore, it is often necessary to treat for bacterial endometritis along with or after treatment for fungal endometritis [117].

1.4.6 Alternative non-antibiotic treatments

1.4.6.1 N-Acetylcysteine

N-acetylcysteine (NAC) is a mucolytic agent with antioxidant properties [10]. Oral administration didn't show any effect on viscosity of uterine mucus, but an anti-inflammatory effect was observed [118]. However, intrauterine infusion of a 3.3% solution of NAC decreased the extracellular mucus thickness and staining intensity in healthy mares but not in mares with active bacterial endometritis, achieving a good pregnancy rate [119]. NAC was also suggested to decrease biofilm biomass and reduce bacteria within biofilms of *E. Coli* isolates *in vitro* but not of other bacteria [81,120]. Furthermore, in a recent report, NAC infusion was suggested as a technique to enhance sensitivity in the diagnosis of bacterial endometritis [121].

1.4.6.2 Hydrogen peroxide

Hydrogen peroxide showed good *in vitro* antimicrobial activities on microorganisms isolated from mares with endometritis [121]. Furthermore, it proved also to be effective on biofilm *in vitro* on isolates of *E. coli* and *K. pneumoniae* [120]. However, reports of the efficacy of hydrogen peroxide treatment are anecdotal and controlled *in vivo* studies evaluating its use in the equine uterus are lacking [122].

1.4.6.3 Povidone-iodine

Povidone iodine is an antimicrobial commonly added to lavage fluids. After. Uterine lavage with 0.05% povidone-iodine is considered safe, while infusion of 1% povidone-iodine could cause chronic endometrial inflammatory changes [122,123].

1.4.6.4 DMSO

The infusion of 30% dimethyl sulfoxide (DMSO) solution determined an improvement in the endometrial biopsy score, and it led to a tendency toward a higher pregnancy rate compared with mares infused with saline [124]. Furthermore, DMSO solutions of 2.5–30% DMSO are also reported to be bacteriostatic for *Staphylococcus aureus*, *E. coli*, *Streptococcus* spp. and many Gram-negative bacteria [2], but in a recent report, it didn't show a significant antimicrobial activity [99]. DMSO is also capable of disrupting biofilm [2,125].

1.4.6.5 Kerosene

Intrauterine infusion of kerosene is performed as a chemical curettage of the endometrium [122]; in a report, 5 out of 11 barren mares were able to carry a foal to term despite being previously classified as infertile due to the uterine severe degenerative endometrial changes after a single intrauterine infusion of kerosene [126]. Kerosene was recently proposed as a treatment to hasten the regression of

endometrial cups. However, although it didn't cause an increase in fibrosis or degenerative changes, it was not successful compared to the control group. Furthermore, it is not a risk-free treatment, since it was reported to cause chemical vaginitis in the mare and chemical dermatitis in the operator [127].

1.4.6.6 Tris-EDTA

Tris-EDTA is a chelating agent. The mechanism of action is not completely clear, but it alters the integrity and permeability of the bacterial cell wall by chelation of the calcium and/or magnesium on the outer membrane [10,52]. In bovine endometritis Tris-EDTA showed the ability to reverse antimicrobial resistance to some antibiotics and produced good pregnancy rates after treatment [128]. Furthermore, the combination of ceftiofur and tris-EDTA resulted more effective at treating biofilm-associated experimentally induced *Pseudomonas aeruginosa* uterine infections compared with ceftiofur or tris-EDTA alone [129].

1.4.6.7 Ozone therapy

Ozone is a highly unstable gaseous molecule made of three oxygen atoms with a cyclic structure that forms naturally from oxygen by the action of ultraviolet light and electrical discharges in the atmosphere [9,10]. Due to its structure, it is a strong oxidising agent, but it is also an unstable gas that cannot be stored and should be used immediately, since it has a half-life of 40 minutes at 20°C [11]. It is produced by ozone generators through a high-voltage gradient, starting from pure oxygen, to obtain a gas mixture containing at least 95% oxygen and up to 5% ozone.

Its antimicrobial properties are based on the high oxidative potential of this gas, which is able to attack the components of cell membranes, cell envelopes, cytoplasm, spore envelopes and viral capsids, leading to the destruction of bacterial cell walls and cytoplasmic membranes [10]. This causes an increase in permeability, which ultimately results in the entry of ozone into the cells [10]. In addition, ozone therapy is also capable of damaging nucleic acid and stimulating the innate immune system to fight microorganisms [10,11].

The first reports of the use of ozone date back to the 20th century [130], and already during the First World War it was used as a disinfectant to treat purulent wounds [130]. Nowadays, because antimicrobial resistance (AMR) is one of the top global public health [131] and the development of new antibacterial agents is insufficient, alternate bactericide treatments are necessary [132]. A promising article was recently published by Roth et al. [133], that described the use of a wearable adjunct ozone and antibiotic therapy system for treatment of Gram-negative dermal bacterial infection. The association of low doses of ozone with antibiotics drastically increased the sensitivity of Gram-negative bacteria to common antibiotics by using oxidative ozone to bypass resistances created by the bacterial cell wall. Results showed complete eradication of Gram-negative bacteria with antibiotics typically

used only for Gram-positive bacteria, which showed the strength of ozone as an enabling adjunct treatment option to sensitize bacteria strains to otherwise ineffective antibiotics. Therefore, it was shown that a combination of ozone and antibiotics can treat antibiotic resistance infections and prevent the development of new infections [133].

In animal reproduction, until recent years, most of the literature was referred to the cow and small ruminants. This is probably due to the usage without prescription and the absence of residues in milk, meat, and other tissues [134]. In the dairy cow, preventive use of a commercial ozonated oil-based therapy (Riger spray®) in post-partum led to an improvement in reproductive efficiency, since treated cows became pregnant earlier and had better conception rate [135]. Cows with retention of fetal membranes treated with intrauterine ozone (Riger spray®) had similar or enhanced reproductive performances compared to the control group of cows without membranes retention [136]. The same result was observed in two studies on goats [137] and sheep [138], where intrauterine ozone treatment in animals with placental retention attained similar results to healthy control groups and standard antibiotic treatment. Furthermore, Escandon et al. [139], described that intrauterine therapy with ozone reduced the rate of subclinical endometritis and uterine inflammation, while improved reproductive performance in postpartum dairy cows.

In equine reproduction, ozone therapy is a new field of research, as the few published articles are still very recent. Camargo Ferreira et al. [140] observed that intrauterine ozone therapy effectively stimulates local angiogenesis of the endometrium. This finding underlines the potential of ozone as a treatment for subfertility, as it has been shown that there is a close correlation between hemodynamic disturbances and impaired uterine function in mares [141,142]. Avila et al. [143] treated mares with infectious endometritis with gaseous ozone and ozonated oil. Compared to the control group, ozone therapy determined significant a reduction in the number of mares with a positive culture and in the number of PMN/field. Kohne et al. [144] compared the efficacy of intrauterine ozone, systemic trimetoprim-sulphadimethoxine and uterine air insufflation to treat infectious endometritis. They observed that ozone and antibiotic were more effective in reducing growth of gram-negative bacteria as compared to air, however, both treatments did not result in a complete absence of intrauterine bacterial growth. No effects on the number of PMN were observed.

In conclusion, although ozone therapy proved to be a promising treatment, the considerable variability in the adopted practices limits standardization and it can justify the different findings found in the literature [145]. Further controlled *in vitro* and *in vivo* studies are therefore required.

1.5 Reproductive microbiome

Traditionally, it has been widely accepted that while the vagina harbors a resident microbial community, the uterus is a sterile organ. However, if bacteria or sperm are not effectively eliminated after breeding, the presence of inflammatory products from the uterus leads to endometritis and decreased fertility [146]. Furthermore, as previously reported, several bacterial genera such as *Streptococcus*, *Escherichia*, *Staphylococcus* and *Pseudomonas* can cause infectious endometritis, resulting in pregnancy loss and infertility [4,27]. The clinical literature continues to describe the uterus of a healthy mare as a site that does not host a resident microbial community [4,38]. With the advent of 16S rRNA gene-based bacterial detection and identification techniques, this dogma of the sterility of the uterus was challenged [147].

Conventional techniques for identifying and characterizing microorganisms require growing isolates on culture plates. While these methods have contributed to numerous crucial discoveries, they have limitations. Specifically, several microbial species cannot grow in standard laboratory culture settings. The advantage of microbiota analysis is that even bacteria that cannot be cultured, either because they are dead or because the culture conditions are not right or because their growth is masked by other species, can be identified using the 16S technique [46]. This allows us to get a picture of the entire endometrial microbiota and not just the few bacteria that can grow in vitro that are identified with the culture technique (about 1% [148]), which leads to an underestimation of the diversity of microbes in a given niche [149]. The 16S ribosomal RNA (rRNA) gene is a small, highly conserved gene in prokaryotes, that codes for the 16S subunit of the ribosome. This gene can be found in all bacteria and archaea, but it also contains highly variable regions that can be used for taxonomic classification. This makes the 16S rRNA gene a valuable molecular marker for identifying different bacterial populations [150].

The first phase of microbiome analysis is obtaining sample specimen of the target biological niche [151]. One of the crucial points of this phase is the sterility: Next-generation sequencing has allowed to investigate microbial communities with higher sensitivity than ever before. However, this sensitivity is a double-edged sword because these tools also efficiently detect contaminant DNA which can confound the interpretation of microbiome data [152]. Proper handling of the samples is also essential to avoid microbial growth that could result in biased microbiomes. Extended times at room temperature or hotter would be expected, indeed, to promote the replication of aerobic microbes, while anaerobic microbes would not grow [153]. The current standard is to freeze the samples at -20 or -80° avoiding repeated freeze-thaw cycle [154].

The second stage involves library preparation, which is the process of preparing DNA for sequencing. Once the DNA has been isolated, the subsequent procedure involves amplifying the 16S rRNA gene by polymerase chain reaction. To do this, it is important to use primers that specifically target a certain

hypervariable region (V1-V9) of the 16S rRNA gene. The selection of the hypervariable region can have an impact on the outcomes, so it is crucial to choose a region that is appropriate for the specific type of sample being analyzed [155]. For example, Walker et al. [156] stated that the off-target amplification of host DNA is an underreported artifact associated with 16S rRNA gene sequencing. V3–V4 primers align to a region within the human mitochondrial DNA and, when the sample has a small biomass and the ratio of host: bacterial DNA is overwhelming, human mitochondrial DNA can be amplified by primers targeting this 16S rRNA gene region. In their study, they analysed different samples from different body sites using primers targeting regions V3-V4 and subsequently V1-V2. They concluded that especially from sites with a ratio of host: bacterial DNA elevated, using primers for V1-V2 resulted in 80% less host genome aligning reads [156].

Subsequently, once the library has been prepared, the next step is sequencing. During this stage, the DNA libraries undergo amplification and sequencing. The obtained data produces short DNA sequences that can be aligned with databases to determine the taxonomic classification. The last phase is the analysis of the data in order to identify and classify the microorganisms present in the sample. This process is carried out utilizing bioinformatics pipelines, which clean up sequencing data by eliminating host and contaminant reads as well as sequencing errors. The purified reads are then aligned with public databases, such as the National Center for Biotechnology Information (NCBI) database. Finally, various statistical methods can be used to assess similarities and differences between different samples.

Nowadays, although there is a better understanding of the microbiome in the human reproductive tract, knowledge of the microbial communities in the reproductive tract of animals is far more limited and most of the studies have been conducted on cows [146].

One crucial concept in the studies on the microbiome of healthy and diseased subjects is dysbiosis. According to Petersen and Round [157], dysbiosis is broadly defined as “any change to the composition of resident commensal communities relative to the community found in healthy individuals”. Furthermore, they described three types of dysbiosis that however are not mutually exclusive and may all occur concurrently: 1) loss of beneficial microbial organisms, 2) expansion of pathobionts or potentially harmful microorganisms, and 3) loss of overall microbial diversity.

Studies including hundreds of women undergoing assisted reproductive techniques have been performed. Moreno et al. in 2016 [158] investigated uterine microbiome of 35 infertile patients undergoing in vitro fertilization. They classified the microbiome of the different patients according to sequencing results as a *Lactobacillus*-dominated microbiota (>90% *Lactobacillus spp.*) or a non-*Lactobacillus*-dominated microbiota (<90% *Lactobacillus spp.* with >10% of other bacteria). The conclusion was that the presence of a non-*Lactobacillus*-dominated microbiota was associated with

significant decreases in implantation, pregnancy, ongoing pregnancy, and live birth rates. Furthermore, in 2022 a larger study was published on 342 infertile patients undergoing assisted reproductive treatments [159]. The results of the previous work were confirmed since *Lactobacillus* was consistently enriched in patients with live birth outcomes, while the presence of *Atopobium*, *Bifidobacterium*, *Chryseobacterium*, *Gardnerella*, *Haemophilus*, *Klebsiella*, *Neisseria*, *Staphylococcus* and *Streptococcus* was associated with unsuccessful outcomes [159]. Therefore, they concluded that endometrial microbiota composition before embryo transfer represents a useful biomarker to predict reproductive outcome, offering an opportunity to further improve diagnosis and treatment strategies [159].

The microbiome of 130 infertile women with chronic endometritis was also investigated [160]. It was observed that chronic endometritis was associated with a significantly higher abundance of 18 bacterial taxa in the endometrial cavity (including *Dialister*, *Bifidobacterium*, *Prevotella*, *Gardnerella*, and *Anaerococcus*), while the median relative abundance of *Lactobacillus* was 1.89% and 80.7% in the chronic endometritis and non-chronic endometritis microbiotas, respectively [160].

In the dairy cow, Pascottini et al. [161] described a different uterine microbiome from animals with clinical endometritis compared to healthy and sub-clinical cows. At phylum level *Bacteroidetes* and *Fusobacteria* were significantly more abundant in cows with clinical endometritis, and at genus level, relative abundance of *Trueperella* was greater. Furthermore, the uterine microbiota was similar between sub-clinical endometritis and healthy, but the microbiome in cows with clinical endometritis had a loss of bacterial diversity.

Although there is presently no agreed-upon definition of a consensus on the 'healthy' or 'core' uterine microbiota, certain generalizations can be drawn from the existing data at the phylum and genus levels for cows diagnosed with metritis [162]. Research has shown that metritic cows had a higher proportion of *Fusobacteria* and *Bacteroidetes*, suggesting that these two phyla of bacteria may play a significant role in the development of the disease [162–164]. At the genus level, *Bacteroides*, *Fusobacterium*, and *Porphyromonas* were consistently identified as being associated with metritis [162,163]. Conversely, failure to successfully treat metritis was correlated with an increase in the relative abundance of these three genera associated with a decreased diversity of uterine microbiota [162,165].

In the mare the literature on the uterine microbiome is sparse. In 2022 Holyoak et al. [147] described the uterine microbiome of 54 healthy mares from 4 different geographical locations. They observed that microbial diversity, richness, and evenness of the equine uterine microbiome varies according to the geographical location. The core microbiome at genus level was composed by *Lactobacillus*, *Escherichia/Shigella*, *Streptococcus*, *Blautia*, *Staphylococcus*, *Klebsiella*, *Acinetobacter*, and *Peptoanaerobacter*. However, *Lonsdalea*, that was the most abundant genus in the

samples of one region was not found in any other region. Furthermore, *Pseudomonas* dominated the microbiome of the samples of two different regions but not the others.

Another recent article was published by Heil et al. [166]. In this study, they aimed to describe the uterine microbiome of 16 healthy mares during estrus and to compare different sampling techniques (namely, double-guarded endometrial and cervical swab, endometrial biopsy, and uterine low-volume lavage). The most abundant phyla were Proteobacteria, Firmicutes and Bacteroidota, while at genus level, microbiome was dominated by *Klebsiella*, *Mycoplasma*, *Aeromonas*, and *Citrobacter*. Furthermore, a significant difference was found in alpha diversity (Chao1) between low-volume lavage and endometrial biopsy, suggesting that low-volume lavage is superior at detecting rare taxa [149].

Finally, two recent abstracts were published on this topic. The first one compared the endometrial microbiome of mare in estrus and anestrus and concluded that the equine uterine microbiome in estrus has a low diversity, low richness, and a low abundance at phylum level, while in anestrus it has a higher diversity, higher richness, and higher abundance. The possible explanation given by the authors for the difference was the variance in immune responsiveness due to estrogens during estrus. The second one aimed to observe if there is an association between fertility and uterine microbiome. They described that the overall bacterial abundance was higher in mares that were barren and failed to become pregnant when compared to successfully bred barren mares and maiden mares [167].

In conclusion, a thorough understanding of the resident microbiota of the reproductive tract of a healthy mare is necessary to identify these commensal bacteria and understand their role in preventing infection and, on the other hand, to understand the effects of dysbiosis on fertility. In addition, the effects of broad-spectrum antibiotics on the uterine microbiome and the role of different therapies in restoring a healthy balance in the uterine microbiome are necessary topics for future research.

CHAPTER 2

Thesis aims

2. AIMS OF THE THESIS

This thesis aims to deepen the knowledge in the field of equine endometritis in terms of **diagnosis** and **treatment** of this pathology:

Diagnosis

- The uterine microbiome of the healthy mare and differences between follicular and luteal phase
- The uterine microbiome of the mare with endometritis
- Influence of intrauterine fluid detection in the recipient on pregnancy rate and early embryonic loss in a commercial embryo transfer program

Treatment

- The effects of antibiotic treatment on the endometrial microbiome of mares with endometritis
- Safety and effects of a commercial ozone foam preparation on endometrial environment and fertility of mares
- In vitro effects of different ozone preparations on microorganisms responsible for endometritis in the mare

CHAPTER 3

The endometrial microbiome of the mare

3.1 Introduction

In veterinary clinical literature, the uterus of a healthy mare has been considered sterile or to have a transient non-resident microbiota [25,168]. However, this opinion was based on the classical culture-based approach, which can miss the great microbial diversity present in a specific environment [169] focusing only on culturable bacteria. Culture-based techniques can only confirm the presence of a microorganism that can grow on a specific media [170], while it is estimated that only a small fraction of microbes can be cultured in a laboratory [171,172]. With the advent of the non-culture-based sequencing techniques targeting the 16S rRNA gene, the dogma of a sterile uterus has been challenged [147,173] and it has been shown that the uterus of healthy mares hosts a resident microbiota [146,147]. In women and other animals, especially cows and to a lesser extent donkeys and mares, specific shifts of the reproductive tract's microbiome have been associated with fertility/infertility and uterine affections [161,167,174–182]. Unfortunately, knowledge on the endometrial microbiome of the mare is still scarce, even though infectious endometritis, diagnosed through the detection of pathogenic bacteria in the uterus using microbial culture, is one of the major causes of subfertility and leads to a substantial economic loss in the equine industry [18,183]. Therefore, understanding more deeply the microbiome of healthy and unhealthy mares is needed and could open new frontiers in the diagnosis and treatment of uterine affections. Since the 16S rRNA sequencing approach for the description of the endometrial microbiome is a relatively new area of research in the equine reproduction field, improvements in the protocols are necessary, especially because, as constantly demonstrated in human medicine, the uterine microbiome contains only a very small biomass of bacteria compared with the lower genital tract [178,184] and its investigation could be therefore extremely difficult.

Regarding potential differences in the reproductive tract microbiome during different phases of the estrous cycle in the mare, only a few contrasting reports exist, while in humans and other animals it has been shown that the reproductive tract flora is not stable during the sexual cycle [184–187]. One of the reasons for this fluctuation is the presence of the cervix, which is a dynamic organ that relaxes during estrus to allow the entrance of semen and the drainage of uterine fluid while it closes tightly during diestrus [188]. Furthermore, the influence of hormones, especially estrogens, that fluctuate during the cycle can affect the reproductive tract microbiome [184]. In the mare, in a recent abstract, Heil et al [189] described strong dissimilarity in the uterine microbiome between anestrus and estrus, with a higher diversity, richness, and abundance detected in the first phase. In contrast, Barba et al did not find significant changes in vaginal microbiota composition during estrus and diestrus [169].

Regarding associations between uterine microbiome and uterine pathologies, many studies have been performed in the woman, as reviewed by Toson et al. [178]. In the cow, furthermore, endometritis has

been associated with a lack of bacterial diversity and richness of the endometrial microbiome [161,181]. However, up to date, no studies have been performed on the mare.

The aims of the present study were to a) describe the uterine microbiome of healthy mares; b) compare the uterine microbiome between follicular (estrus) and luteal (diestrus) phase; c) describe the uterine microbiome of mares with endometritis; d) observe the effect of the intrauterine antibiotic treatment (ceftiofur sodium) on uterine microbiome.

3.2 Materials and Methods

3.2.1 Animals and experimental design

3.2.1.1 Experiment 1

Eleven healthy mares aged between 3 and 15 years old were enrolled in this study during the 2022 breeding season. The mares were used as embryo recipients at Keros Embryo Transfer Center in Passendale, Belgium.

All the mares were Standardbreds and pluriparous and the sampling process occurred at least 9 months after the last foaling. The mares were housed in single or shared stalls and fed with hay and corn silage; water was supplied ad libitum. The mares were resident at Keros for more than 3 months.

All the mares were clinically normal with no history of reproductive problems. A breeding soundness exam was performed before the study in order to include only reproductively normal mares with no clinical evidence of endometritis. A double-guarded swab for bacterial culture was collected and only culture-negative mares were enrolled.

The mares were submitted every other day to transrectal ultrasound examination in order to determine the phase of the cycle and the day of ovulation. The sample for the follicular phase was collected when all these findings were observed: follicle >35 mm, uterine edema > 2/4 (on a subjective scale ranging from 0, no edema, to 4, marked edema), open and relaxed cervix. The samples for the luteal phase were collected 6 to 9 days after ovulation when a corpus luteum was clearly visible, there was an absence of uterine edema and the presence of a good uterine tone, while the cervix was tightly closed.

Two samples were collected for each mare, one during follicular phase and one during luteal phase.

3.2.1.2 Experiment 2

Out of the mares that were admitted for infertility to the Veterinary Teaching Hospital (OVU) of the University of Turin (Italy) during the breeding seasons 2022 and 2023, 5 mares with clinical signs of endometritis, positive to bacteriological culture and treated with the same antibiotic were included in this study.

The mares were aged 5-15 years. One mare was an Arabian, 1 was a Standardbred and 3 were Thoroughbreds. The mares were housed in shared paddocks, fed with hay and pellet feed and water was supplied ad libitum. The mares lived in 3 different farms in Vigone (Turin, Italy) and Cercenasco (Turin, Italy).

The mares underwent three or more unsuccessful natural mating/artificial insemination attempts with fertile semen during the reproductive season and showed clinical signs of endometritis (excessive uterine edema, intrauterine fluid accumulation >2 cm, shortened inter-oestrus interval [18]). They didn't receive any antibiotic treatment in the previous three months.

During early follicular phase, determined as the presence of a follicle >30 mm and uterine edema > 1/4, a double guarded cytobrush for microbiota analysis immediately followed by a double guarded swab for bacteriological analysis were collected.

Subsequently, during the follicular phase, the mares were treated with intrauterine infusions of ceftiofur sodium (1g daily for 3 days, Wondercef, FATRO, Italy). The day after every infusion, a uterine lavage with sterile saline solution was performed until the efflux was clear.

During the subsequent follicular phase (18-26 days after the first sampling), the sampling for microbiota and cultural examination were repeated.

3.2.2 Bacterial culture

The perineum was washed three times with water and povidone-iodine soap (Betadine; MEDA PHARMA SpA, Milan, Italy) and then dried with paper towel.

Wearing a sterile glove, a double-guarded uterine swab (Minitube GmbH, Tiefenbach, Germany) was used to collect a sample for bacteriological culture avoiding contaminations from the vagina. Briefly, the instrument was introduced in the vagina and carried to the external cervical os, where the inner tube and subsequently the brush were pushed out. The tip of the swab was rolled against the endometrium of the uterine body for 30 seconds.

Subsequently, the swab was inserted in AMIES transport medium (Minitube GmbH, Tiefenbach, Germany) and transported, within 2-8 hours at refrigeration temperature, to the Microbiology Laboratory of OVU for the examination. Each uterine swab was resuspended in 3 ml of a non-selective enrichment medium (Brain Heart Infusion Broth Oxoid) and incubated overnight at 37 °C. Then, each cultural sample was streaked on agar with 5% sheep blood plates for a further 24 hours at 37 °C. The isolates were identified by matrix assisted laser desorption ionization time of flight/ mass spectrometry (MALDI-TOF/MS) (Bruker, Germany). Furthermore, the bacteria were tested *in vitro* for their antimicrobial susceptibility to the following panel of antimicrobials (Oxoid Ltd., Basingstoke, UK): amikacin (30µg),

amoxicillin (10µg), ampicillin (10µg), amoxicillin/clavulanic acid (30µg), ceftiofur (30µg), clindamycin (2µg), gentamicin (10µg), penicillin (1U), tetracycline (30µg), trimethoprim-sulfamethoxazole (25µg), enrofloxacin, (5µg) and marbofloxacin (5µg; Fatro S.p.A.,Ozzano Emilia, IT). The disk diffusion method was performed and interpreted for each species according to Clinical and Laboratory Standards Institute (CLSI) M27-A3 (2018) and to EUCAST guidelines.

3.2.3 16S rRNA gene sequencing

The sampling procedure was repeated as previously described for bacteriological examination, but using a double-guarded cytobrush (Minitube GmbH, Tiefenbach, Germany).

After the sampling, the tip of the brush was cut using sterile scissors and stored into a sterile 2 ml Eppendorf tube (Sigma-Aldrich, St.Louis, USA) and kept refrigerated until arrival at the lab within 2 hours. The samples of experiment 1 were stored at -20° for up to one month, while the samples of experiment 2 were stored at -80° for up to 8 months.

Sequencing of the bacterial 16S rRNA gene was performed on 22 uterine brushes in experiment 1 and 10 in experiment 2. DNA extraction from the frozen and thawed brushes was performed using the E.Z.N.A[®] Soil DNA Kit (Omega Bio-Tech). Laboratory reagents were used as negative controls to assess possible contamination from the extraction kit. Furthermore, two clean sterile brushes served as negative control to check for possible contamination from the swabs. DNA was quantified using the NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific). 16S V1-V2 were used because they significantly reduce off-target amplification of host mitochondrial DNA at low microbial biomass, since they have lower similarity with mammalian mitochondrial genome [156,190], as described in vaginal swabs from cats [190]. V1-V2 primers were tailed with i5 and i7 Nextera adapters allowing barcoding with a second amplification step.

PCR was performed in a 25 µL volume reaction containing 12.5 µL Accustart II PCR ToughMix 2X (Quanta Bio), 1.25 µL EvaGreen[™] 20X (Biotium), 1 µL 16S-i5-XT-27F primer (5'- TCG TCG GCA GCG TCA GAT GTG TAT AAG AGA CAG AGV GTT YGA TYM TGG CTC AG, 10 µM), 1 µL 16S-i7-XT-338R primer (5'- GTC TCG TGG GCT CGG AGA TGT GTA TAA GAG ACA GTG CTG CCT CCC GTA GGA GT, 10 µM) and 2 µL (50 ng) DNA template. PCR was performed in a CFX 96[™] PCR System (Bio-Rad) with a real-time limited number of cycles of 94°C for 30 s, 55°C for 20 s, 72°C for 30 s and a final extension of 72°C for 5 min. All amplicons were checked for quality and size by running 2 µL on 2% agarose gel electrophoresis and visualising a ≈ 350 bp band. They were then sent to an external laboratory (BMR Genomics, Padua, Italy) for barcoding, and sequencing using the Illumina MiSeq platform (Illumina, San Diego, CA).

3.2.4 Data analysis

Sequencing data were initially processed and analyzed using CLC Microbial Genomics Module version 23.0.3. Raw reads were quality filtered to remove adapters and low-quality sequences with a quality score threshold of 0.03. Paired-end reads were merged, and primers were trimmed. Paired-end reads were denoised into Amplicon Sequence Variants (ASVs) using the DADA2 algorithm [191] implemented in the CLC Microbial Genomics software. The resulting ASVs were taxonomically classified using the SILVA database version 138.1 with a confidence threshold of 97%. All sequences of non-bacterial origin (i.e., chloroplasts, and sequences not assigned to Bacteria kingdom) were removed.

Data analyses were further conducted using R ver. 4.2.2 (Vienna, Austria) using the packages phyloseq, vegan, ggplot2 and microbiome. Data were rarefied based on minimum library size. Alpha diversity was calculated by Observed species, Chao 1, Shannon and Simpson indexes using a pairwise Wilcoxon test; beta diversity was estimated using Bray-Curtis analysis. Permutational multivariate analyses of variance (PERMANOVA) were run based on Bray-Curtis dissimilarity to assess differences based on cycle phase (experiment 1), and the effect of the treatment (experiment 2). Significance was considered for $P < 0.05$.

3.3 Results

3.3.1 Experiment 1

A total of 2,581,412 pair-end reads were obtained. After the filtering step, 2,187,428 reads remained resulting in 24,388 ASVs.

Rarefaction curves show that the sequencing depth was sufficient to observe the total diversity present in the microbial community (Figure 2).

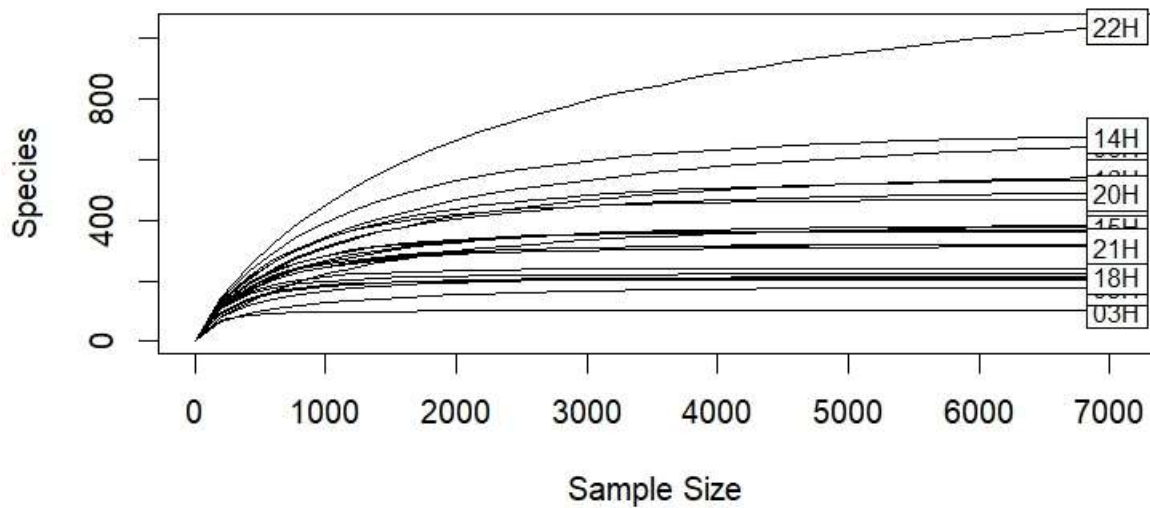


Figure 2: Taxonomy rarefaction curves for the endometrial samples included in Experiment 1.

Twenty bacterial phyla were observed in diestrus and 23 during estrus. Twenty-four phyla in total were identified (Figure 3).

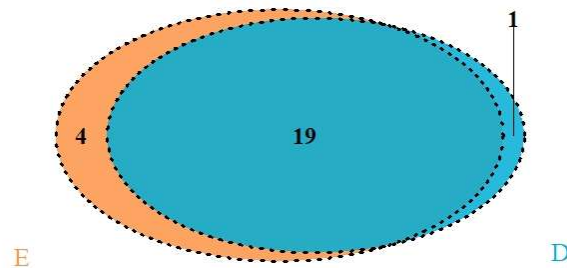


Figure 3: Total number of phyla detected in estrus and diestrus.

The most abundant phylum overall was Firmicutes (E: 38.8%, D:39.2%), followed by Proteobacteria (E:22.7%; D:29.8%), Bacteroidota (E:16.6%; D:14.0%) and Actinobacteriota (E:16.2%; D:12.1%). These Phyla accounted for nearly 95% of relative abundance, while the remaining phyla showed a relative abundance <1,5%.

Relative abundances at Phylum level are shown in Figures 4 and 5. Relative abundances of all the 24 phyla are shown in the supplementary materials (S1).

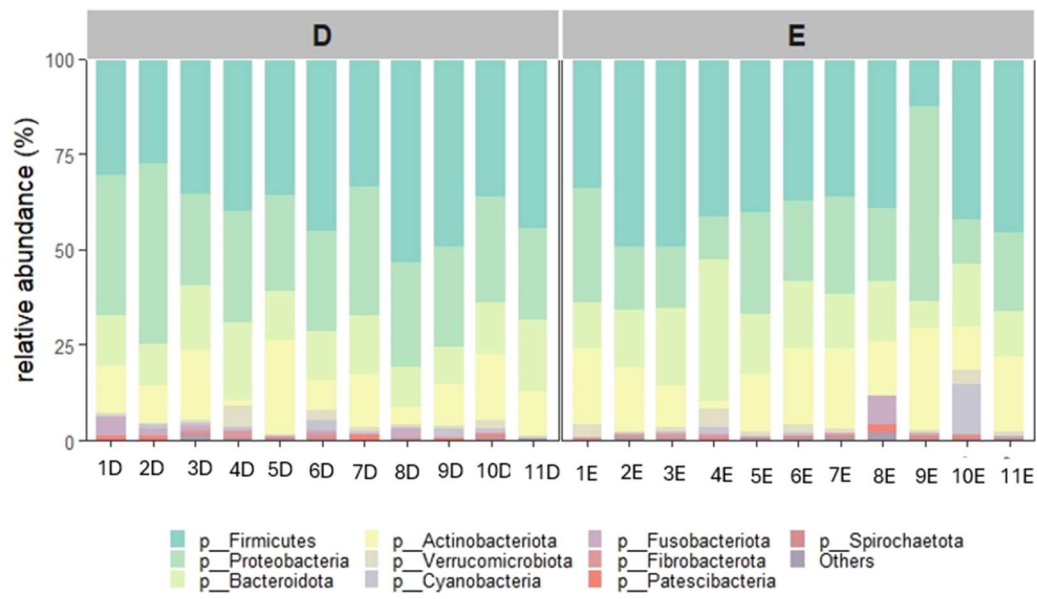


Figure 4: Relative abundance of the 10 most abundant phyla detected in the 11 mares during estrus (E) and diestrus (D).

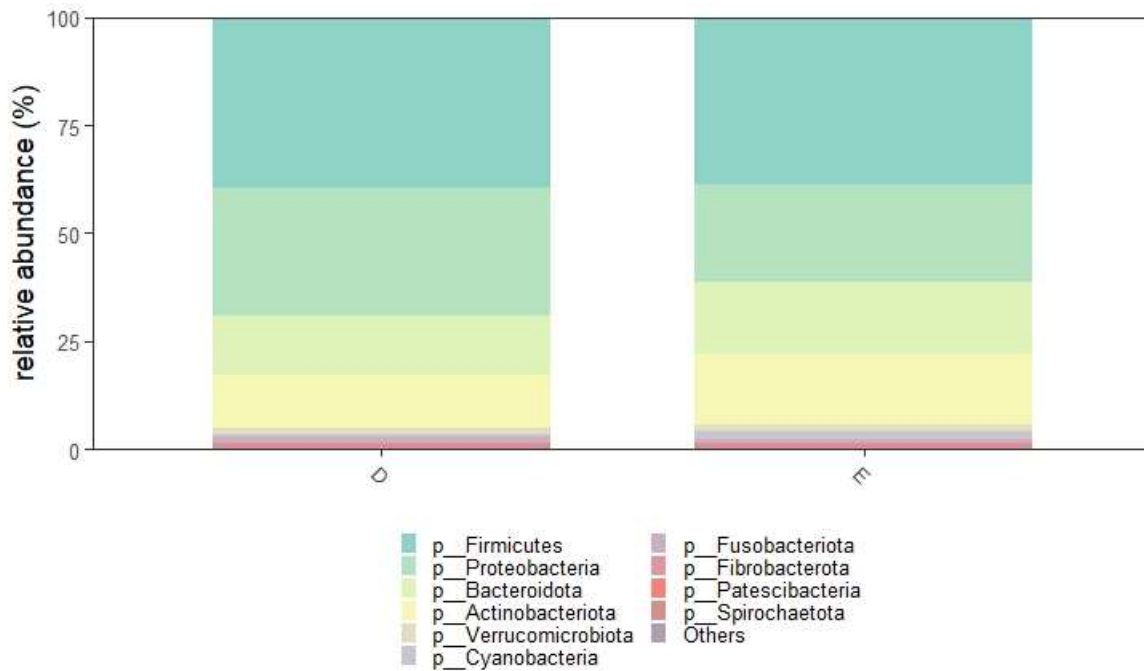


Figure 5: Relative abundance of the 10 most abundant phyla grouped according to cycle phase. Estrus (E) and diestrus (D).

At genus level, 567 different genera were observed (Figure 6)

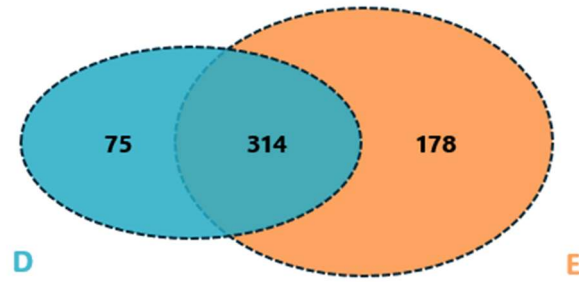


Figure 6: Total number of genera detected in estrus and diestrus.

The most abundant was *Staphylococcus* (E:8,2%; D:7,8%), followed by *Acinetobacter* (E:5,3%; D:7,2%), *Sphingomonas* (E:4,7%; D: 6,8%), *Corynebacterium* (E:6,6%; D:4,6%), *Streptococcus* (E:6.6%; D:4.4%), *Rikenellaceae* (E: 4,2%; D: 3%), *Clostridium* (E:4,2%; D: 2%), *Pseudomonas* (E: 3,1%; D: 2,2%). Furthermore, *Porphyromonas* accounted for 1% and 1,9 % in estrus and diestrus, respectively, while *Escherichia-Shigella* only for 0,4% and 0,8%.

Relative abundances at genus level are shown in Figures 7 and 8. Relative abundances of the 40 most abundant genera are shown in the supplementary materials (S2).

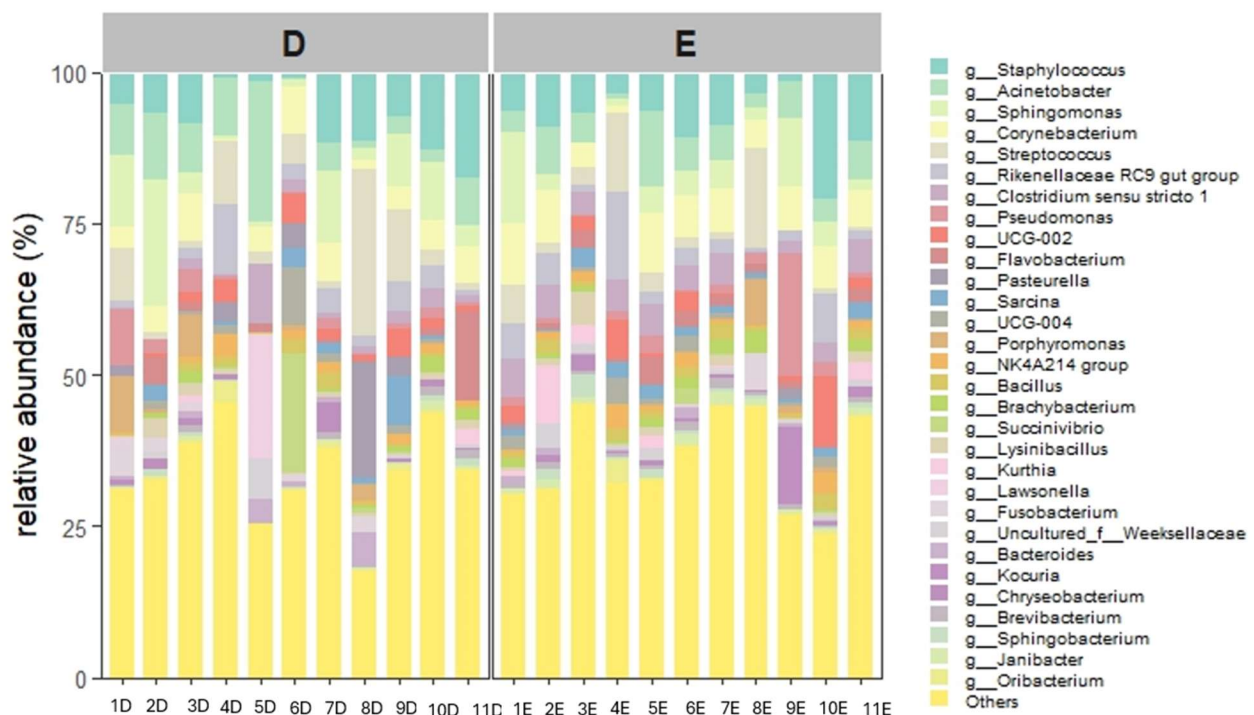


Figure 7: Relative abundance of the 30 most abundant genera detected in the 11 mares during estrus (E) and diestrus (D).

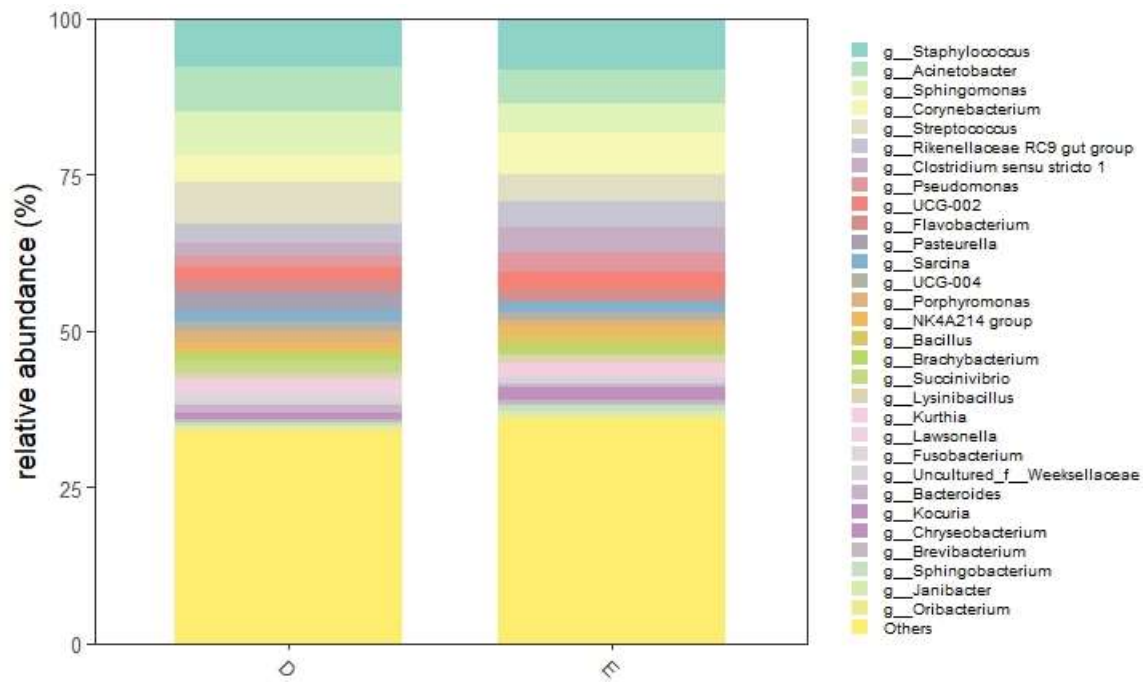


Figure 8: Relative abundance of the 30 most abundant genera detected grouped according to cycle phase. Estrus (E) and diestrus (D).

3.3.1.1 Alpha and Beta diversities

An increase in alpha diversity was observed during the follicular phase, as described through Shannon ($p=0.023$) and Simpson indexes ($p=0.047$). For Observed species and Chao 1 Index p values were respectively 0.065 and 0.066 (Figure 9).

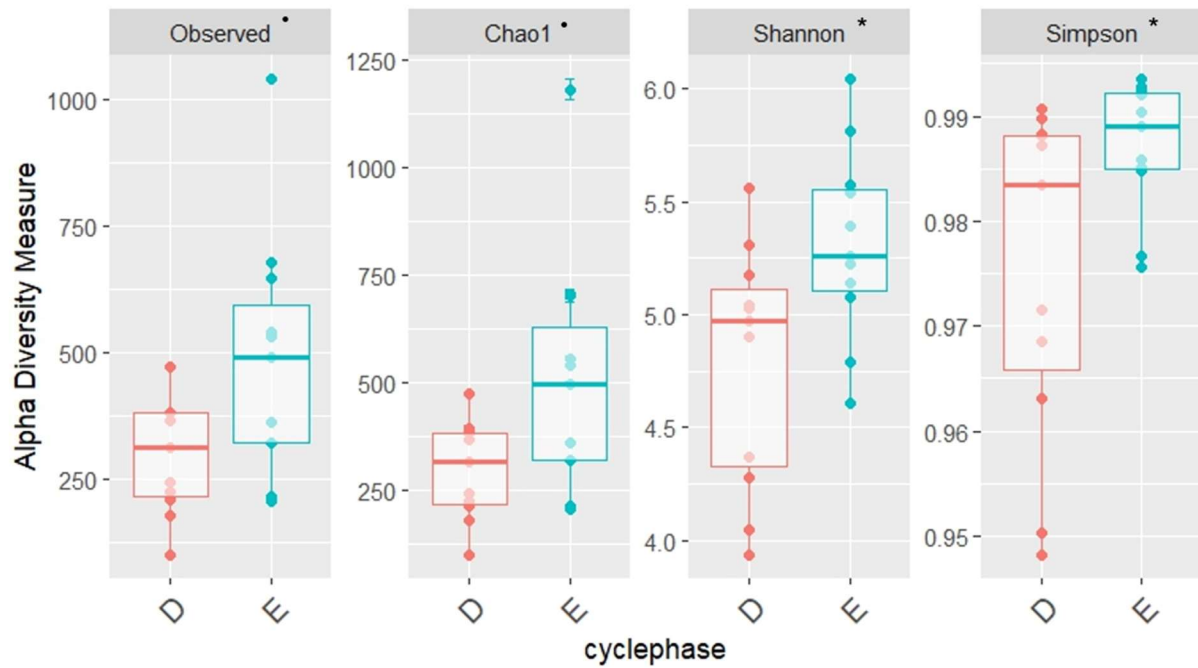


Figure 9: Variation in endometrial microbial diversity based on the cycle phase (estrus – E, diestrus – D) assessed by observed richness, Shannon, Simpson, and Chao 1. * indicates a p -value < 0.05 , while ' indicates $0.1 < p < 0.05$.

Beta diversity did not differ between the two phases ($p < 0.1$) (Figure 10).

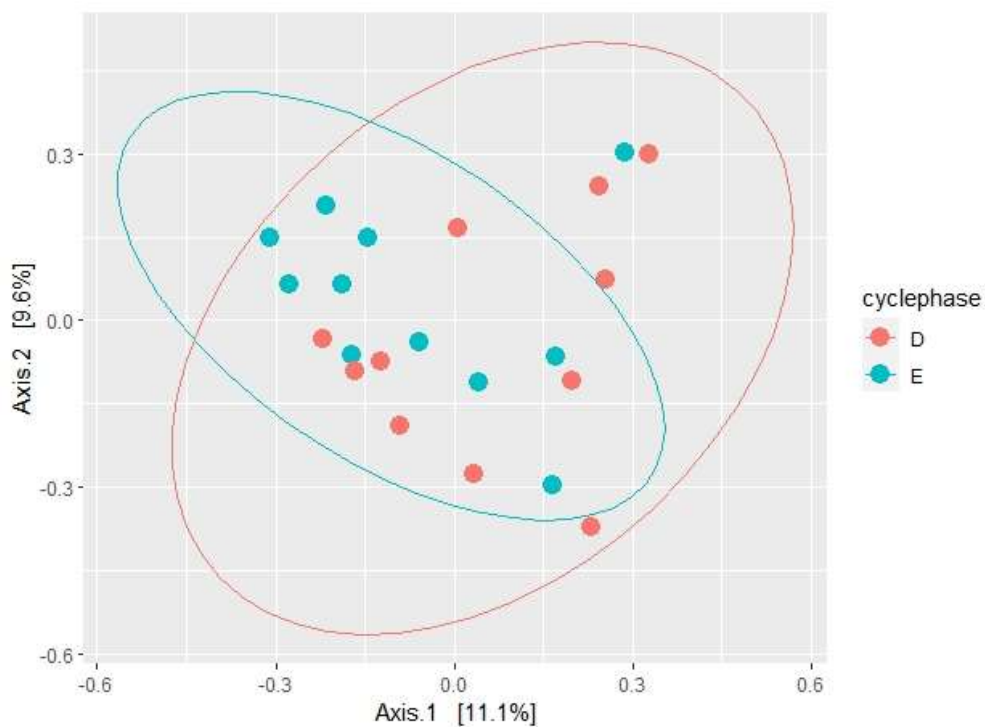


Figure 10: Two dimensions plot by Bray-Curtis based on cycle phase. The samples clustered together and significant differences were not observed.

3.3.2 Experiment 2

3.3.2.1 Bacterial culture

According to bacterial culture, 4 mares were positive for *Streptococcus equi zooepidemicus* (mares number 1,2,3 and 5), while 1 for *Escherichia coli* (mare number 4). After the treatment, 3 mares were negative (mares 1,2 and 5), 1 was positive for *Staphylococcus spp* (mare number 3) and 1 remained positive for *Escherichia coli* (mare number 4) (Table 3).

Mare nº	Before (T0)	After (T1)
1	<i>S. equi zooepidemicus</i>	/
2	<i>S. equi zooepidemicus</i>	/
3	<i>S. equi zooepidemicus</i>	<i>Staphylococcus spp</i>
4	<i>E. coli</i>	<i>E. coli</i>
5	<i>S. equi zooepidemicus</i>	/

Table 3: Results of bacterial culture before (T0) and after (T1) antibiotic treatment.

3.3.2.2 16S rRNA sequencing

A total of 922,028 pair-end reads were obtained. After the filtering step, 790,350 reads remained resulting in 4,124 ASVs.

Rarefaction curves show that the sequencing depth was sufficient to observe the total diversity present in the microbial community (Figure 11).

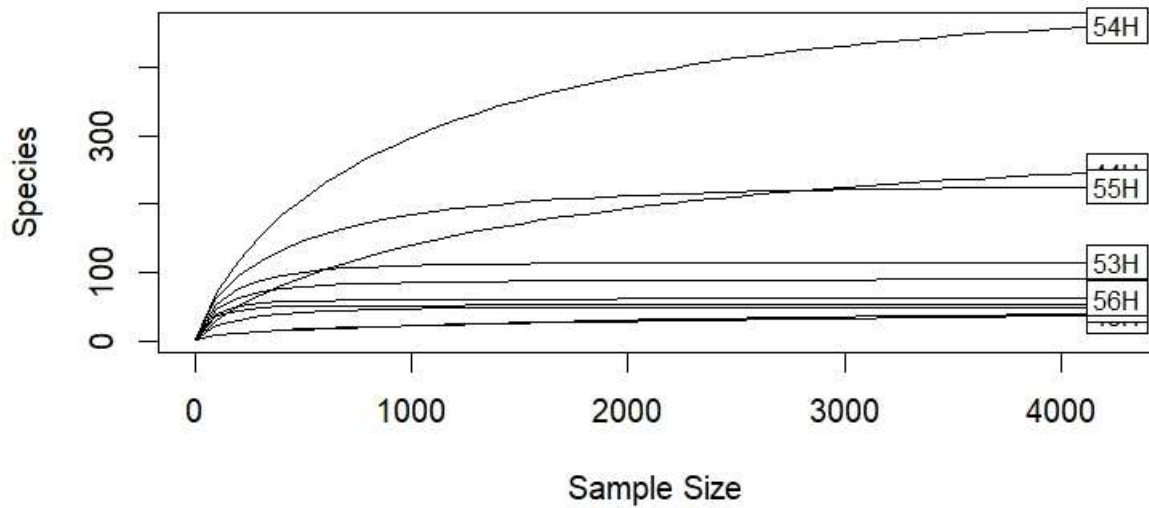


Figure11: Taxonomy rarefaction curves for the endometrial samples included Experiment 2.

In total, bacteria belonging to 18 phyla were observed. Specifically, 18 phyla were detected after the treatment (T1) while only 14 before (T0) (Figure 12).

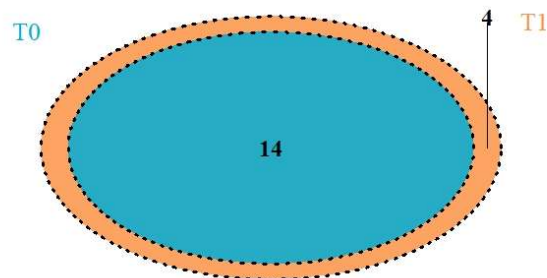


Figure12: Total number of phyla detected before (T0) and after treatment (T1).

At genus level, 299 different genera were identified. Among these, 92 were in common, while 173 and 34 were observed only after and before the treatment respectively (Figure 13).

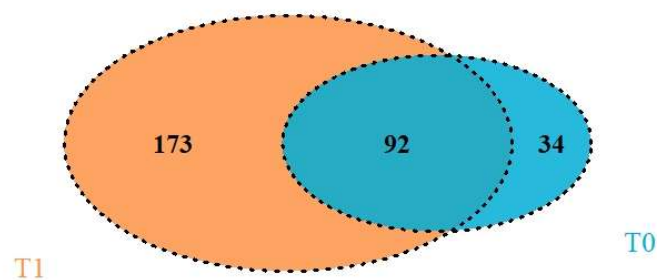


Figure 13: Total number of genera detected before (T0) and after treatment (T1).

The most represented phylum was Proteobacteria (Total: 36.8%; T0:34.1%; T1: 39.6%), followed by Firmicutes (Total: 33.2%; T0:38.7%; T1: 27.7%), Actinobacteriota (Total: 14%; T0:14.8%; T1: 13.2%), Bacteroidota (Total: 9.9%; T0:8.9%; T1: 11.1%) and Cyanobacteria (Total: 3.5%; T0: 2.9%; T1: 4.1%). These Phyla accounted for over 95% of the relative abundance, while the remaining Phyla showed a relative abundance <1,5%. Relative abundance at phylum level is shown in Figures 14 and 15. Relative abundances of all the 18 phyla are shown in the supplementary materials (S3).

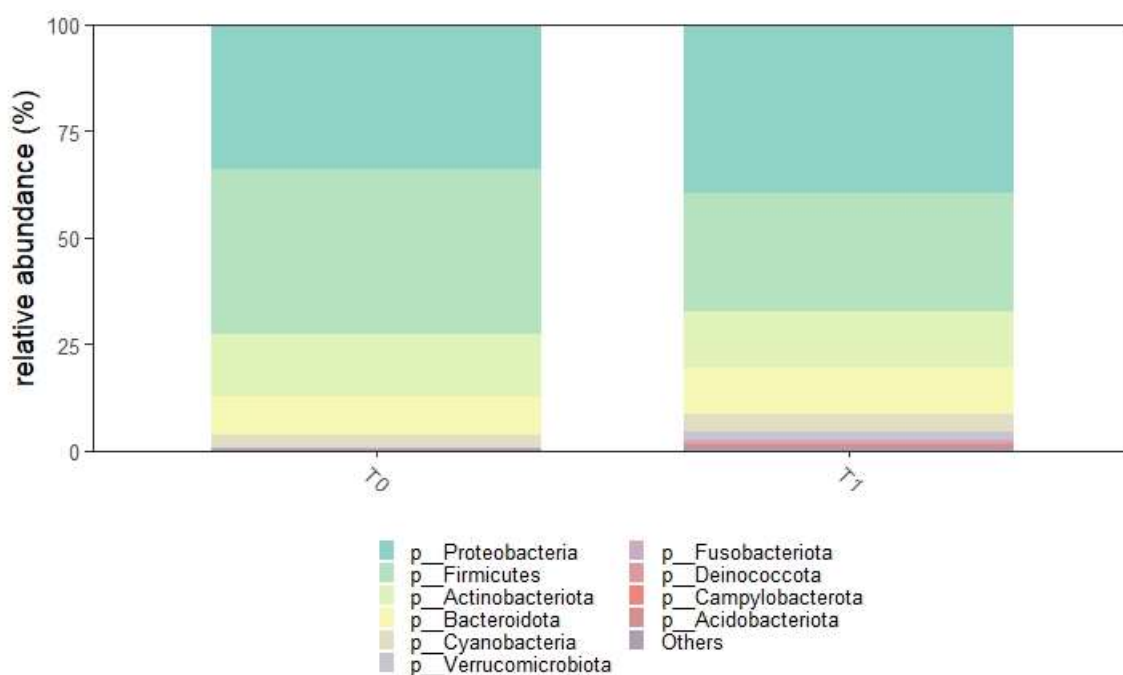


Figure 14: Relative abundance of the 10 most abundant phyla detected before (T0) and after (T1) treatment grouped according to time point.

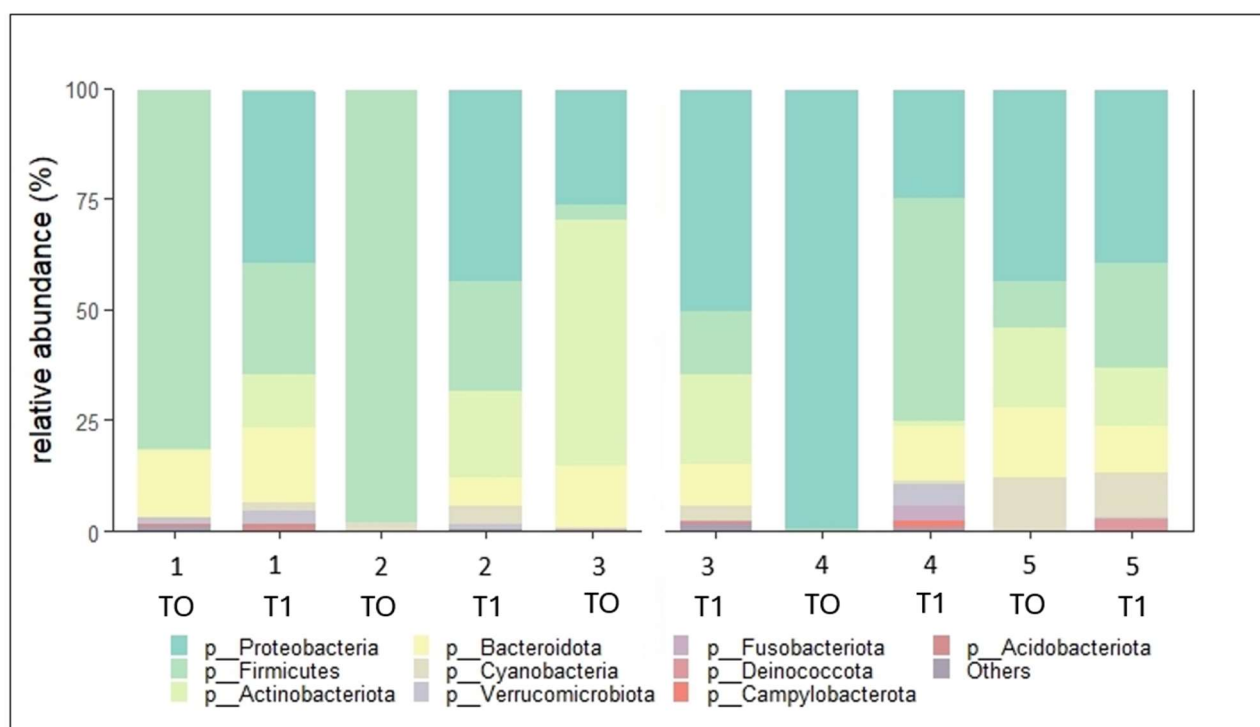


Figure 15: Relative abundance of the 10 most abundant phyla detected before (T0) and after (T1) treatment in each mare.

The most represented genus was *Streptococcus*, which accounted for 36.5% of total abundance before the treatment and dropped to 0.7% after the antibiotic. The same trend was observed for the genus *Escherichia-Shigella* (T0: 19.9%; T1: 1.9%) and *Corynebacterium* (T0: 10.1%; T1: 3.1%). On the contrary, relative abundance of *Staphylococcus* (T0: 2.9%; T1: 4.9%), *Pseudomonas* (T0: 1.6%; T1: 4.6%) and *Acinetobacter* (T0: 0.3%; T1: 5.4%) increased after the treatment. Relative abundance at genus level is shown in Figures 16 and 17. Relative abundances of the 40 most abundant genera are shown in the supplementary materials (S4).

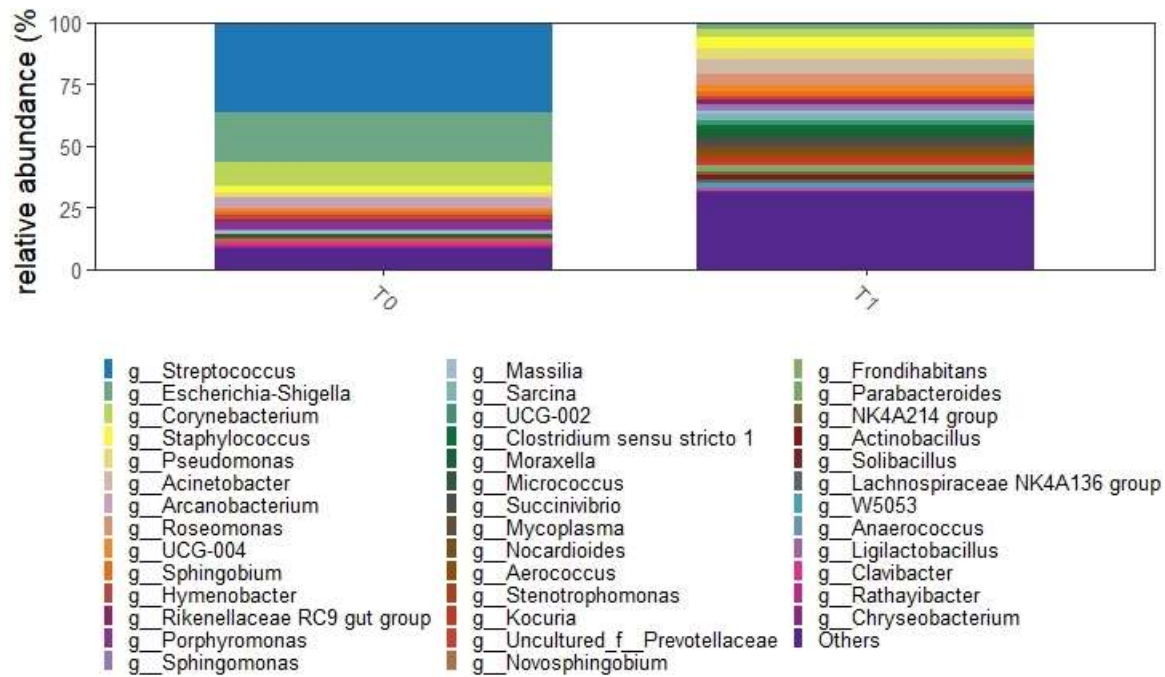


Figure 16: Relative abundance of the 40 most abundant genera detected before (T0) and after (T1) treatment grouped according to status.

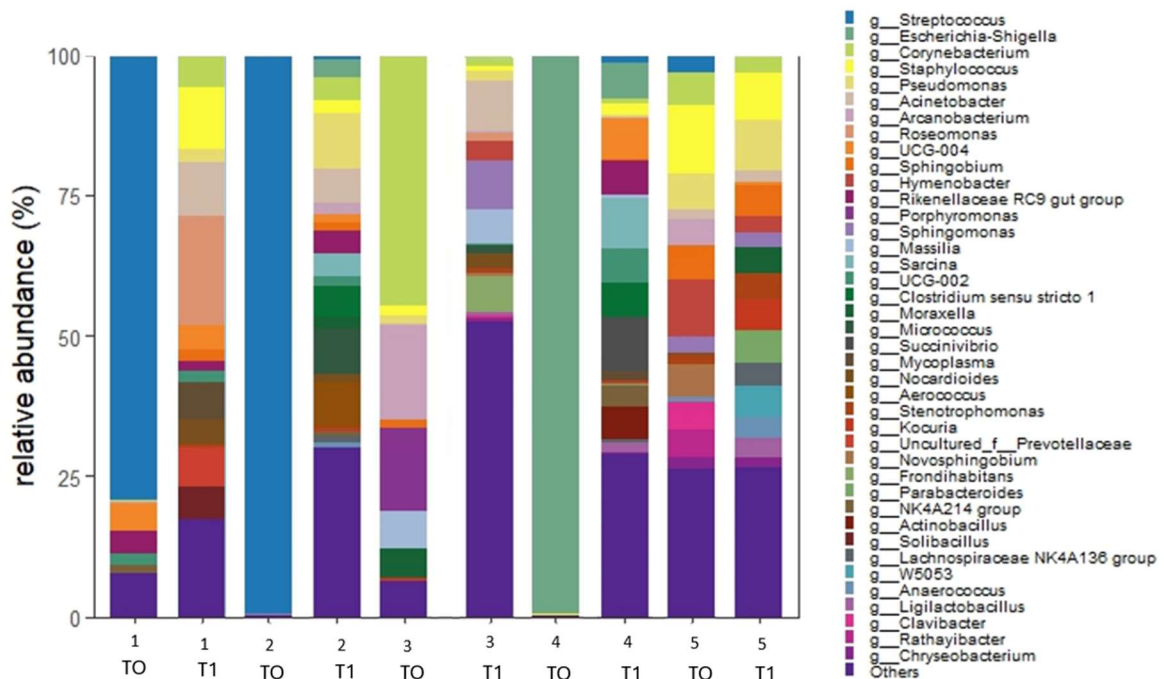


Figure 17: Relative abundance of the 40 most abundant genera detected before (T0) and after (T1) treatment in each mare.

Individually, we see that *Streptococcus* dominated the microbiome of mares 1 and 2, showing a relative abundance of 79% and almost 99% respectively. However, after antibiotic treatment, it decreased to less than 1% in both mares. The same trend was observed for the mare number 4, in which *Escherichia-Shigella* dropped from 99% to 6%. In Mare number 3, the relative abundance of *Streptococcus* at T0 is

<1%, and the microbiota is dominated by *Corynebacterium* (44%). At T1, *Streptococcus* abundance remained below 1%, while *Corynebacterium* drastically decreased (1.3%). Relative abundance of the genus *Staphylococcus*, that was isolated using the culture method, was <1%. In mare number 5, the genus *Streptococcus* accounted for 3.4% of relative abundance at T0, while it was not detected at T1.

3.3.2.2.1 Alpha and Beta diversities

An increase in alpha diversity was observed after the antibiotic treatment, as described through Shannon ($p=0.032$) and Simpson indexes ($p=0.032$). For Observed species and Chao 1 Indexes, the p -value was 0.35 (Figure 18)

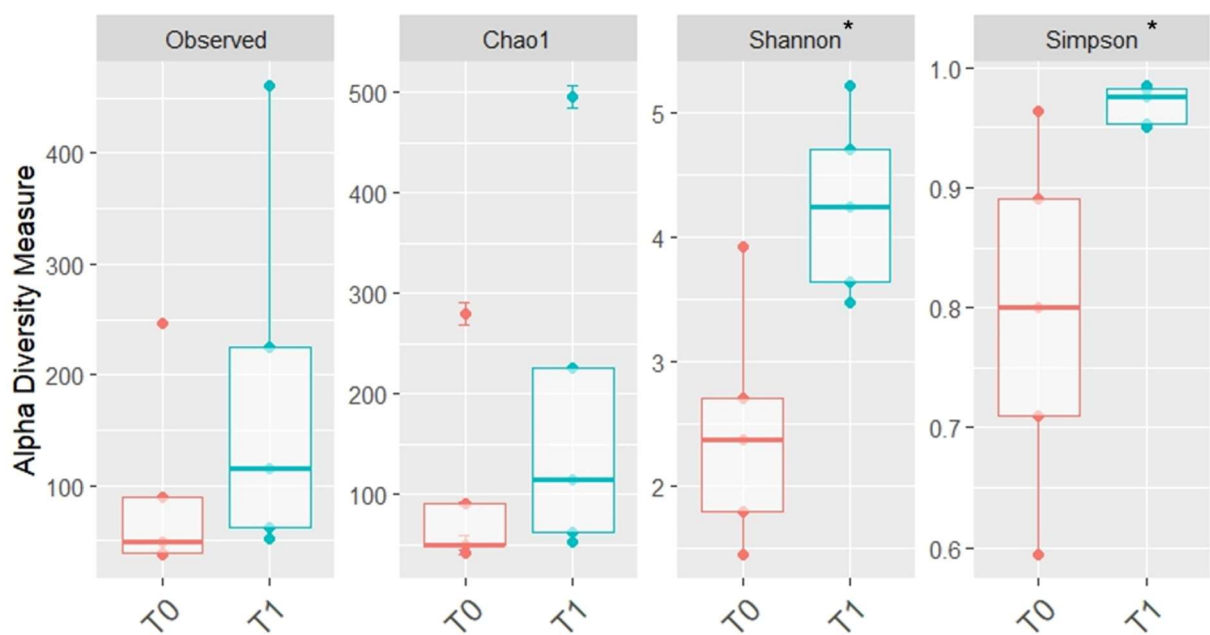


Figure 18: Variation in endometrial microbial diversity based on time point (before antibiotic treatment - T0, after antibiotic treatment - T1) assessed by observed richness, Shannon, Simpson, and Chao 1. * indicates a p -value < 0.05.

Beta diversity did not differ between the two phases of the study ($p=0.6$) (Figure 19).

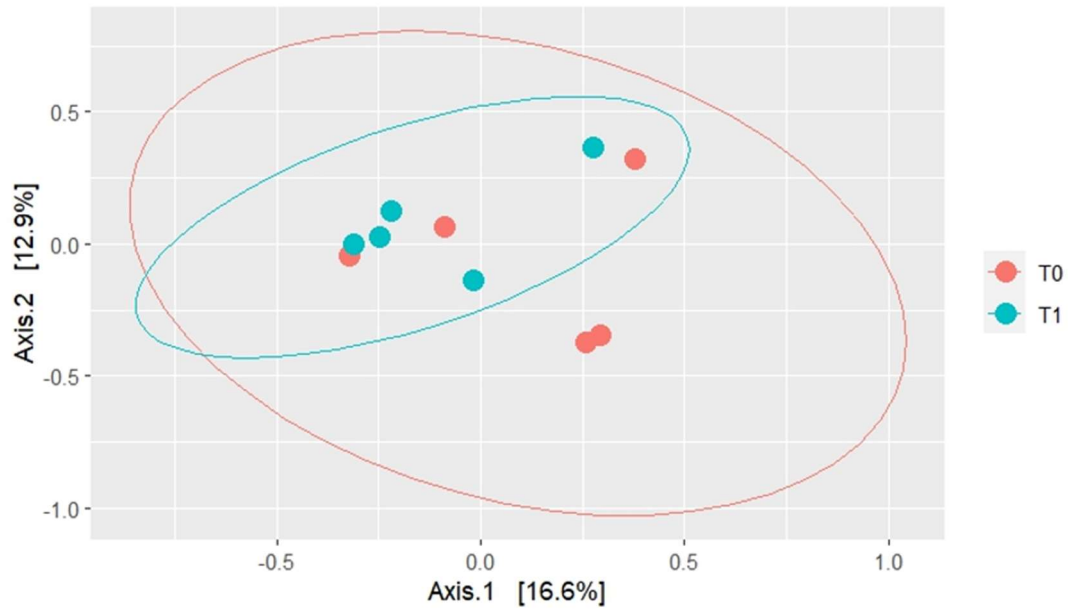


Figure19: Two dimensions plot by Bray-Curtis based on time point. The samples clustered together and significant differences were not observed.

3.3.3 Comparison between healthy and diseased mares

To compare healthy mares and mares with endometritis, only the samples from the healthy mares during the follicular phase were selected, since also the samples from mares in experiment 2 were collected during this phase. For mares with endometritis, only the samples before the treatment were used.

Alpha diversity was significantly higher in healthy mares (Shannon index, $p=0.018$; Simpson index, $p=0.02$; Observed species, $p=0.004$) compared to mares with endometritis (Figure 20).

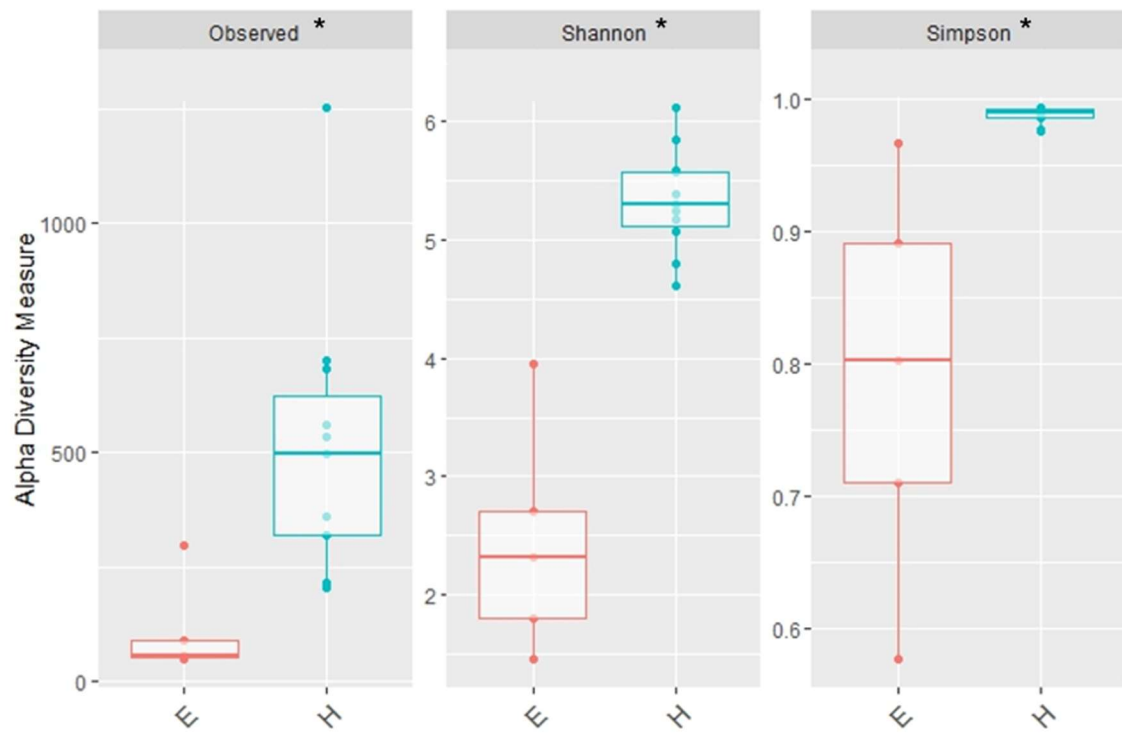


Figure 20: Variation in endometrial microbial diversity based on status (endometritis – E, healthy – H) assessed by observed richness, Shannon and Simpson. * indicates a p-value < 0.05.

Even beta diversity was significantly different ($p=0.001$), forming two separate clusters indicating two distinct bacterial communities (Figure 21).

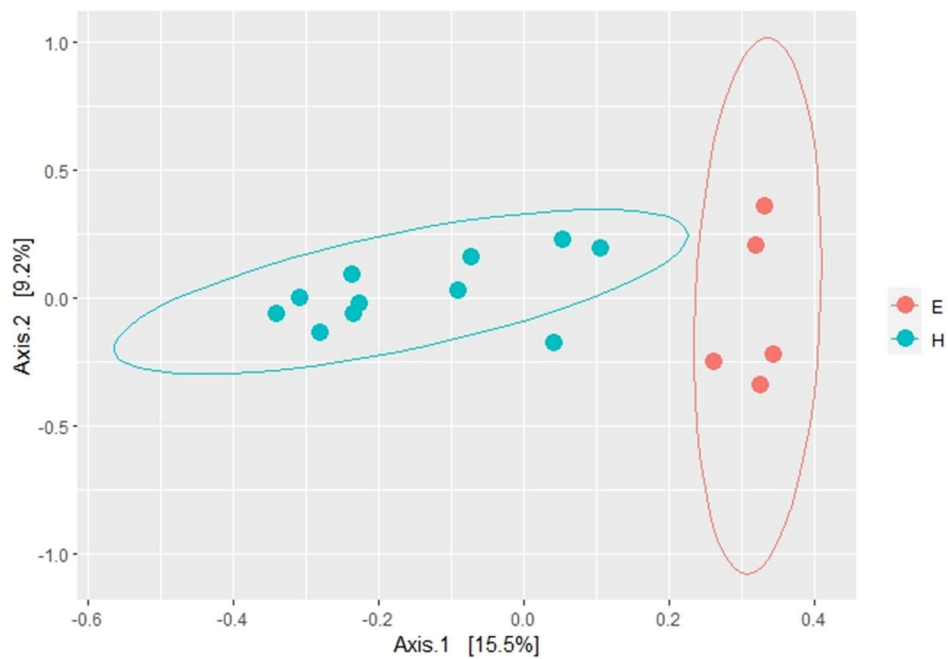


Figure 21: Two dimensions plot by Bray-Curtis based on status (endometritis – E, healthy – H). The samples form two separate clusters, showing significant differences.

3.4 Discussion

This study was divided into two different experiments: The first aimed to describe the microbiota of healthy mares and to observe whether there are differences between follicular and luteal phases. The second part investigated the microbiota of mares with endometritis diagnosed by clinical symptoms, reproductive history and positive cultural exam for known intrauterine pathogens.

However, two limitations of this study must be mentioned: the small sample size, especially in trial 2, and the different geographical origin of the mares in trials 1 (healthy) and 2 (endometritis), which did not allow any clear conclusions to be drawn about differences in the uterine microbiome of healthy and affected mares. In fact, Holyoak et al. [147] described that the uterine microbiome of mares from different geographical regions differs. Therefore, the analysis of differences between healthy and affected mares is only briefly discussed in the present study.

In experiment 1, we identified 24 different phyla, 20 during luteal phase and 23 during follicular phase. Furthermore, 567 different genera were identified, 492 during estrus and 389 during diestrus, showing an higher number of bacterial phyla and genera observed during follicular phase.

The microbiome of healthy mares was dominated by Firmicutes (39%), Proteobacteria (26.2%), Bacteroidota (15.3%) and Actinobacteriota (14.1%), which accounted for over 95% of relative abundance. It is difficult to compare different studies, however, some differences were observed with the recent papers of Holyoak et al. [147] and Heil et al. [149,192] where the most abundant phylum was Proteobacteria, followed by Firmicutes. On the other hand, as in our study, Firmicutes was the most represented Phylum in the vagina of healthy mares [169].

The most abundant genera were *Staphylococcus*, *Acinetobacter*, *Sphingomonas*, *Corynebacterium*, *Streptococcus*, *Rikenellaceae*, *Clostridium*, *Pseudomonas* and *Porphyromonas*. *Escherichia-Shigella* had a relative abundance of less than 1%. This result is particularly interesting because the most abundant genera colonizing the uterus of healthy mares include genera that are considered in the clinical literature to be common pathogens causing endometritis in horses, such as *Staphylococcus*, *Streptococcus*, *Pseudomonas* and *Escherichia* [4,149]. However, our findings are supported by the study of Holyoak et al. [147], who identified *Pseudomonas*, *Escherichia-Shigella*, *Streptococcus*, *Staphylococcus* and *Acinetobacter* among the most abundant genera in the uterus of healthy mares. Therefore, these genera are probably not only responsible for endometritis, but are normal constituents of the uterine microbiome of healthy mares. According to another recent paper by Heil et al. [166], *Klebsiella*, *Mycoplasma*, *Aeromonas* and *Citrobacter* were identified to dominate endometrial microbiome, accounting for 53–59% of the total at the genus level. These genera were not identified in

our study (*Klebsiella*, *Citrobacter*) or had a relative abundance <0.01% (*Mycoplasma*, *Aeromonas*). This is probably due to the different geographical origins, housing conditions and diet of the mares in our study [147]. In addition, different sampling methods, extraction kits and hypervariable regions were used in these two studies - all factors that can affect the consistency of results between different studies and make it difficult to compare different articles on microbiome [155,193,194].

This is the first study to investigate possible differences in the microbiome of the mare between estrus and diestrus. It was observed that the endometrial microbiome is different in the two phases of the cycle and a higher diversity characterizes the follicular phase. This result is in line with a recently published work on women, in which a higher diversity was described during the follicular phase [195]. Furthermore, variations in the vaginal microbiome have also been described in other animal species [196–200]. The changes we observed in the endometrial microbiome could be mainly explained by two factors. The first is hormonal fluctuation during the estrous cycle, a well-known factor that influences the overall structure and function of the reproductive tract microbiome [195]. The second is the presence of the cervix: this dynamic organ acts indeed as a barrier between the vagina and uterus to prevent the entry of infectious agents [201,202] during diestrus and pregnancy, while it is open during estrus, allowing the entrance of semen and elimination of mucus, excess spermatozoa, and contaminants introduced by breeding [203]. Therefore, if cervical ascension of bacteria through the cervix is the driving force shaping the uterine microbiome, this could explain the observation that the microbiome changes during the estrous cycle and that, as the cervix is open, a higher diversity is observed during the follicular phase [195].

These results may contradict the work by Barba et al. [169], who described the vaginal microbiota of Arabian mares in estrus and diestrus and found no statistically significant differences in alpha or beta diversity, from which they concluded that the vaginal microbiome is stable during the estrus cycle. It is worth noting that higher bacterial counts have been observed during estrus using culture-dependent methods. However, this work investigated the vaginal microbiome, which is not dependent on cervical opening and closing, and this factor could explain the stable microbiome, in contrast to our study that investigated the endometrial environment.

Furthermore, another recent abstract by Heil et al. [192] investigated the differences between the uterine microbiome in estrus and anestrus and found lower diversity and richness at the phylum level during estrus. They attributed this difference to the variance in immune responsiveness of the endometrium, that is under the influence of estrogens in the follicular phase, which enables a strong immune response and possibly a less diverse microbiome. These results are not consistent with our study, but it is worth noting that different cycle phases were sampled (anestrous in the study of Heil et al. vs diestrus in our study) and that the time-interval between the two samples was very different in the

two studies (maximum two weeks in our study, as opposed to several months in the study by Heil et al., that collected the first sample during the breeding season and the second in the subsequent non-breeding season). Further studies are needed to better understand the endometrial microbiome during the estrous cycle in the mare.

Regarding experiment 2, to the best of our knowledge, this is the first work that investigated and described the endometrial microbiome of mares with endometritis. Furthermore, we expanded further the research and investigated also the effects of antibiotic treatment, since the role of therapies for uterine infections in restoring a healthy balance in the uterine microbiome has not been studied yet in horses [4]. Antibiotic treatment is indeed a necessary treatment when bacterial endometritis is diagnosed [4]. The route of administration can be systemic or intrauterine, but there are conflicting data in the literature and both routes are used [2,4]. In the past, intrauterine administration was the method of choice [2,110], as the effects of antibiotic treatment on the intestinal flora are low, the local concentration is higher and the amount of antibiotic required is lower than with systemic administration [2,4]. However, systemic treatment is currently also widely used because the risk of iatrogenic contamination is lower, since repeated invasions of the cervix are avoided, treatment is possible regardless of the phase of the oestrus cycle and the antibiotic is evenly distributed in the uterine mucosa [2,111].

In the present study, the mares were treated with an intrauterine infusion of ceftiofur sodium, a drug of common choice for endometritis [117]. Although it is sometimes used empirically [117], it is a third-generation cephalosporin and should only be used in the presence of clinical signs, positive cultures and antimicrobial susceptibility testing [2], as was done in this study.

At T0, before the treatment, a total of 14 different phyla and 126 genera were identified, compared to 18 phyla and 265 genera after the treatment (T1). This data already gives an indication about an increased number of different bacteria in the endometrium at T1.

At T0 Firmicutes was the most abundant phylum (38.7%), followed by Proteobacteria (34.1%), Actinobacteriota (14.8%) and Bacteroidetes (8.9%). At T1, however, the most abundant was represented by Proteobacteria (39.6%), followed by Firmicutes (27.6%), Actinobacteriota (13.2%) and Bacteroidetes (11.1%). This drop in relative abundance of Firmicutes could be better understood by looking at results at the genus level, since *Streptococcus* is the main contributor to the relative abundance of this phylum.

At the genus level, the two most abundant genera at T0 were *Streptococcus* (36.5%) and *Escherichia-Shigella* (19.9%), confirming the results of bacterial culture that isolated bacteria belonging to these genera (namely, *Streptococcus equi zooepidemicus* and *Escherichia coli*) in the 5 mares. Furthermore,

Corynebacterium (10%), *Arcanobacter* (4.4%), *Staphylococcus* (2.9%), *Porphyromonas* (2.9%), *Pseudomonas* (1.6%) were identified as the most abundant genera.

After treatment, the relative abundance of *Streptococcus*, *Escherichia-Shigella* and *Corynebacterium* decreased to 0.7%, 1.9% and 3%, respectively. In contrast, we observed an increase in *Staphylococcus* (4.9%), *Pseudomonas* (4.5%) and *Acinetobacter* (5.4%). This decrease in the abundance of bacteria that were isolated by cultural examination and that dominated the microbiota at T0 shows the effectiveness of targeted antibiotic treatment performed according to the antimicrobial sensitivity test.

It is worth describing the situation of each mare individually, as each endometrial microbiome is specific and provides new and different information in this recent field of research.

Mares 1 and 2 were positive for *Streptococcus equi zooepidemicus* according to bacterial culture at T0 and this finding was confirmed by 16S analysis as the relative abundance of the genus *Streptococcus* was 79 and 99% of the total abundance, respectively. At T1, the abundance decreased to less than 1% and 1.1%, respectively, and the culture test was negative.

Mare number 4, which tested positive for *Escherichia coli*, had a relative abundance of over 99% of the genus *Escherichia-Shigella* at T0. At T1, the relative abundance decreased to 6.4%, while the result of the culture examination remained positive. The antibiotic treatment was therefore effective, even if it did not reduce the abundance to a minimal level, as was the case with mares 1 and 2. However, it is important to note that we were able to demonstrate the effect of the antibiotic treatment using 16s sequencing, whereas we were unable to do so with the culture test. The advantage of microbiota analysis is that even bacteria that cannot be cultured, either because they are dead or because the culture conditions are not right or because their growth is masked by other species, can be identified using the 16S technique [46]. This allows us to get a picture of the entire endometrial microbiota and not just the few bacteria that can grow in vitro that are identified with the culture technique [148], which leads to an underestimation of the diversity of microbes in a given niche [149].

The result of mare number 3 was quite surprising, since the endometrial microbiota at T0 was dominated by the genus *Corynebacterium* (44.4%). This genus, as we showed in trial 1, is a common constituent of microbiome of healthy mares. However, there are increasing reports associating *Corynebacterium uterequi* with endometritis and pregnancy complications, suggesting a potential role of *Corynebacterium* strains in the disease [39,40]. Therefore, clinical signs and subfertility that led the mare to be included in the study could have been due to *Corynebacterium* infection rather than *Streptococcus equi zooepidemicus* (relative abundance of *Streptococcus* was less than 1% at T0) that was identified by bacterial culture. The antibiotic treatment was, however, effective also against *Corynebacterium* since relative abundance decreased to 1.3% at T1.

Finally, mare number 5, also positive for *Streptococcus equi zooepidemicus*, showed a modest relative abundance of *Streptococcus* of 3.4% at T0 while it was not detected at T1.

Investigating the overall diversity described through alpha diversity measures, we showed that after the antibiotic treatment, the microbiome is more diverse, according to Shannon and Simpson indexes. At T0, there is a dominance of one or few species that account for most of the abundance in the environment. At T1, on the other side, there is more evenness, since the bacterial are more equally distributed to form a more balanced microbiota.

Therefore, from experiment 1 and previous studies [147,166], we can affirm that even bacteria that are commonly considered uterine pathogens are normal residents of the microbiota of healthy mares, such as *Streptococcus*, *Staphylococcus*, *Escherichia-Shigella*, *Pseudomonas*, *Corynebacterium*. However, in mares with endometritis, these bacteria are overrepresented and dominate the uterine microbiome, that is therefore in a state of dysbiosis.

If we compare the mares from trial 1 (healthy) with the mares from trial 2 at T0 (endometritis), we can see a decrease in bacterial abundance and diversity in the affected mares. As already mentioned, this comparison is severely limited by the different geographical origin, which is why the results cannot be regarded as clear and reliable evidence of differences between the two groups. However, they are confirmed by many studies conducted on the cow and recently reviewed by Çomlekcioglu et al. [162]. Although the specific composition of a healthy uterine microbiota is still controversial, we now know that bacterial diversity is essential for the maintenance of uterine health. In general, loss of bacterial diversity (characteristic of a state of dysbiosis) contributes to the development of uterine infections, which are associated with an increased relative abundance of known intrauterine pathogens [161,162]. Becker et al. describing the uterine microbiota of healthy and affected cows also observed lower bacterial diversity in affected animals, with the microbiome dominated by one or a few genera (including *Escherichia-Shigella*, *Histophilus*, *Porphyromonas* and *Bacteroides*), which accounted for up to 99% of the relative abundance [181], as was also observed in our study.

In the present study, we observed an increase in overall bacterial diversity associated with antibiotic treatment and a decrease in the abundance of uterine pathogens that dominated the microbiome at T0 (*Streptococcus*, *Escherichia* and *Corynebacterium*). These results are consistent with studies conducted in cows: Jeon et al. described that a single subcutaneous injection of ceftiofur crystalline free acid in animals with metritis resulted in an increase in uterine bacterial diversity, as measured by the Shannon index, and a reduction of *Fusobacterium*, a known uterine pathogen [204]. In another study on cows with metritis treated intramuscularly with ceftiofur, ampicillin trihydrate or untreated, success in curing metritis was associated with an increase in the diversity of the uterine microbiome and a decrease in the relative abundance of pathogenic bacteria such as *Bacteroides*, *Porphyromonas* and

Fusobacterium [165]. In agreement with the results of previous studies, we can therefore confirm that intrauterine treatment with ceftiofur, when administered to mares with clinical endometritis and according to the antimicrobial sensitivity test, is beneficial for the uterus in a state of dysbiosis due to endometritis by effectively restoring bacterial diversity and decreasing abundance of pathogens in the uterus.

Finally, comparing the results of bacterial culture and 16S, in most of the mares we saw a substantial agreement, since the bacteria isolated in culture also dominated the uterine microbiota according to sequencing technique. However, in one mare positive for *S. zooepidemicus* the relative abundance of the genus *Streptococcus*, was low and not different from the one observed in healthy mares. On the contrary, a high relative abundance of *Corynebacterium* was detected with 16S but not with culture technique. This result could be explained by the differences in techniques, as sequencing can identify dead or fragmented microbial DNA in addition to living organisms and thus possibly detect dormant or biofilm-producing bacteria. On the contrary, dead and fragmented DNA does not replicate in the female reproductive tract and is therefore not detected by traditional culture methods [149]. Another explanation could lie in the sampling technique, as only a small segment of the uterus is sampled with uterine swabs or brushes and therefore focal infections may have been missed by one or the other sample, leading to different results between the two techniques [4,66]. A solution could be represented by sampling with low-volume lavage, which, although used in previous studies [147,149], is more difficult to perform, time-consuming and susceptible to contamination if specific double-guarded systems are not used [147].

3.5 Conclusions

In conclusion, we demonstrated that the equine uterus hosts a resident microbiota during estrus and diestrus. In estrus, the microbiota is more diverse and tends to be richer, probably due to hormonal fluctuations during the cycle and the presence of the cervix, which opens during the follicular phase and allows cervical ascension of bacteria from the vagina. We also demonstrated that bacterial genera commonly considered pathogenic are normal components of the uterine microbiota of healthy mares (e.g. *Streptococcus*, *Escherichia-Shigella*, *Corynebacterium*). However, in mares with endometritis, they are overrepresented and dominate the endometrial microbiome, which is in a state of dysbiosis characterized by a reduction in bacterial diversity. Treatment with intrauterine infusions of ceftiofur sodium resulted in the restoration of a more diverse microbiome.

CHAPTER 4

Ozone therapy

4.1 Introduction

Although subfertility in mares has been extensively researched and discussed, it still represents a complex diagnostic and therapeutic challenge for practitioners and researchers involved in horse breeding industry and results in significant economic losses [10]. Unfortunately, even when fertile semen is used and multiple inseminations are performed according to good veterinary practice, many mares fail to become pregnant every year (the so-called “barren mares”) [205]. It is often difficult to identify a single cause for the reduced fertility of these mares, but certainly the status of the endometrium is one of the main factors [18,206]. In addition, both clinical and subclinical endometritis are the main causes of mare infertility [18].

Infectious endometritis is considered to be one of the main causes of infertility and can affect up to 60% of infertile mares [18]. It is characterized by the presence of one or more microorganisms in the reproductive tract, which were isolated from 31% of over 8000 uterine samples examined in a large study [2,27]. The etiologic agents commonly isolated in equine endometritis are *Streptococcus equi* subsp. *zooepidemicus*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Klebsiella pneumoniae* [4]. In addition, yeasts, more commonly *Candida spp*, and fungi can also cause endometritis, and although these infections account for only 1 to 5% of endometritis in mares, they can be extremely difficult to resolve [42]. Traditional treatment of infectious endometritis involves the administration of antibiotics and/or alternative non-antibiotic therapies in conjunction with uterine lavage and ecboic agents [2]. As traditional therapies are not always effective [6] and considering the increasing evidence of AMR, the development of alternative therapies such as ozone is a compelling field of research in the treatment of mare endometritis. Indeed, according to the World Health Organisation, antimicrobial resistance is one of the greatest global threats to human public health [131], and there is growing evidence that AMR is widespread in bacteria isolated from the equine reproductive tract [27,207].

Ozone was discovered in 1839 by Schönbein [130], and medical applications of ozone date back to the end of the nineteenth century: in 1881 it was already mentioned as a disinfectant in a book on diphtheria [208] and in 1892 an article was published describing the administration of ozone for the treatment of tuberculosis [209]. In addition, ozone was used during the First World War to treat German soldiers affected by gaseous gangrene and to prevent and suppress wound infections [210].

Ozone is a highly unstable gaseous molecule made of three oxygen atoms with a cyclic structure that forms naturally from oxygen by the action of ultraviolet light and electrical discharges in the atmosphere [211,212]. It is 1.6 times denser and 10 times more soluble in water than oxygen. It is the third strongest oxidising agent after fluorine and persulphate. Ozone is an unstable gas that cannot be stored and should be used immediately as it has a half-life of 40 minutes at 20°C and 140 minutes at 0°C [213]. It

is produced by ozone generators through a high-voltage gradient, starting from pure oxygen, to obtain a gas mixture containing at least 95% oxygen and up to 5% ozone. For clinical purposes, the ozone concentrations range is usually between 10–45 µg/ml [208,214].

Its antimicrobial properties are based on the high oxidative potential of this gas, which attack the components of cell membranes, cell envelopes, cytoplasm, spore envelopes and viral capsids, leading to the destruction of bacterial cell walls and cytoplasmic membranes [212]. This causes an increase in permeability, which ultimately results in the entry of ozone into the cells [212]. In addition, ozone therapy is also capable of damaging nucleic acid and stimulating the innate immune system to fight microorganisms [212,213]. In this scenario, ozone could represent an important resource to reduce or circumvent the need for antibiotics thanks to its antimicrobial properties [213,214].

The function of ozone is similar to that of a prodrug, as it is modified upon reacting with molecules to develop more active substrates, triggering an endogenous reaction cascade [215]. Indeed, ozone therapy induces moderate oxidative stress when it interacts with lipids, and this interaction increases endogenous antioxidant production, local perfusion, and oxygen delivery, and enhances immune responses [215,216]. Due to its multiple properties, ozone therapy has been shown to strengthen the immune system [217], optimize tissue regeneration mechanisms, stimulate granulation and epithelialization processes and reduce inflammation in various pathological conditions [218–221].

In recent years, ozone has become increasingly popular in veterinary and human medicine due to its effectiveness in the treatment of reproductive disorders that could affect female fertility [222]. In addition, ozone therapy is also used to treat mastitis, osteoarthritis, laminitis, loin pain, wounds, herniated discs and eye diseases [223].

Riger Spray® (Novagen, Podenzano, PC, Italy) is a product consisting of ozonized sunflower oil. Its efficacy in improving reproductive performance and treating common reproductive diseases such as endometritis and retained placental membranes in cows, goats and ewes has been widely reported [134–138,224–229].

However, the term “ozone therapy” includes a wide range of treatments and pharmaceutical forms with different mechanisms of action such as gas, foam, beads, ozonated water solutions and oil. This heterogeneity of protocols and formulations limits standardization and may be the reason for the different and sometimes contradictory results reported in the literature [145].

Two different studies were therefore carried out to investigate the effects of ozone therapy *in vivo* and *in vitro* in more detail.

The first study aimed to describe the safety and effects of the application of an ozone foam spray treatment (Riger Spray®, Novagen, Podenzano, PC, Italy) on the endometrium of subfertile mares, as to our knowledge no articles on the effects of treatment with this ozone foam spray in mares have been published to date.

The aim of the second study was to evaluate the *in vitro* antimicrobial activities of different ozone formulations, either as gas, ozonated water or ozonated oil, against six different microorganisms involved in equine endometritis, namely *Escherichia coli*, *Staphylococcus aureus*, *Streptococcus equi* subsp. *zooepidemicus*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae* and *Candida albicans*, in order to provide insights for clinical use in equine endometritis.

4.2 Safety and effects of a commercial ozone foam preparation on endometrial environment and fertility of subfertile mares

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4.2.1 Materials and methods

4.2.1.1 Animals and clinical records

Thirty-three Standardbred mares with an history of subfertility were referred to the Veterinary Teaching Hospital (OVU) of the University of Turin (Italy) and were submitted to a complete examination of the reproductive tract, including transrectal palpation, ultrasound examination (MyLabOne, Esaote, Italy), vaginal speculum examination, bacteriological, cytological and histological evaluation. Only subfertile mares negative to bacterial culture were included in this study because they were privately owned and therefore, due to the will of the owners, positive mares with a diagnosis of infectious endometritis were treated with gold standard therapy, antibiotic therapy, according to the results of antimicrobial sensitivity tests.

These mares had an average age of 14.08 ± 3.80 years (median: 14 years; range: 8-19 years), an average weight of 478.12 ± 41.32 kg (median: 479 kg, range: 410-548 kg) and a body condition score between 2.5 and 4 [230].

The mares were pluriparous and were determined to be subfertile because failed to get pregnant during the previous reproductive season after three or more inseminations performed according to good veterinary practices with semen from fertile stallions or underwent early embryonic loss [231].

The mares were housed in two different racehorse farms near Turin (Italy) under the management of the same veterinary team. They were kept in outdoor paddock and were fed with hay and pellet feed, and water was supplied *ad libitum*.

4.2.1.2 Treatment

The treatments were performed between February and April 2022 during the follicular phase.

An ultrasound examination was performed every two days to detect the presence of estrous, established through the detection of a dilated cervix, a >35 mm follicle, the absence of a corpus luteum, endometrial oedema, and a relatively soft uterus on palpation [232].

Mares were randomly divided into two groups:

Control group (CG): 8 mares were treated with an intrauterine infusion of lactated Ringer's solution.

Ozone group (OG): 16 mares were treated with an intrauterine infusion of an ozone-based foam spray product (Riger Spray®, Novagen, Italy).

Before all the treatments, the rectal ampoule was gently emptied, the tail was wrapped and the perineum washed with water and povidone iodine (Betadine; MEDA PHARMA SpA, Milan, Italy) for three times, then dried with paper towel.

Subsequently, for the mares in CG, 20 ml of lactated Ringer's solution were instilled in the uterus through a syringe and an insemination catheter.

For the mares in OG, Riger Spray®, was instilled in the uterus with a sterile insemination catheter and a special 10-cm long fitting, keeping the applicator of the can pressed for five seconds, as described for the treatment of bovine endometritis [135].

The mares in the two groups were homogeneous for age (CG: 13.25 ± 4.40 years; OG: 14.50 ± 3.60 years), parity, and body condition score.

4.2.1.3 Experimental design

In order to assess the safety of ozone foam spray treatment on uterine environment, uterine swabs for bacteriological examination were collected before the ozone treatment (T0), and after a period of 18 to 27 days (T4), when the subsequent estrous phase was detected. Subsequently, samples for endometrial cytology were collected at T0, 24 h after the treatment (T1), after one week (T2), after two weeks (T3) and at T4. Finally, endometrial samples for histological examination were collected at T0 and T4. Ultrasound examination was performed at T0, T1, T2, T3 and T4.

To determine the effect of ozone treatment on reproductive parameters of subfertile mares, the mares were inseminated at the cycle after the treatment. After the detection of a follicle bigger than 35 mm and the presence of endometrial edema, 1500 IU of Human Chorionic Gonadotropin (Corulon, MSD Animal Health S.r.l., Segrate Milan, Italy) were administered intravenously to induce ovulation. Artificial insemination with chilled semen of stallion of known fertility was performed 24 h after ovulation induction. The number of mares that successfully became pregnant after the first insemination, at the end of the 2022 reproductive season, the number of inseminations required for pregnancy and the episodes of early embryonic loss were recorded.

4.2.1.4 Cultural examination

The samples for cultural examination were collected with a double guarded uterine swab (Minitube GmbH, Tiefenbach, Germany). Briefly, the instrument was introduced into the vagina and through the cervix. Once the tip reached the uterine body, the outer tube was retracted enough to expose the swab, that was gently rotated against the uterine wall. Finally, the swab was retracted into the outer tube prior to removal from the uterus. Subsequently, the swab was inserted in AMIES transport medium (Minitube GmbH, Tiefenbach, Germany) and transported, within 2-8 hours at refrigeration temperature, to the Microbiology Laboratory of OVU for the examination. Each uterine swab was resuspended in 3 ml of a non-selective enrichment medium (Brain Heart Infusion Broth Oxoid) and incubated overnight at 37°C. Then, each cultural sample was streaked on agar with 5% sheep blood plates for a further 24 hours at 37°C. The isolates were identified by MALDI-TOF/MS (Bruker, Germany).

4.2.1.5 Cytological examination

The samples for cytological examination were collected with a double guarded uterine cytobrush (Minitube GmbH, Tiefenbach, Germany) as previously described [64]. Briefly, the instrument was introduced into the vagina and through the cervix. Once the tip reached the uterine body, the outer tube was retracted enough to expose the cytobrush, that was gently rotated against the uterine wall. Finally, the cytobrush was retracted into the outer tube prior to removal from the uterus. The cytobrush was gently unrolled on a glass slide and dried at air. The slides were brought to the Animal Reproduction Laboratory of the Veterinary Teaching Hospital (OVU) of the University of Turin (Italy).

The cell smears were fixed and stained using Diff-Quick stain (Medion Diagnostics AG, Düringen, Switzerland), and evaluated, blindly, by a pathologist at 400X magnification over multiple fields. Ten microscopic fields were randomly selected in multiple areas of the slide and the average number of PMN/hpf was determined [28].

4.2.1.6 Histological examination

The samples for histological examination were collected transcervically from the utero-cornual junction, using a sterilized forceps for uterine biopsy (Equivet, Kruuse, Marselv, Denmark), as previously described [76]. Uterine biopsy samples were immediately placed in 10% buffered formalin. Formalin fixed biopsies were paraffin embedded; 5µm sections were then stained with Haematoxylin and Eosin, according to the standard procedure. Histological observation was performed, blindly, by a pathologist, and biopsy samples were graded according to Kenney and Doig classification [76].

4.2.1.7 Statistical analysis

For statistical evaluation the data were analyzed with the software GraphPad Prism for Mac, ver.9.4.1. The analysis was considered significant with a P value <0.05 . Data were tested for normality using Kolmogorov and Smirnov test. Differences in number of PMN/hpf and inseminations required for pregnancy within and between groups were compared by means of Friedman and Mann-Whitney tests, respectively. Differences between the two groups in the number of mares pregnant at the first insemination and at the end of the season were tested by means of Chi-squared test. Regarding the histological classification, differences within the two groups were tested by means of McNemar's Chi-squared test.

4.2.2 Results

4.2.2.1 Uterine culture

Out of 33 mares sampled, 9 (27.30%) were positive to bacterial culture. Therefore, they were excluded from the study. In the remaining 24 mares, the uterine culture was negative at T0 and the animals were enrolled in this study. All 24 mares resulted also in culture negative at time T4.

4.2.2.2 Cytology

The number of PMN/hpf at the different time points is shown in Figure 22.

At T0, 14 mares (OG n=9; CG n= 5) showed a normal to mild grade of inflammation (PMN/hpf between 1 and 2) and 10 mares (OG n= 7; CG n= 3) showed a mild to moderate grade of inflammation (PMN/hpf between 3 and 5) [48]. No differences were observed between the two groups at T0 ($P>0.1$).

At T1, 24 hours after treatment, there was a statistically significant increase of endometrial inflammation in the OG mares compared to T0 ($P<0.05$) and compared to CG at the same time point ($P<0.05$).

Also in the CG mares, the number of PMN/hpf slightly increased, but the difference between T0 and T1 was not statistically significant ($P>0.1$).

Already at T2, at one week after treatment, the resolution of inflammation was noted in OG compared to T1 ($P<0.05$) and at T2, T3 and T4 the number of PMNs was not different from T0 in both OG and CG (OG: $P>0.1$; CG: $P>0.1$).

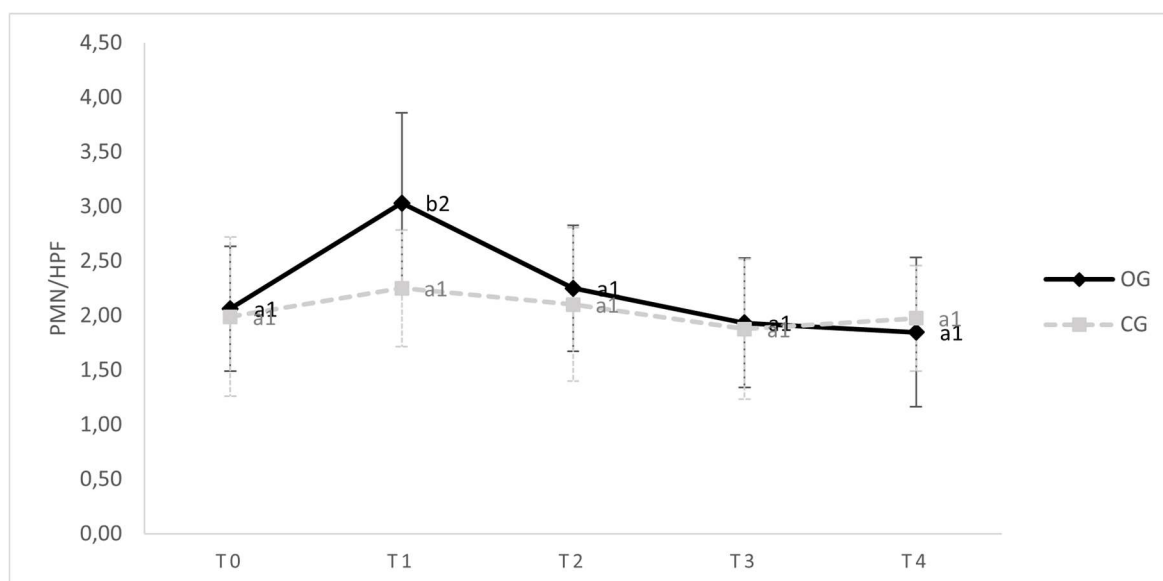


Figure 22: Number of PMN/hpf observed with uterine cytology in mares treated with ozone (OG) and in the control group (CG) at different time points. a, b Different letters indicate a statistical difference in the same group ($P < 0.05$). 1, 2 Different numbers indicate a statistical difference between two groups ($P < 0.05$).

4.2.2.3 Histology

All the mares' endometria were graded as Kenney-Doig score IIA (OG n=9; CG n=4) or IIB (OG n=7; CG n=4). An increase in the severity in the endometrial Kenney-Doig score at the estrus successive to the treatment was not observed ($P > 0.1$).

In addition, no differences were observed in the endometrial histological classification between the two groups ($P > 0.1$) neither before nor after treatment ($P > 0.1$). Besides, no signs of fibrosis, necrosis or endometrial degeneration were observed after the treatment.

4.2.2.4 Effects on clinical and reproductive parameters

After the treatment with ozone or with Lactated Ringer solution, no mare showed signs of discomfort or pain. In 6/16 mares (37.50%) of the OG in the day after treatment the presence of 1-2.5 cm of grade II (semi-echogenic) or III (hypoechoic) [49] fluid in the lumen of the uterus was observed, but it was not noticeable at the second examination, after one week (T1).

At the end of the season, 13/16 mares (81.30%) in the OG and 5/8 mares (62.50%) in the CG successfully became pregnant, but no statistically significant difference has been observed ($P > 0.1$). At the first insemination after treatment, 6 mares in the OG (33.30%) and 1 in the CG became pregnant (12.50%) ($P > 0.1$). Regarding the number of inseminations required for pregnancy, it tended to be lower in the OG mares (1.69 ± 0.06) than in CG mares (2.60 ± 0.89) ($P = 0.0711$). No early embryonic losses were observed.

4.2.3 Discussion

To the best of the authors' knowledge, the safety and the effects on equine endometrium of Riger Spray® have never been described, although in cows, ewes and goats this ozone formulation has been shown to improve fertility rate, due to a healthier endometrial environment [134].

One of the major causes of fertility reduction in the mare is generally identified in endometritis, the local inflammation of the lumen of the uterus [18], that generally develops because of the establishment of microbial contamination of the organ, but also because of the inability of the mare to clear the physiological inflammatory response after breeding [4]. Furthermore, chronic inflammation or infection of the endometrium could also lead to the development of other endometrial diseases, such as endometrosis (fibrosis of endometrial glands) [233] and angiosis (degenerative diseases of vessels), that are important causes of subfertility as well [74].

In our study, to assess the effects of ozone on the uterine environment, endometrial culture, cytology and histological examination were performed, all procedures that are routinely used for the diagnosis of uterine illnesses in mares [75,234,235]. Positive bacterial cultures have been reported in 12-60% of barren mares [25,28,235,236] and the results of our study are in accordance with these authors, since 27.3 % of the mares presented a bacterial endometritis.

However, the failure to isolate bacteria does not eliminate the presence of endometritis, since this disease may be difficult to diagnose as not all the mares show positive bacteriological and/or cytological examination and evident clinical signs. Cytology is considered to be twice as sensitive than culture in the diagnosis of endometritis, and endometritis has to be suspected in mares with a history of subfertility [237]. The mares in the present study, although negative to bacterial culture, had a previous history of subfertility and showed a normal to moderate grade of endometrial inflammation.

The aim of Riger Spray® and other products composed of ozonated oil is to obtain formulations containing ozone that are practical and easy to store and use in field conditions, since ozonated oil improves the short half-life of ozonated water or saline and O₂-O₃ gas mixture [238,239], avoiding the need of an ozone generator equipment. In the present study we decided to use ozonated oil in a single treatment, as previously described for bovine endometritis and recommended by the producer [134,227]. However, this decision is supported by the findings of Camargo Ferreira et al. [61] that observed the *in vitro* germicidal action of a [240] single treatment with ozonated oil against *Pythium insidiosum* isolated from horse. Interestingly, to obtain the same effect with O₂-O₃ gas mixture, it was necessary to perform the treatment for three consecutive days, since a single exposure to gas was ineffective. Furthermore, since the amount of peroxide in the oil after the ozonation process is generally considered to be correlated with the antimicrobial action, it was observed that only ozonated oil with a

peroxide index higher than 500 mEq kg⁻¹ was able to inactivate *P. insidiosum*. Also Ávila et al. [143] recently proved the in vivo antimicrobial effect of a single intrauterine treatment with ozonated oil with peroxide index of 600 mEq kg⁻¹. Riger Spray® is made with a sunflower ozonated oil that has a peroxide index higher than 4000 mEq kg⁻¹.

Only a few studies have been performed about the use of ozone in the mare in the previous years, but recently, due to the ever-increasing attention that this molecule is eliciting in the equine reproduction field, different studies have been published: Camargo Ferreira et al. [140] proved the ability of uterine insufflation with an oxygen–ozone gas mixture to induce endometrial angiogenesis and the same effect has been observed in healthy women treated with ozonated saline flushing [241]. Therefore, it can be useful in cases of subfertility since blood supply is necessary for hormonal signaling, uterine contractility, placentation and feto-endometrial interactions [18] and blood flow is diminished in mares with endometrial degeneration [141].

Almeida et al. [242] observed that intrauterine ozone therapy can cause controlled and transient systemic oxidative stress in healthy mares. This result is promising since ozone therapy is described to act inducing a controlled oxidative stress that stimulates an adaptive antioxidant response [210,243] and oxidative stress has been shown to be associated with endometritis in the mare [244].

Furthermore, Ávila et al. [143] have shown the effectiveness of ozone therapy in the treatment of endometritis. In particular, although the ozonated sunflower oil was not as effective as the O₂-O₃ gas mixture in the reduction of inflammation (the number of PMNs did not change in the cycle after treatment), its antimicrobial property against the bacteria present in the equine uterus has been proved. Mazzuchini et al. [245] observed that the number of PMNs tended to be higher 24 hours after the treatment with ozonated saline solution compared to the control group.

The results of our study are in accordance with Mazzuchini et al. [245] and Ávila et al. [143], since in the mares treated with ozone an increase in the endometrial inflammation has been observed the day after treatment, but it completely resolved after one week, and the number of PMN was not different in the cycle after the treatment compared to the previous one. This inflammation, that is a natural response of the equine uterus to the introduction of exogenous materials such as semen, extender, bacteria, debris and also air [246,247], is probably due to the fact that Riger Spray® is an oil-based product, that is more aggressive on endometrium [248]. Therefore, this was not able to reduce the number of PMNs, as described for the gaseous form [143] and the ozonated distilled sterile solution [139].

However, Riger Spray®, despite its foam nature, is well tolerated by the equine uterus and no adverse reactions had been observed in the mare, such as a persistent exaggerated inflammatory response or

abdominal discomfort. Furthermore, it did not predispose to a uterine unhealthy environment since no bacterial infections were detected after treatment.

The safety of Riger spray® was also investigated from the histological point of view and we assessed that this ozone presentation is not dangerous and detrimental for intrauterine use in the mare and do not cause chronic inflammatory changes in the endometrium, as previously described for other treatments like povidone-iodine [249] or enrofloxacin [250].

Riger Spray® has proved to be safe also according to clinical and reproductive parameters, since after treatment 81% of the mares successfully got pregnant, without any episode of early embryonic loss. No statistically significant differences between the two groups in the number of mares pregnant at the first cycle and at the end of the season were noticed, but the number of inseminations required to obtain the pregnancy tended to be lower in OG mares. Therefore, further investigations are needed increasing the sample size to observe if Riger spray® is not only safe but also effective in enhancing reproductive parameters, since many variables are involved in mare's infertility.

Furthermore, due to the many proven beneficial properties of ozone and the practicality of this ozone preparation, it's worth performing additional investigations to establish the best protocol for ozone therapy and to demonstrate the efficacy of this product in mares with clinical endometritis and positive to bacterial culture, given the wide use of intrauterine antibiotics in the equine industry [107], the increase of antimicrobial resistance [251] and the worldwide effort to develop effective alternative antibiotic-free therapies to treat endometritis.

4.2.4 Conclusions

Intrauterine ozone therapy with Riger spray® is a safe treatment in the mare since no adverse effects were noted in terms of bacterial culture, endometrial cytology, histology and pregnancy outcomes. Furthermore, this ozone formulation is practical to use under field condition, although future studies are needed to assess antimicrobial activity and positive effects on fertility in mares with bacterial endometritis.

4.3 *In vitro* effects of different ozone preparations on microorganisms responsible for endometritis in the mare

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4.3.1 Materials and methods

4.3.1.1 Sample collection

Mares admitted for infertility to the Veterinary Teaching Hospital (OVU) of the University of Turin (Italy) during the breeding seasons 2021, 2022 and 2023 underwent a complete examination of the reproductive tract, including transrectal palpation, ultrasound examination (MyLabOne, Esaote, Genova, Italy), microbiological and cytological evaluation. Those that exhibited one or more clinical signs of endometritis (intrauterine fluid accumulation, short inter-oestrous intervals, excessive edema [18]) and the evidence of inflammatory uterine cytology were recruited for this study and double-guarded uterine swab samples (Minitube GmbH, Tiefenbach, Germany) were collected to research bacteria and yeasts.

4.3.1.2 Isolation of bacterial and yeast strains

Swab samples were streaked on Columbia agar with 5% sheep blood (CA-SB) and on Sabouraud dextrose agar (SDA) (Thermo Fisher Diagnostics, Altrincham, UK). CA-SB plates were incubated for 24/48 h at 37°C with or without 5% CO₂ atmosphere, whereas SDA plates were incubated for 48 h at 35°C. Isolates were identified by MALDI-TOF/MS (Bruker Daltonik GmbH, Bremen, Germany).

Three antibiotic-resistant strains belonging to each of the following species, *E. coli*, *S. aureus*, *S. equi* subsp. *Zooepidemicus*, *P. aeruginosa*, *K. pneumoniae* and *C. albicans* were selected for this study. Each strain was isolated from a different mare (n=18) and was selected if there was evidence of resistance to one or more antibiotics. To determine this parameter, the bacteria were tested *in vitro* for their antimicrobial susceptibility to the following panel of antimicrobials (Oxoid Ltd., Basingstoke, UK): amikacin (30µg), amoxicillin (10µg), ampicillin (10µg), amoxicillin/clavulanic acid (30µg), ceftiofur (30µg), clindamycin (2µg), gentamicin (10µg), penicillin (1U), tetracycline (30µg), trimethoprim-sulfamethoxazole (25µg), enrofloxacin, (5µg) and marbofloxacin (5µg; Fatro S.p.A., Ozzano Emilia, IT).

The disk diffusion method was performed and interpreted for each species according to CLSI M27-A3 (2018) and to EUCAST guidelines.

4.3.1.3 Experiment 1: antimicrobial activity of gaseous ozone

The action of gaseous ozone was tested on three strains for each microbial species. A starting inoculum of bacterial cells was prepared by inoculating three or four colonies grown on CA-SB plates in sterile saline solution and adjusting the bacterial suspension to a 0.5 McFarland turbidity standard, corresponding to ≈ 1 to 2×10^8 colony forming units /ml (CFU/ml) [252]. One hundred microlitres of this suspension was inoculated onto either Tryptic soy agar plates (TSA, Thermo Fisher Diagnostics, Altrincham, UK) (for *E. coli*, *P. aeruginosa*, *S. aureus* and *K. pneumoniae*) or CA-SB plates (for *S. equi* subsp. *zooepidemicus*). For *C. albicans* the starting yeast inoculum of 0.5 McFarland corresponding to $\approx 5 \times 10^6$ cells/ml was diluted in sterile saline to yield a final suspension of 0.5 - 2.5×10^3 CFU/ml [253]. Again, 100 μ L of this suspension was inoculated onto SDA plates. The inoculum size was checked by plating serial dilutions on TSA for bacteria and on SDA for yeasts and determining the colony counts in triplicate after incubation at 37°C for 24/48 h.

Three clinically relevant strains per each species were exposed to the following different treatments and all the examinations were performed in duplicate:

- Group 1: samples were exposed to a continuous flow of gaseous ozone at a concentration of 15 and 40 μ g/ml for 1, 3 and 5 minutes.
- Group 2: samples were exposed to a continuous flow of 100% O₂ for 3 minutes (negative control)
- Group 3: samples weren't exposed to neither ozone nor oxygen (negative control)

Samples were exposed to the O₂O₃ gas mixtures (Group 1) and O₂ (Group 2) inside a glass hermetic box with a polypropylene lid. This box was coupled to an ozone generator (VetBo3x, Eco3 S.r.l., Brandizzo, Italy) (Group 1) through a silicon tube, while another silicon tube was used to let the gas out [254] (Figure 23). For Group 2, the glass hermetic box was coupled to an oxygen cylinder.

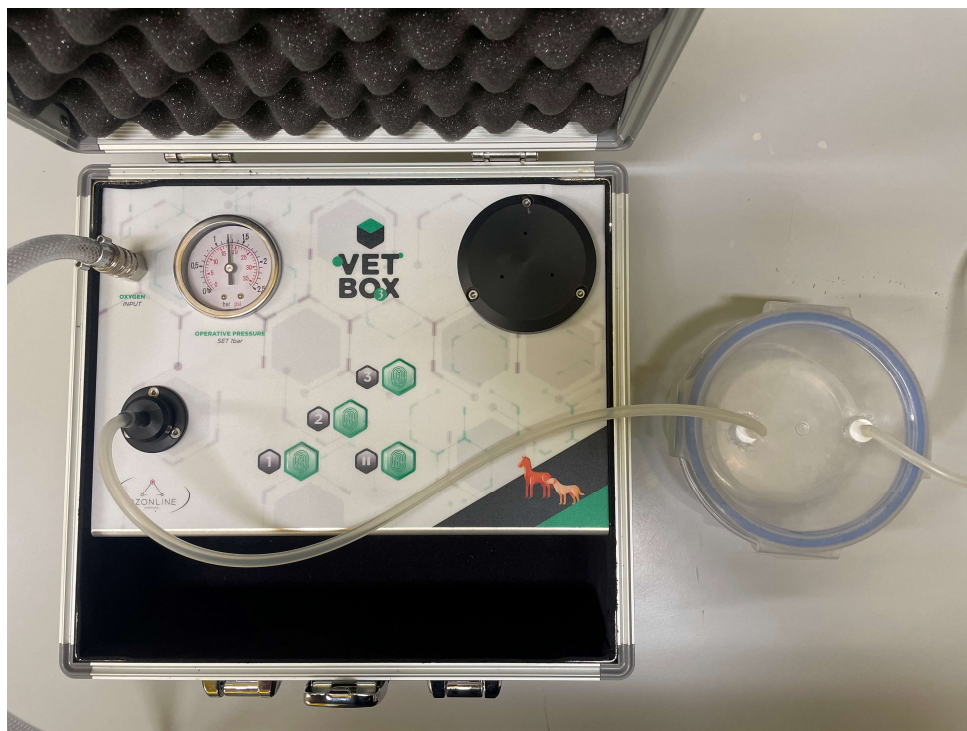


Figure 23: Ozone generator (Vet Bo3x, Eco3 S.r.l, Brandizzo, Italy) coupled to a glass hermetic box containing a tested sample.

All samples were subsequently incubated at 37°C for 24h or 48 h (*C. albicans*) and the microbial count (CFU/ml) was performed. The CFU/ml observed after the exposure to O₂O₃ gas mixtures (Group 1) were compared with the count observed in the Group 2 and 3 to evaluate the effect of gaseous ozone. Furthermore, differences between the effect of the various times and concentrations of O₂O₃ gas mixtures were assessed.

4.3.1.4 Experiment 2: antimicrobial activity of the ozonated water and oil

The action of ozone dissolved in liquid matrices was tested on three strains for each microbial species and all the examinations were performed in duplicate. The ozonated water was obtained by continuously bubbling 100 mL of sterile distilled water for 10 minutes using O₂O₃ gas mixture (concentration 40 µg/mL). After preparation, ozonated water was immediately used. The ozonated oil was a commercial ozonated sunflower oil, Neozone 4000 (Cosmoproject, Parma, Italy) with a peroxide index of 4000 mEqO₂/Kg. Non-ozonated distilled water and sunflower oil were also tested in this experiment as control groups.

Minimum inhibitory concentrations (MICs) and minimum bactericidal/fungicidal concentrations (MBCs/MFCs) were determined by broth dilution method according to the CLSI. The inoculum was 10⁵ CFU/ml for bacteria and 10³ CFU/ml for *C. albicans*. Doubling concentrations of ozonated oil, ozonated water, sunflower oil and water controls in the Mueller Hinton broth medium (Thermo Fisher Diagnostics,

Altrincham, UK) for bacteria and RPMI 1640 broth medium (Sigma, Milan, Italy) for yeasts were used (range 50% v/v – 6.25% v/v).

MICs were visually read as the lowest concentration at which no bacterial/fungal growth was observed, determined as the absence of turbidity in the tubes after incubation [255].

MBCs/MFCs were read as the lowest concentration resulting in no growth on subculture and corresponding in the death of 99.9% or more of the initial inoculum [255].

4.3.1.5 Statistical analysis

In Experiment 1 the data are expressed as mean number of CFU/ml $\pm$ standard error of the mean (SEM). The results of Experiment 2 are expressed as modal values and ranges of MIC and MBC/MFC (% v/v).

Data were analysed using GraphPad Prism, 10.1.1 for macOS, (GraphPad Software, La Jolla, USA). Data were tested for normality using Shapiro-Wilk test. Friedman test and Dunn's as post hoc were performed to assess differences in CFU/ml between the different groups and the different conditions of Experiment 1. Fisher's exact test applying Bonferroni corrections for multiple comparisons was performed to assess differences in MIC values between the groups of Experiment 2.

4.3.2 Results

4.3.2.1 Experiment 1

For every microorganism, a statistically significant decrease in the number of CFU/ml was observed in the treated group (Group 1) compared to the control groups (Groups 2 and 3) ($p < 0.001$) (Table 4 and Figure 24). For all the bacterial species, whether Gram + or Gram -, a reduction of over 99.9% of the bacterial load was observed at every time and concentration tested. Furthermore, regarding *C. albicans*, exposure to O₂O₃ gas mixtures determined a reduction between 93.5% and 98.3% in the number of CFU/ml, depending on the experimental condition evaluated (respectively 15 μ g/ml for 3 minutes and 40 μ g/ml for 3 minutes).

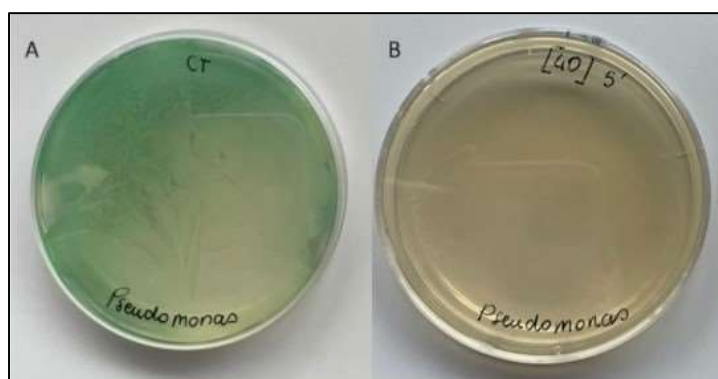


Figure 24: An example, Petri plates of *Pseudomonas aeruginosa* after 24 h of incubation. A: control untreated plate (group 3). B: plate exposed to gaseous ozone at a concentration of 40 μ g/ml for 5 min (group 1).

Despite the very high antimicrobial activity of gaseous ozone in all tests, this experiment stresses also a time-dependent effect. Indeed, considering the microbial counts of all the microorganisms for each different time, a significantly lower number of CFU/ml was observed at both concentrations after 3 minutes of exposure (15 µg/ml, average microbial count of $2.1 \times 10^2 \pm 3.1 \times 10$ CFU/ml; 40 µg/ml, average microbial count of $9.8 \times 10 \pm 1.8 \times 10$ CFU/ml) compared to 1 minute (15 µg/ml, average microbial count of $7.9 \times 10^2 \pm 1.6 \times 10^2$ CFU/ml, $p < 0.01$; 40 µg/ml, average microbial count of $2.8 \times 10^2 \pm 3.9 \times 10$ CFU/ml, $p < 0.01$). The same result was observed when comparing 5 minutes vs 3 minutes at the concentration of 15 µg/ml ($8 \times 10 \pm 1.8 \times 10$ CFU/ml, $p = 0.004$), while no difference was observed at 40 µg/ml ($5.7 \times 10 \pm 1.3 \times 10$ CFU/ml, $p > 0.1$). Furthermore, the antimicrobial effect of ozone resulted also concentration-dependant at 1 and 3 minutes of exposure since at a higher concentration the number of CFU/ml was significantly lower (15 vs 40 µg/ml at 1 and 3 minutes, respectively $p < 0.05$ and 0.02), while no differences were observed between the two ozone concentrations after 5 minutes of exposure ($p > 0.1$).

Microorganism	15 µg/ml 1'	15 µg/ml 3'	15 µg/ml 5'	40 µg/ml 1'	40 µg/ml 3'	40 µg/ml 5'	100 % O2	Control untreated
	CFU/ml							
E. coli (n = 3)	$1.8 \times 10^2 \pm 4.3 \times 10$	$1.4 \times 10^2 \pm 6.7 \times 10$	$2.5 \times 10 \pm 7.6$	$1.6 \times 10^2 \pm 5.1 \times 10$	$5.7 \times 10 \pm 3 \times 10$	$3 \times 10 \pm 1.3 \times 10$	$1-2 \times 10^8$	$1-2 \times 10^8$
S. aureus (n = 3)	$2.4 \times 10^3 \pm 5.5 \times 10^2$	$3.8 \times 10^2 \pm 9 \times 10$	$1.7 \times 10^2 \pm 7.1 \times 10$	$4.4 \times 10^2 \pm 6.3 \times 10$	$1.8 \times 10^2 \pm 5.2 \times 10$	$9.2 \times 10 \pm 5.1 \times 10$	$1-2 \times 10^8$	$1-2 \times 10^8$
S. zooepidemicus (n = 3)	$7.8 \times 10^2 \pm 2.9 \times 10^2$	$1.6 \times 10^2 \pm 7.2 \times 10$	$3.7 \times 10 \pm 1.7 \times 10$	$1.5 \times 10^2 \pm 7.5 \times 10$	$1.3 \times 10^2 \pm 6.4 \times 10$	$6.8 \times 10 \pm 3.9 \times 10$	$1-2 \times 10^8$	$1-2 \times 10^8$
P. aeruginosa (n = 3)	$6.8 \times 10^2 \pm 9.6 \times 10$	$2.7 \times 10^2 \pm 7.9 \times 10$	$1.5 \times 10^2 \pm 5.2 \times 10$	$5.8 \times 10^2 \pm 8.7 \times 10$	$7.8 \times 10 \pm 2 \times 10$	$6.3 \times 10 \pm 3 \times 10$	$1-2 \times 10^8$	$1-2 \times 10^8$
K. pneumoniae (n = 3)	$6.8 \times 10^2 \pm 9.7 \times 10$	$2.1 \times 10^2 \pm 6.6 \times 10$	$6.2 \times 10 \pm 1.9 \times 10$	$2.6 \times 10^2 \pm 7.2 \times 10$	$1.1 \times 10^2 \pm 4.2 \times 10$	$5.8 \times 10 \pm 3.1 \times 10$	$1-2 \times 10^8$	$1-2 \times 10^8$
C. albicans (n = 3)	$8.7 \times 10 \pm 5.4 \times 10$	$9 \times 10 \pm 5.7 \times 10$	$1.9 \times 10 \pm 2.4 \times 10$	$7.2 \times 10 \pm 4.5 \times 10$	$2.3 \times 10 \pm 1.7 \times 10$	$3.0 \times 10 \pm 2.4 \times 10$	$1.4 \times 10^3 \pm 5 \times 10$	$1.3 \times 10^3 \pm 6.1 \times 10$
Significance*	a	b	c	b	c	c	d	d

Table 4: Mean of CFU/ml $\pm$ SEM of each species after exposure to oxygen-ozone gas mixture (Group 1), 100% O2 (Group 2) or left untreated (Group 3). Three strains for each microbial species were tested and two independent replicates per each strain were performed. The presence of different letters in the same row indicates a statistically significant difference ($p < 0.05$).

4.3.2.2 Experiment 2

The absence of turbidity was only observed in the tubes containing ozonated oil, which showed an inhibition of bacterial growth that was significantly different from that of other liquid matrices (ozonated water, non-ozonated oil and non-ozonated water, all p values < 0.001), with MIC values comprised between 12.5 and 25% (v/v). Furthermore, ozonated oil showed bactericidal activity (MBC/MFC) at a concentration between 12.5 and 50% (v/v) depending on the pathogen. Ozonated oil was effective against all the tested microorganisms. On the other hand, ozonated water, non-ozonated water and non-ozonated oil did not show any inhibitory effect on bacterial growth even at a concentration of 50%

(v/v), therefore MBC/MFC were not evaluated. The MIC and MBC/MFC values of ozonated and non-ozonated oil and water are reported in Table 5.

Microorganism		Ozonated oil		Ozonated water		Non-ozonated oil		Non-ozonated water	
		MIC (% v/v)	MBC/MFC (% v/v)	MIC (% v/v)	MBC/MFC (% v/v)	MIC (% v/v)	MBC/MFC (% v/v)	MIC (% v/v)	MBC/MFC (% v/v)
<i>E. coli</i> (n=3)	modal value	12.5	25	>50%	nt*	>50%	nt	>50%	nt
	range	12.5/25	25/50	-/-		-/-		-/-	
<i>S. aureus</i> (n=3)	modal value	12.5	12.5	>50%	nt	>50%	nt	>50%	nt
	range	-/-	12.5/25	-/-		-/-		-/-	
<i>S. zooepidemics</i> (n=3)	modal value	12.5	25	>50%	nt	>50%	nt	>50%	nt
	range	12.5/25	12.5/25	-/-		-/-		-/-	
<i>P. aeruginosa</i> (n=3)	modal value	25	25	>50%	nt	>50%	nt	>50%	nt
	range	12.5/25	-/-	-/-		-/-		-/-	
<i>K. pneumoniae</i> (n=3)	modal value	25	25	>50%	nt	>50%	nt	>50%	nt
	range	-/-	-/-	-/-		-/-		-/-	
<i>C. albicans</i> (n=3)	modal value	12.5	12.5	>50%	nt	>50%	nt	>50%	nt
	range	-/-	-/-	-/-		-/-		-/-	

Table 5: MIC and MCB/MFC values (% v/v), presented as modal values and range of ozonated oil, ozonated water and controls (non-ozonated oil and water) on antibiotic-resistant bacteria and *C. albicans*. Three strains per species and two independent replicates per strain were performed. nt*=non tested

4.3.3 Discussion

To the best of the author's knowledge, this study described for the first time the in vitro antimicrobial effect of different ozone preparations on clinical isolates responsible for equine endometritis.

In this experiment ozonated distilled water didn't have any observable bactericidal effect. These findings are in contrast to literature describing the bactericidal effect of ozonated water solutions [256,257]. On the other side, the activity of water-dissolved-ozone is controversial, indeed in the equine reproduction field, two recent studies have stated that ozonated saline and Ringer do not have any significant antimicrobial or antibiofilm activity versus microorganisms responsible for endometritis [37,99]. These apparent controversial data could be explained by the action of different variables that may ultimately affect the final concentration of ozone in a liquid matrix, which is rarely measured and reported and that mostly depends on the protocol used to ozonize the water solution [258]. The lack of a precise measurement of the concentration of ozone dissolved in water constitutes indeed a limitation of the present study. Thus, it can be hypothesized that the protocol we used to ozonize the water (40 µg/mL for 10 minutes), was not sufficient to reach or maintain the critical ozone concentration

needed to kill the microorganisms. Again, this underpins the need of standardized protocols for ozone administration that should have a satisfactory bactericidal effect and can be performed in field conditions.

On the other hand, ozonated sunflower oil showed antimicrobial activity against all the species tested, while the control non-ozonated sunflower oil didn't show any effect. Ozonation of oils is a procedure carried out to improve the short half-life of gaseous ozone or water-based solutions. Briefly, ozone reacts with carbon-carbon double bonds of unsaturated fatty acids present in triglycerides of vegetable oils. This reaction produces several oxygenated products, such as ozonides, hydroperoxides, aldehydes, peroxides, diperoxides and polyperoxides, that are responsible for the antibacterial and fungicidal activity [259,260]. To give an objective meaning to the word "ozonated", however, it is necessary to describe the quantity of peroxides in the solution [217]. The peroxide value is a number that expresses in milliequivalents of active oxygen the quantity of peroxides contained in 1000g of the substance and is generally considered to be correlated to its antimicrobial activities [217,259]. Diaz et al. observed that at higher peroxide indexes corresponded a higher antimicrobial activity [260], while Ferreira and coauthors [240] noted that only ozonated oil with a peroxide value higher than 500 mEq kg⁻¹ was able to inactivate the growth of *Phytium insidiosum* *in vitro*. The oil we used in the experiment is Neozone 4000 (Cosmoproject, Parma, Italy). It is a commercial standardized product, with a measured peroxide index (over 4000 mEq kg⁻¹) that maintains satisfactory stability at different storage conditions [259]. This ozonated oil is the base of different ozone preparations, including Riger Spray®, that was widely used with good results in cows, sheep and goats to treat endometritis, metritis, and placental retention [223]. Therefore, it can be speculated that the beneficial effects on fertility reported in the previous studies could have been due to the bactericidal effect of the ozonated oil, among other properties of ozone therapy. The use of ozonated oil to treat endometritis in the mare was recently reported *in vivo* by Ávila et al. [143], who observed that the treatment did not cause an increase in uterine inflammation or adverse effects more serious than mild and transient discomfort, but it was effective in reducing the rate of positive uterine culture. The data we obtained using ozonated oil in a larger number of species of uterine pathogens support its use as a therapeutic agent. Moreover, the evidence that it acts on bacteria with a high resistance pattern to antibiotics enforces its use as an alternative to antibiotics therapy in equine reproduction. In conclusion, ozonated oil showed a potent bactericidal effect and represents an effective ozone formulation to treat mares with endometritis under field conditions, bypassing the use of gaseous ozone which needs an ozone generator and an oxygen cylinder that can be expensive and not easily available or utilizable in the field.

Also gaseous ozone proved to be a strong bactericidal agent, since all the concentrations and times of exposure achieved a reduction in the number of CFU/ml of over 99.9% on all the bacteria tested,

whether Gram + or Gram -. Furthermore, regarding *C. albicans*, a reduction between 93.5% and 98.3% in the number of CFU/ml depending on the experimental condition was observed.

These results are in agreement with previously published data: Fontes et al. [252] observed that the growth of different bacterial isolates was completely inhibited after 5 minutes of exposure to an ozone-oxygen mixture at a concentration of 20 µg/mL. Lillo et al. [254] performed a similar experiment on clinical isolates of bacteria responsible for bovine metritis and stated that already after 1 minute of exposure to ozone at a concentration of 20 µg/mL the growth was minimal or totally inhibited. In the present study, a higher concentration of bacteria was inoculated on the plate (10^7 CFU vs 10^3 CFU). Furthermore, we performed a dose-dependent analysis reaching 15 µg/mL of O₂O₃, in the attempt to identify a lower effective antibacterial dose. A minimal effective dose of gaseous ozone for topical application has not been clearly determined yet and ozone, due to its mechanism of action based on its oxidating properties, if delivered in high doses could have a detrimental effect, overwhelming the antioxidant system of the cells and organism and resulting in tissue damage [133,210,223,254,261]. Lowering the ozone supply, either reducing the time of administration or its concentration, has the purpose of identifying a standard therapy able to reduce these risks. It was recently proved, moreover, that the intrauterine administration of gaseous ozone in the mare at a concentration of 39 µg/mL and a total dose of 125 µg/ kg causes a systemic oxidative stress evidenced by decreased total antioxidant capacity and increased total oxidant capacity [242]. However, it is important to perform more studies to understand whether this effect is beneficial or not, in order to adjust the doses [242].

According to the results of the present study, 15 and 40 µg/mL achieved a similar inhibition of bacterial growth after 5 minutes of exposure. This finding is particularly interesting, especially considering the results of two recently published in vivo studies. In the first one, Ávila et al. [143] performed 10 minutes of continuous intrauterine insufflation of ozone at a concentration of 40 µg/mL, repeated for two consecutive days, and 8 out of 9 mares showed a negative bacteriological culture after the treatment. In the second study, Kohne et al. [144], reported that 5 out of 9 mares showed a negative culture after the insufflation of 240 ml of ozone at a concentration of 80 µg/mL repeated twice at a 48-h interval. These results prove that a higher concentration is not always more effective, but probably the volume of O₂O₃ mixture insufflated plays an important role. Two-hundred forty ml is, indeed, a volume generally not sufficient to fill the entire uterine cavity and probably ozone could not reach all the surface of the uterus and does not have enough contact time with endometrium to carry out its antimicrobial action, especially due to the gaseous nature of the molecule and its short half-life. Thus, it can be hypothesized that focalized infection could be missed [4]. Therefore, the insufflation of lower concentrations of ozone in the mixture (up to 15 µg/mL, which proved to be effective in this study) but using higher volumes/time of exposure could be a successful strategy to achieve optimal antimicrobial activity while minimizing

the total dose of ozone administered to the mare and therefore potential local cytotoxicity and systemic impact [261].

Another aspect to consider is that in mares with clinical infectious endometritis, sometimes not only a microorganism is involved but mixed infections do occur and may be the cause of therapeutic failure [110]. In this work, however, ozone, due to its mechanism of action that is based on the oxidation of biological components of bacteria [252], proved to be effective against all the pathogens tested, whether Gram +, Gram – or yeasts. Ozone therapy could therefore bypass this problem and be effective even when the infection is caused by multiple pathogens.

Finally, in the present study, ozone showed its bactericidal effect against antibiotic-resistant bacteria. The phenomenon of antibiotic resistance is considered one of the top ten public health risks facing humanity by the World Health Organization [131] and it is widespread also in the equine reproduction field [27,207]. However, the use of antibiotics is very common in the mare and in a recent descriptive study, Mouncey et al. [104] described that post-covering antibiotics are still routinely administered, even in the absence of clinical signs of endometritis, bacterial culture and antimicrobial sensitivity test, a dangerous habit that may lead to an increase of antibiotic-resistant bacterial strains. In this scenario, since the development of novel antibacterial agents is insufficient to counter this growing threat, alternative bactericide treatments are required [132] and ozone could play an important role in the future in the treatment of numerous infective pathologies, including equine endometritis. It was recently described in human medicine the strategy of using micro doses of ozone in association with antibiotics to improve the performance of both therapies, especially against resistant strains of Gram-negative bacteria [133]. The principle is that ozone, oxidizing the outer membrane of the bacterial cell, is able to create holes on the surface allowing an increased diffusion of antibiotics into the bacteria, being effective in reducing the dosage of both antibiotics and ozone while obtaining a complete eradication of the pathogen [133]. Furthermore, in a case report, Kempchen et al. showed that intrauterine gaseous insufflation successfully resolved endometritis caused by methicillin-resistant *Staphylococcus aureus* in two mares [262]. In our study, we confirmed and expanded this finding since ozone proved to be effective *in vitro* against all the antibiotic-resistant strains tested. These results, obtained on clinical isolates responsible for equine endometritis, create a solid scientific rationale for its use as antimicrobial agent *in vivo* that could contribute to prevent or at least to scale down the use of antibiotics.

4.3.4 Conclusions

In the present study ozonated distilled water didn't show a significant antibacterial effect, discouraging its use as an antimicrobial agent for the treatment of endometritis.

On the other hand, both gaseous ozone and ozonated oil showed antimicrobial activity against antibiotic-resistant bacterial and yeast strains. Furthermore, even lower than previously reported concentrations of gaseous ozone showed efficacy *in vitro*. Thus, ozone therapy proved to be a promising treatment for infectious endometritis and the results of this study create a solid scientific rationale for its use as an antimicrobial agent in an effort to scale down or prevent the use of antibiotics in equine reproduction.

CHAPTER 5

Influence of IUF detection on pregnancy rate and early embryonic loss in a commercial ET program

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5.1 Introduction

Horse embryo transfer (ET) was first successfully performed in the mid-1970s [263,264]. Since then, it has become a popular technology that enables the production of multiple foals from a single mare during a breeding season, as well as the pregnancy of older mares with reproductive issues or foals from competing mares, late-foaling mares, and 2-year-old mares [265–267]. The International Embryo Transfer Society estimates that 26,145 fresh embryo transfers and 44,906 embryo flushes were carried out on horses globally in 2020 [268]. After transfer, the pregnancy rate (PR) can range from 65% to 84%, and the incidence of early embryonic loss (EEL) occurring before 50 days can range from 10% to 15% [269–274].

Since the advent of ultrasound, the detection of IUF has been reported both in estrus and diestrus, and the awareness of the frequency of this abnormality has increased [49,50]. A small quantity of anechoic IUF is a common finding within the uterine lumen of healthy mares during estrus [1] and generally is considered not to affect pregnancy rates, while a fluid volume exceeding 2 cm has been associated with decreased pregnancy rates [53,275,276]. Even small accumulations of anechoic IUF during the immediate post ovulation period could possibly adversely affect fertility [1], and it has been shown that free fluid within the uterine lumen during diestrus should be considered abnormal because it is associated with endometritis and indicates an inflammatory process and/or impaired uterine clearance [58,59]. In fact, inadequate uterine drainage is a cause of subfertility in old pluriparous mares and in some maiden mares that do not completely dilate their cervix during oestrus [275]. However, to the best of the authors' knowledge, the effect of the detection of IUF in recipient mares before and after ovulation on PR and EEL in an ET program has never been described.

The aim of this study was to determine if detection of IUF during follicular phase and after the ovulation affected the pregnancy rate and/or early embryonic loss in a commercial embryo transfer program. It was hypothesized that: 1) the presence of IUF before ovulation is not pathological and does not lead to reduced PR or EEL. 2) Detection of IUF post ovulation, being a sign of endometritis and/or impaired uterine clearance, is associated with reduced fertility.

5.2 Materials and Methods

This study was performed retrospectively based on data of 1222 transfer performed at Keros Embryo Transfer Center, Passendale (Belgium) during 2021 and 2022 breeding seasons.

5.2.1 Donor mares and embryo flush

Donor mares' insemination and flushing for embryo recovery were performed at the centre or at distant locations. In this case, embryos were shipped to the centre in different holding media and cooling

devices, according to practitioners' preferences. For the mares inseminated at the centre, after the detection of a follicle larger than 35 mm and the presence of endometrial oedema, 1500 IU of Human Chorionic Gonadotropin (Chorulon®, MSD Animal Health SRL, Boxmeer, Netherlands) were administered intravenously to induce ovulation. Artificial insemination with fresh and chilled semen was performed 24 h after ovulation induction. If ovulation did not occur, mares were inseminated again 48 h after the first insemination. When frozen semen was used, insemination was performed within 6 h after the ovulation. The stallions used for insemination were recorded. Donor mares showing fertility problems were treated accordingly.

Uterine flushes for embryo recovery were performed between days 8 and 9 post ovulation (after the first ovulation for mares with multiple ovulations). For flushes performed at the centre, the procedure is briefly described. The rectum was gently emptied, the tail was wrapped, and the perineum washed three times with water and povidone iodine, then dried with paper towel. The uterus was flushed using a three-way silicon tubing system connected to a silicon cuffed 36 CH Bivona catheter (IMV Technologies, L'Aigle, France) and to an EZWay Filter (EZ-Way, A&E Int'l, Alta Vista, KS, USA). The catheter was inserted transcervically into the uterus. Once into the uterine body, a cuff on the end of the catheter was inflated with 40-60 mL of air and then pulled caudally to ensure a tight seal against the internal os of the cervix. Donors were flushed three times with 1 to 3 L of a commercial Lactated Ringer's solution (Baxter SpA, Rome, Italy) depending on uterus size. After the second and third flush, in order to maximize the recovery of the fluid, the uterus was massaged transrectally. Finally, the air cuff was deflated, and the catheter was removed from the mare. 2 mg of Alfaprostol (Gabbrostim, CEVA, Libourne, France) or 250 mcg of Cloprestenol (Estrumate, MSD-Animal Health SRL, Rahway, NJ, USA) were injected IM to the donor mares in order to induce luteolysis, prevent unwanted pregnancies and accelerate the return in estrus.

Embryos were searched, graded [277] and measured using a stereomicroscope (Olympus SZX10, Olympus Corporation, Tokyo, Japan) equipped with an eyepiece micrometer, then washed three times in Embryo Holding Medium (IMV Technologies, L'Aigle, France).

5.2.2 Recipient mares and ET

Recipient mares, aged 3 to 17 years (average: 9.02 ± 3.30 ; median: 9 years), were housed in single or shared stalls, fed with hay and corn silage; water was supplied ad libitum.

During follicular phase, the recipients were submitted every two days to transrectal palpation and ultrasound examination in order to detect the ovulation. 4 to 5 days after the detection of ovulation, transrectal palpation and ultrasound examination was performed and only mares with a good uterine and cervical tone, absence of uterine edema, absence of IUF accumulation and presence of a morphologically normal CL were selected to receive an embryo. If the mare was not used as a recipient,

6 to 8 days after the detection of ovulation 250 mcg of Cloprostenol (Estrumate, MSD Animal Health SRL, Boxmeer, Netherlands) were administered IM to induce luteolysis and the mare was scanned 3 to 4 days later.

If intrauterine fluid accumulation was observed during follicular phase or the day of detection of ovulation (therefore indicating the presence of intrauterine fluid within 0 and 48 hours after ovulation), the data was recorded. However, a given mare was used as a recipient in that cycle only if IUF was not present anymore the day of the transfer or the day before. If the fluid detected in any moment of the cycle was more than grade I (anechoic) [49] and >2 cm, the recipient mare was removed from use.

Mares that didn't get pregnant after the first transfer received a second embryo or a third embryo during the breeding season and the data was recorded.

The recipient mares, synchronized alongside the donors, received an embryo between 4 and 7 days post ovulation. The mares were held in a palpation stock and sedated with 0.01-0.02 mg/kg of detomidine hydrochloride IV (Medesedan, Virbac Belgium SA, Leuven, Belgium) alone or in association with butorphanol (Dolorex, MSD Animal Health SRL, Boxmeer, Netherlands) (0.01-0.03 mg/kg IV) if needed. The embryo was loaded into a 0.25 ml or 0.5 ml straw (depending on the size of the embryo) and the straw loaded into a stainless steel Cassou gun (IMV Technologies, L'Aigle, France), covered by a disposable sterile sheath and an outer sterile chemise (IMV Technologies, L'Aigle, France). All the transfers were performed by the same veterinarian with the "double glove" manual technique. Briefly, the tip of the instrument was held by a hand wearing a sterile sleeve and covered by a second sterile obstetrical sleeve and introduced in the cervix. Once the tip was inserted in the cervical os, the chemise was broken and pulled caudally. Subsequently, the operator used transrectal manipulation to ensure that the pipette entered the uterus minimizing manipulation of the cervix and finally the embryo was deposited into the uterus, as deep as possible into one uterine horn. Before or after the transfer the recipients didn't receive any treatment.

Transrectal ultrasound examination (MyLabOne, Esaote, Italy) was performed 4 to 6 days after ET in order to detect the pregnancy (this data indicates PR at 14 days). Subsequently pregnancy examinations were performed every one to two weeks and a final one was performed between 45 and 50 days after ET (PR at 45 days). Early embryonic loss (EEL) was calculated as the number of embryo losses between the first and last pregnancy check, divided by the number of mares pregnant at the first pregnancy check.

5.2.3 Statistical analysis

Data were analysed using the software R 4.2.2. Three generalized linear models were created to observe the effect of different factors on PR at 14 and 45 days and EEL (dependent variables). For all the models, the factors investigated were: 1) Detection of fluid during follicular phase, 2) Detection of fluid after the

ovulation, 3) Age of the recipient mare (4 groups: 3-5 years, 6-9 years, 10-13 years, 14-17 years), 4) Number of transfers during the season on the same recipient, 5) Embryo size (4 groups: <300µm, 300-600µm, 601-1500µm, >1500µm), 6) Embryo quality grade (3 groups: grade I, grade II, grade III). For data showing differences < 0.05, Pearson's Chi-squared test, applying Bonferroni's correction in the case of multiple comparisons, was used to compare differences between levels. Pearson's Chi-squared test was used to observe the relation between detection of IUF before and after ovulation and age.

The aim of this article is to observe the effect of IUF before and after ovulation (factors 1 and 2) on the success of equine ET. Factors 3 to 6 are known to influence the outcome of embryo transfer as already described by other authors [270,273,274,277] and were therefore included in the statistical analysis. Nevertheless, the results are only briefly discussed.

5.3 Results

An overall PR at 14 days of 77,1 % was achieved and EEL had an incidence of 11,7%. PR at 45 days was 68.1%.

Grade I (anechoic) [49] and <2 cm fluid during the follicular phase was detected in 155 cycles (12.7%). Pregnancy rate at 14 or 45 days was not statistically different between recipient mares with IUF and mares without (respectively PR 14= 74.8% and 77.4%, $p>0.1$; PR 45= 63.2% and 69%, $p>0.1$) and neither EEL was affected ($p>0.1$). Detection of fluid during follicular phase was not related to the age of the recipient ($p>0.1$).

Grade I (anechoic) [49] and <2 cm fluid after ovulation was observed in 38 cycles (3.1%) and negatively affected the PR 14 (60.5% in mares with fluid vs 77.6% in mares without fluid, $p=0.013$), but not the EEL or PR 45 ($p>0.1$) (Table 6).

The fluid was detected mostly in young (3-5 years, 6.5%) and old mares (14-17 years, 7.3%), compared to the other two groups (6-9 and 10-14 years, respectively 2.6% and 1.2%; $p<0.001$).

A given mare was not used as embryo recipient in that cycle because intrauterine fluid was detected the day of transfer or the day before on 53 occasions.

	PR 14	EEL	PR 45
IUF during estrus			
Yes (n=155)	74.8% <i>a</i>	15.5% <i>a</i>	63.2% <i>a</i>
No (n=1067)	77.4% <i>a</i>	10.9% <i>a</i>	69% <i>a</i>
IUF after ovulation			
Yes (n=38)	60.5% <i>a</i>	8.7% <i>a</i>	55.3% <i>a</i>
No (n=1184)	77.6% <i>b</i>	11.9% <i>a</i>	68.4% <i>a</i>

Table 6: Different factors affecting pregnancy rate and early embryonic loss. Within a column, different letters (*a,b*) indicate a significant difference ($p<0.05$).

The age of the recipient mare didn't influence the PR at 14 or 45 days but affected the EEL, since mares in the 3-5 years old group had a lower EEL than mares between 10 and 13 years (respectively 6.4% and 15.6%, $p=0.03$).

No differences were observed between mares receiving one or two embryos in one year (respectively $n=893$, PR 14=78.8% and $n=252$, PR 14=75.4%; $p>0.1$; PR 45=70.1 % and 66.7%; $p>0.1$), but the PR 14 and 45 of the third transfer of the season ($n=61$) is significantly lower compared to the first one (PR 14=65.6%, $p=0.04$; PR 45=54.1%, $p=0.026$).

The size of the embryo affected PR 14 and PR 45. Embryos $<300\mu\text{m}$ had a lower PR 14 than embryos between 300 and $600\mu\text{m}$ (respectively 67.4% and 78.2%, $p=0.047$) and between 601 and $1500\mu\text{m}$ (79.6%, $p=0.026$). Regarding PR at 45 days, it was significantly lower in embryos $<300\mu\text{m}$ than in embryos between 300 and $600\mu\text{m}$ (respectively 55.6% and 69.5%, $p=0.01$) and between 601 and $1500\mu\text{m}$ (72.1%, $p<0.01$). PR 45, moreover, was lower in embryos $>1500\mu\text{m}$ than in embryos between 601 and $1500\mu\text{m}$ (51.3%, $p=0.04$).

The grade of the embryo influenced both PR 14 and PR 45, but not EEL. Grade I embryos had a statistically significant higher PR 14 than grade II (respectively 78.8% vs 63.7%, $p<0.001$) and grade III (38.9%, $p<0.0001$). PR 45 was higher in Grade I embryos than grade II or III (respectively 70.1%, 55% and 38.9%, $p=0.015$ and 0.01).

5.4 Discussion

Hypotheses 1 and 2 are based on whether the detection of IUF in the recipient mares in different moment of the estrus cycle has a clinical significance, affecting PR or EEL, and should therefore be

considered a parameter to disqualify a mare from receiving an embryo in that cycle. IUF is one of the symptoms associated with delayed uterine clearance and endometritis and is a useful tool in the diagnosis of this pathology [58,278]. However, the relationship between the presence of fluid, the moment of the cycle when fluid is detected, and the success of transfer has never been studied in the recipient mare.

The findings of this study confirmed hypothesis 1, which states that an IUF accumulation less than 2 cm during the follicular phase is not pathological and does not result in a decreased PR and EEL.

Intrauterine fluid originates from secretions of endometrial glands, from transudation and from failure of the mechanical uterine clearance [55]. Detection of small quantities of IUF during the follicular phase does not necessarily indicate inflammation, but it can also be transudate from the vessels, associated with the same mechanism that causes normal estrous edema. In fact, estrogens increase endometrial secretion, edema, and the permeability of the endothelium of uterine vessels during estrus [48,55,188,279]. Furthermore, in many cases, the IUF detected during estrus is sterile and does not contain inflammatory cells [279,280]. However, it is unclear how much fluid should be deemed significant, but smaller quantities during estrus in inseminated mares do not affect PR [48,278,280], in contrast to larger (>2cm) collections of fluid [49,53,275,279].

Therefore, even if the presence of IUF during estrus is generally considered an important factor that can negatively affect the selection of the recipient mare [277], our study confirmed the hypothesis that the presence of an anechoic IUF accumulation <2 cm during estrus is not a pathological finding and does not negatively affect PR and EEL. This result suggests that mares with small quantities of IUF during estrus can be safely used as embryo recipients.

The results of this study also confirmed hypothesis 2, as the presence of IUF accumulation in the recipient mare after ovulation was associated with a reduced PR at 14 days. However, it is important to note that the present study did not assess the depth of IUF accumulation, except for being less than 2cm. This hypothesis is based on the assumption that the presence of fluid inside the uterus after ovulation and during diestrus is considered a clinical sign of endometritis and inflammation, identifies mares with delayed uterine clearance and is related to subfertility [1,48,58,279].

In our study, the identification of intrauterine fluid within 2 days after ovulation indicated mares that were unable to evacuate the fluid from the uterus or accumulated fluid after ovulation. inadequate uterine clearance is considered a factor contributing to reduced fertility in older pluriparous mares, as well as in some young and older mares that do not completely relax their cervix during estrus. Our study confirmed this finding, as we observed the presence of fluid mostly in the group of very young (3-6 years) and older (14-17 years) mares. Furthermore, in a previous study, it was observed that the incidence of intrauterine fluid during diestrus was associated with the presence of an inflammatory process, as

indicated by a high biopsy score, reduced progesterone concentrations, and a shorter inter-ovulatory interval, resulting in a lower PR and higher EEL [59,278]. Therefore, it is considered a clinical sign of endometritis [58]. Due to the retrospective nature of this study, cytological, bacteriological, or histological examinations were not conducted to diagnose uterine inflammation and endometritis. However, the presence of IUF in the early luteal phase may indicate the presence of endometritis, which could explain the decrease in PR. Endometritis is an important factor that must be taken into account while managing recipient mares for embryo transfer since it can have a major influence on the success of the procedure [50]. It is a multifactorial disease and physical clearance through the cervix plays an important role in the resistance of mares to uterine infection, since it has been demonstrated that mares susceptible to endometritis have a delayed uterine clearance [55,281]. Furthermore, IUF accumulations provide an appropriate environment for the growth of bacteria [277,281].

In ET programs, individuals undergoing embryo transfer are typically examined on the day of the procedure to evaluate many factors, including the absence of IUF [277]. This is because the presence of even small volumes of fluid at this stage reduces the chances of a successful embryo transfer [281]. However, no research has been conducted to examine the relationship between the presence of fluid within the first two days following ovulation and the success rate of ET. Some practitioners suggest scanning recipients for at least one day after ovulation to assess the absence of fluid in the uterus during diestrus [282], as this finding may have a negative impact on fertility. Nevertheless, this study is the first to validate this hypothesis and reveals a detrimental association between the identification of IUF following ovulation and pregnancy PR in recipient mares in a commercial ET program. Therefore, mares with IUF after ovulation should be disqualified as embryo recipients in that cycle.

The hypothesis 3 that PR and EEL are influenced by the age of the recipient was partially supported by this study. The PR at 14 and 45 days was not different between mares belonging to different age groups, but EEL was increased in mares between 10 and 13 years compared to mares very young, aged between 3 and 5 years. This result is in accordance with Carnevale *et al.* [274] who observed no differences in PR between younger (2-9 years) and older recipients (10-18 years) but noticed a tendency to an increase in EEL in the group of older mares (13.3 % vs 20.5%).

Hypothesis 4, that mares at the third transfer of the season have a lower PR was substantiated by the results of this study, since we observed a decreased PR at 14 and 45 days in mares at the third transfer compared to mares at the first one. This finding is in accordance with previous literature [277].

Finally, although the main aim of the study was to observe if IUF detection in different phases of the cycle can influence the success of ET (hypothesis 1-2), we had to include and consider also other factors related to the embryo that may have had a significant impact on the results. We observed that small embryos had a lower PR 14 and 45. Similar results have been already described by different

authors and probably the reason underlying the decreased success is not the small size per se, but because they are “small for age”, showing a delay in the development [277]. Furthermore, we noticed that also larger embryos have a lower PR at 45 days, and this could probably be explained because they are more fragile and could be more easily damaged during handling or transfer [277,283]. The embryo grade also influenced the success of ET in our study, since embryo grade II and III had significantly lower PR 14 and 45 compared to embryo grade I, as previously reported by other authors [270,274,283].

5.5 Conclusions

In conclusion, the detection of IUF after ovulation negatively affected PR at 14 days, while the detection during estrus did not affect PR or EEL. Therefore, according to the results of our study, in order to increase the success rate of an embryo transfer program, mares with IUF during estrus can be safely used as embryo transfer recipients while mares with IUF after ovulation should possibly be disqualified.

CHAPTER 6

General conclusions

In conclusion, this study investigated endometritis from different angles in order to gain a deeper understanding of this widespread pathology. As the title suggests, it focused on both the diagnosis and treatment of endometritis: for the diagnosis, it investigated a potential new diagnostic technique such as 16S sequencing and the impact of the detection of clinical signs associated with this pathology in a commercial ET program; for treatment, it observed the effect of antibiotics on the uterine microbiome and investigated, through *in vitro* and *in vivo* studies, the possibilities of a new promising treatment, namely ozone therapy.

Regarding the microbiome, we decided to perform a first experiment on healthy mares because there are few studies in the literature on this topic and understanding what is normal and healthy is the first step to further understanding what is pathological. Therefore, we characterized the uterine microbiome of 11 healthy mares using the 16S rRNA sequencing technique. The most abundant genera were *Staphylococcus*, *Acinetobacter*, *Sphingomonas*, *Corynebacterium*, *Streptococcus*, *Rikenellaceae*, *Clostridium*, *Pseudomonas* and *Porphyromonas*. Furthermore, this experiment investigated the differences between estrus and diestrus and showed that the uterus harbours a resident microbiota even during the latter phase, when the cervix is closed. During the diestrus phase, however, the uterine microbiome diversity is reduced due to hormonal fluctuations and the closed cervix, which does not allow the ascension of bacteria from the vagina, which harbors a larger biomass.

In addition, the uterine microbiome of 5 mares with endometritis was described for the first time in the second experiment. The microbiome was dominated by the genera *Streptococcus* and *Escherichia-Shigella*, confirming the results of the culture technique. Furthermore, *Corynebacterium* accounted for most of the relative abundance in one of the mares. However, this genus was not detected with the culture technique, highlighting the differences between the two methods and the potential of 16S in gaining a deeper knowledge of the uterine microbiome as a whole, rather than just focusing on the 1% of bacteria that can grow *in vitro*.

After treatment with an antibiotic molecule, ceftiofur sodium, we observed a drastic decrease in the relative abundance of pathogenic bacterial genera that dominated the microbiome of the affected mares and the restoration of a more diverse and probably healthier environment. Thus, we were able to demonstrate that bacterial genera commonly considered pathogenic are normal components of the uterine microbiota of healthy mares. In mares with endometritis, however, they are overrepresented and dominate the endometrial microbiome, which is in a state of dysbiosis characterized by a reduction in bacterial diversity. Intrauterine antibiotic treatment led to the restoration of a more diverse microbiome.

The second part of this thesis deals with a promising treatment for endometritis, ozone therapy. Initially, we wanted to test the safety and effects of a commercial product based on ozonated oil, Riger Spray®, which was chosen because of the extensive literature demonstrating its beneficial effects in cows,

sheep and goats. However, its use in mares had never been described. Cytological, bacteriological and histological examinations before and after treatment revealed a temporary increase in inflammation of the endometrium, but the product proved to be safe. In addition, it did not have a negative effect on pregnancy rates, but a tendency towards a lower number of inseminations required to achieve pregnancy in the treatment group was observed. However, although subfertile mares were used in this study, since they were from private owners, we could not treat mares with a positive uterine culture as the owners decided to treat them with the gold standard, namely antibiotics.

We have therefore decided to take a step back and demonstrate the antibacterial effect of various ozone preparations on antibiotic-resistant intrauterine pathogens *in vitro* in order to provide a solid rationale for their use *in vivo*. Ozonized oil, gaseous ozone in two different concentrations and three exposure times as well as ozonized water were tested. It is noteworthy that the ozonated oil tested is the same as that used to produce Riger Spray®. In the present study, ozonated distilled water showed no significant antibacterial effect, which discourages its use as an antimicrobial agent for the treatment of endometritis. On the other hand, both gaseous ozone and ozonated oil showed antimicrobial activity against antibiotic-resistant bacterial and yeast strains responsible for endometritis. Moreover, even lower concentrations of gaseous ozone than previously reported showed efficacy *in vitro*. Thus, ozone therapy proved to be a promising treatment for infectious endometritis, and the results of this study provide a solid scientific rationale for its use *in vivo* as an antimicrobial agent to reduce or even avoid the over-use of antibiotics in equine reproduction.

The last study is based on the question of whether a clinical sign associated with endometritis, namely intrauterine fluid accumulation, can affect the results of a commercial ET program and should therefore be considered as a parameter for excluding a recipient from receiving an embryo during that cycle. We have distinguished two phases for the observation of IUF: pre-ovulation (estrus) and post-ovulation (early diestrus). Fluid during estrus is considered physiologic in the literature when the depth is <2cm, and the results of this study confirmed this hypothesis as it did not affect the success of the transfer. On the other hand, fluid during the diestrus phase is generally associated with uterine inflammation and/or delayed uterine clearance. Our retrospective study described a negative impact of post-ovulation IUF detection on transfer success. This seems to confirm the link between IUF in the diestrus phase and endometritis/delayed clearance and suggests that these mares should be avoided as embryo recipients.

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Supplementary Materials

Phylum		D	E	Mean
Firmicutes	39.233151470	3.882554e+01	39.029344109	
Proteobacteria	29.797019690	2.267165e+01	26.234333556	
Bacteroidota	14.017669868	1.663962e+01	15.328643088	
Actinobacteriota	12.108305936	1.616582e+01	14.137061523	
Verrucomicrobiota	1.379685391	1.764459e+00	1.572072122	
Cyanobacteria	0.958679702	1.550100e+00	1.254389958	
Fusobacteriota	1.055680546	8.399243e-01	0.947802407	
Fibrobacterota	0.438209987	3.827719e-01	0.410490968	
Patescibacteria	0.396076426	3.496345e-01	0.372855452	
Spirochaetota	0.232775668	2.604318e-01	0.246603731	
Campylobacterota	0.088902616	2.429175e-01	0.165910064	
Desulfobacterota	0.140320827	4.986424e-02	0.095092534	
Deinococcota	0.051723816	4.896065e-02	0.050342234	
Acidobacteriota	0.039830269	5.611649e-02	0.047973380	
Chloroflexi	0.002780095	7.606252e-02	0.039421308	
Myxococcota	0.013355890	3.829062e-02	0.025823256	
Bdellovibrionota	0.013355890	1.089559e-02	0.012125739	
Gemmatimonadota	0.007645844	9.562969e-03	0.008604407	
Entotheonellaeota	0.015518793	0.000000e+00	0.007759397	
Planctomycetota	0.009311276	7.703507e-04	0.005040813	
Fermentibacterota	0.000000000	6.714940e-03	0.003357470	
Ascomycota	0.000000000	5.135672e-03	0.002567836	
Sumerlaeota	0.000000000	2.390206e-03	0.001195103	
Synergistota	0.000000000	2.379093e-03	0.001189546	

S1: Relative abundances at phylum level of Experiment 1 (diestrus - D, estrus - E).

Genus	D	E	Mean
Staphylococcus	7.7647611532	8.205889918	7.98532554
Acinetobacter	7.1937105031	5.353353502	6.27353200
Sphingomonas	6.7591251694	4.650215233	5.70467020
Corynebacterium	4.5560487431	6.625817643	5.59093319
Streptococcus	6.6296270426	4.392413317	5.51102018
Rikenellaceae RC9 gut group	2.9569160367	4.155256837	3.55608644
Clostridium sensu stricto 1	2.0174811231	4.214939974	3.11621055
Pseudomonas	1.9698245674	3.094396378	2.53211047
UCG-002	1.9296779955	2.689264484	2.30947124
Flavobacterium	2.1800952775	1.723516980	1.95180613
Pasteurella	2.8374013502	0.323706367	1.58055386
Sarcina	1.6380233618	1.505438594	1.57173098
UCG-004	1.5344087778	1.330317736	1.43236326
Porphyromonas	1.8602638144	0.948997004	1.40463041
NK4A214 group	1.0874040259	1.483146210	1.28527512
Bacillus	0.9782121950	1.554783858	1.26649803
Brachybacterium	0.8737721740	1.388930578	1.13135138
Succinivibrio	1.9313136015	0.317995567	1.12465458
Lysinibacillus	0.9443387577	1.217755940	1.08104735
Kurthia	0.4237655485	1.712668323	1.06821694
Lawsonella	1.8858303434	0.023679411	0.95475488
Fusobacterium	1.1951950256	0.634639258	0.91491714
Uncultured	0.8091224821	1.006830131	0.90797631
Bacteroides	1.1211341998	0.494920167	0.80802718
Kocuria	0.1754597860	1.398359219	0.78690950
Chryseobacterium	0.9144255179	0.533474760	0.72395014
Brevibacterium	0.5236572877	0.902976145	0.71331672
Sphingobacterium	0.4302875650	0.915195776	0.67274167
Janibacter	0.3700511503	0.939941248	0.65499620
Oribacterium	0.7592744245	0.540480458	0.64987744
Jeotgaliococcus	0.6051583209	0.687437149	0.64629773
Escherichia-Shigella	0.7889552542	0.441488497	0.61522188
Anaerococcus	0.9885544869	0.187947362	0.58825092
Pedobacter	0.5679000057	0.602430900	0.58516545
Neorhizobium-Pararhizobium	0.6852443446	0.483634031	0.58443919
Uncultured	0.5433123758	0.611513397	0.57741289
Facklamia	0.6838793012	0.447530021	0.56570466
Hymenobacter	0.4016692372	0.721499141	0.56158419
Weissella	0.0691765578	1.044997666	0.55708711
Aerococcus	0.7050325526	0.369311255	0.53717190

S2: Relative abundances at genus level of the 40 most abundant genera of Experiment 1 (diestrus - D, estrus - E).

Phylum	T0	T1	Mean
Proteobacteria	39.641221580	34.048786441	36.845004010
Firmicutes	27.646855113	38.647374441	33.147114777
Actinobacteriota	13.187670525	14.819623791	14.003647158
Bacteroidota	11.047648666	8.886023163	9.966835915
Cyanobacteria	4.096028658	2.869417427	3.482723043
Verrucomicrobiota	1.925588924	0.325673413	1.125631169
Fusobacteriota	0.634045597	0.110093153	0.372069375
Deinococcota	0.592881579	0.002337040	0.297609310
Campylobacterota	0.312460307	0.040328717	0.176394512
Acidobacteriota	0.318381027	0.000000000	0.159190514
Spirochaetota	0.067461334	0.193273474	0.130367404
Patescibacteria	0.164081830	0.008370111	0.086225971
Fibrobacterota	0.142258351	0.028914929	0.085586640
Chloroflexi	0.123883022	0.000000000	0.061941511
Desulfobacterota	0.041653713	0.018262061	0.029957887
Bdellovibrionota	0.037571080	0.001521838	0.019546459
Planctomycetota	0.014216084	0.000000000	0.007108042
Gemmatimonadota	0.006092608	0.000000000	0.003046304

S3: Relative abundances at phylum level of Experiment 2 (before treatment -T0. After treatment - T1).

Genus	T0	T1	Mean
Streptococcus	0.710848724	3.651513e+01	18.612991328
Escherichia-Shigella	1.912169920	1.992122e+01	10.916694921
Corynebacterium	3.049331638	1.004785e+01	6.548592000
Staphylococcus	4.928745111	2.872983e+00	3.900863877
Pseudomonas	4.598987280	1.575645e+00	3.087316237
Acinetobacter	5.422570510	3.496671e-01	2.886118795
Arcanobacterium	0.465847292	4.370124e+00	2.417985496
Roseomonas	4.210219427	0.000000e+00	2.105109714
UCG-004	2.690992779	1.012148e+00	1.851570152
Sphingobium	1.879355987	1.478044e+00	1.678700123
Hymenobacter	1.281689756	2.034339e+00	1.658014493
Rikenellaceae RC9 gut group	2.358838616	8.009023e-01	1.579870448
Porphyromonas	0.000000000	2.955581e+00	1.477790433
Sphingomonas	2.291515436	5.658137e-01	1.428664590
Massilia	1.343171329	1.378132e+00	1.360651724
Sarcina	2.588544392	0.000000e+00	1.294272196
UCG-002	2.008895903	3.986807e-01	1.203788290
Clostridium sensu stricto 1	2.312336456	6.302608e-03	1.159319532
Moraxella	1.285871553	1.009727e+00	1.147799187
Micrococcus	1.942685834	6.237382e-03	0.974461608
Succinivibrio	1.932985853	2.729071e-03	0.967857462
Mycoplasma	1.751653284	0.000000e+00	0.875826642
Nocardioideis	1.650678213	8.525617e-02	0.867967192
Aerococcus	1.627162076	1.560002e-03	0.814361039
Stenotrophomonas	1.211474834	3.017690e-01	0.756621912
Kocuria	1.313392887	1.159328e-01	0.714662856
Uncultured	1.398255368	2.806648e-02	0.713160925
Novosphingobium	0.100049320	1.196670e+00	0.648359728
Frondihabitans	1.290777144	0.000000e+00	0.645388572
Parabacteroides	1.235024890	0.000000e+00	0.617512445
NK4A214 group	0.939764542	2.447046e-01	0.592234593
Actinobacillus	1.170513775	0.000000e+00	0.585256888
Solibacillus	1.159793814	0.000000e+00	0.579896907
Lachnospiraceae NK4A136 group	1.096648858	3.771434e-02	0.567181597
w5053	1.120992277	0.000000e+00	0.560496139
Anaerococcus	0.954251724	1.612903e-01	0.557771023
Ligilactobacillus	1.075427622	6.139543e-03	0.540783583
Clavibacter	0.077502994	9.729448e-01	0.525223922
Rathayibacter	0.019728035	9.963580e-01	0.508042998

S4: Relative abundances at genus level of the 40 most abundant genera in Experiment 2 (before treatment -T0. After treatment -T1).

Research Products

Publications originating from the study:

- 1 Donato GG, Nebbia P, Stella MC, Scalas D, Necchi D, Bertero A, et al. *In vitro* effects of different ozone preparations on microorganisms responsible for endometritis in the mare. *Theriogenology* 2024;219:132–7. <https://doi.org/10.1016/j.theriogenology.2024.02.011>.
- 2 Donato GG, Necchi D, Vandaele H, Vita ME, Bertero A, Vincenti L, et al. Influence of Intrauterine Fluid Detection, Number of Transfers and Age of the Recipient on Pregnancy Rate and Early Embryonic Loss in a Commercial Embryo Transfer Program. *Animals* 2023;13. <https://doi.org/10.3390/ani13111799>.
- 3 Donato GG, Appino S, Bertero A, Poletto ML, Nebbia P, Robino P, et al. Safety and Effects of a Commercial Ozone Foam Preparation on Endometrial Environment and Fertility of Mares. *J Equine Vet Sci* 2023;121. <https://doi.org/10.1016/j.jevs.2023.104222>.

Oral presentations at international congresses:

- 1 Donato GG, Nervo T, Stella MC, Nebbia P, Romano G, Necchi D, Bergamini L, Poletto ML, Scalas D, Robino P. *In vitro* evaluation of antimicrobial effect of ozone against bacteria responsible for equine endometritis
1st European Symposium on Animal Reproduction, Nantes, 21-23 September 2023
Reprod Dom Anim, 58: 203. <https://doi.org/10.1111/rda.14441>
- 2 Donato GG, Necchi D, Bertero A, Vincenti L, Nervo T. Relationship between intrauterine fluid detection in the recipient mare and pregnancy rate in a commercial embryo transfer program.
28° SIVE International Congress, Venice 3-4 February 2023.
- 3 Donato GG, Nervo T, Gionechetti F, Nebbia P, Robino P, Ala U, Pallavicini A. The endometrial microbiome: new insights into equine endometritis.
29° SIVE International Congress, Florence 2-3 February 2024.