Feto-maternal microbiome in Small Animals

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List of abbreviations

16S	16 subunit
ACD	Appenzeller Cattle Dog
AF	amniotic fluid
ARRIVE	Animal Research: Reporting of In Vivo Experiments
ASV	Amplicon Sequence Variants
CA	California
cAMP	cyclic adenosine monophosphate
CEE/EEC	European Economic Community
C-FMU	canine feto-maternal unit
C-section	cesarean section
CFU	colony-forming unit
DAPI	4',6-diamino-2-phenylindole, dihydrochloride
ddH_2O	double-distilled water
DG	days of gestation
D.L.	Decree-Law
DNA	deoxyribonucleic acid
DOHaD	Developmental Origins of Health and Disease
dsDNA	double stranded deoxyribonucleic acid
e.g.	*exempli gratia*
et al.	*et alia*
EU	European Union
EUB-338-Cy3	Eubacteria probe with Cyanine3 dye
FEDIAF	European Pet Food Industry Federation
F-FMU	feline feto-maternal unit
FISH	Fluorescence In Situ Hybridization
FLASH	Fast Length Adjustment of SHort reads
FMU	feto-maternal unit
i.e.	*id est*
Ig	immunoglobulins
IgG	immunoglobulins G
IQR	interquartile range

i.v.	intravenous
LR	Lagotto Romagnolo
M	meconium
MA	Massachussets
MALDI-TOF MS	matrix-assisted laser desorption/ionization coupled to time-of-flight mass spectrometry
n	number
n/a	not available
NGS	Next-Generation Sequencing
NY	New York
OUT	Operational Taxonomic Unit
P	placenta
PA	Pennsylvania
PBS	Phosphate-Buffered Saline
PCoA	Principal Coordinate Analysis
PCR	Polymerase Chain Reaction
PERMNOVA	permutational multivariate analysis of variance
R	rectum
RDP	Ribosomal Database Project
RNA	ribonucleic acid
rRNA	ribosomal RNA
SD	standard deviation
SEM	standard error of the mean
s.r.l.	limited liability company
UK	United Kingdom
UniFrac	Unique Fraction metric
USA	United States of America
UV	ultraviolet
V	vagina
Ver.	version

Chapter 1
General introduction

Adapted from:

The dogma of the sterile uterus revisited: does microbial seeding occur during fetal life in humans and animals?

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1. The microbiome and its role in the Developmental Origin of Health and Disease

The microbiome is defined as "a microbial community occupying a reasonably well-defined habitat with characteristic physio-chemical properties" (Berg *et al.*, 2020). Like a real ecosystem, the microbiome is a dynamic element constantly changing in response to environmental conditions and interacting with its eukaryotic host. These bacterial communities promote the development of its immune and nervous systems, participate in metabolic functions, synthesize useful compounds, and protect the host against potential pathogens. Therefore, it has a crucial role in the health of the individual (Berg *et al.*, 2020; Hou *et al.*, 2022). Although the existence and functions of the microbiome of body niches such as the gastrointestinal tract, skin, some reproductive and respiratory organs have been recognized (Wilson and Hamilos, 2014; Cresci and Bawden, 2015; Yamashita and Takeshita, 2017; Lykke *et al.*, 2021), the presence of microorganisms within the fetal environment during healthy pregnancies is still controversial. This debate revolves around the pioneer colonization of the individual, questioning whether it starts during fetal life (i.e., '*in utero* colonization' hypothesis) or whether it occurs during birth and the early post-natal period (i.e., 'sterile womb paradigm') (Funkhouser and Bordenstein, 2013; Perez-Muñoz *et al.*, 2017). Answering this question is a fundamental step to investigate the contribution of bacteria to the 'developmental origins of health and disease' (DOHaD). This theory, previously known as 'Barker hypothesis' (Barker and Osmond, 1986), has evolved from initially associating malnutrition with hypertension in later human life to encompassing a growing list of factors linked to long-term health (Stiemsma and Michels, 2018). Moreover, the need to extend this concept to companion animals has recently been recognized (Gaillard *et al.*, 2022), as dogs and cats are considered as family members and their welfare represents a priority. Several factors may come into play at early stages of animal development and influence its future health (Gaillard *et al.*, 2022). Among these, birthweight, early growth rate, neonatal and pediatric nutrition, maternal care, drugs administration, and social factors have been associated with risk of future obesity, behavioral problems, and chronic enteropathy in small animals, with species-specific and breed-specific variations (Gaillard *et al.*, 2022). Furthermore, the DOHaD concept can be applied to pre-conception stages, when factors targeting the dam and the sire impact on their general health and gametes development (Gaillard *et al.*, 2022). Among these factors, some have been confirmed, as the role of the gut microbiota of the dam in shaping that of her puppies (Del Carro *et al.*, 2022), whereas others have been reported in other species, such as the effect of antibiotics administration to the mother on the risk of gastrointestinal disease in humans and mice (Schulfer *et al.*, 2018; Agrawal *et al.*, 2021).

Although research on the role of specific factors in shaping future health is needed, what must be acknowledged is that only complex models considering the characteristics of the animal, the window of exposure, the co-existence of multiple factors, and their interactions would allow predicting and preventing future disease, approaching the concept of 'developmental programming' (Sutton *et al.*, 2016). Many studies focus on assessing the risk related to some factors that passively target the animal during early developmental stages (Kondo *et al.*, 2023; Shenassa *et al.*, 2023), but an active programming may represent a powerful tool to prevent future disease (Kenyon and Blair, 2014; Meesters *et al.*, 2024).

The microbiome is a key player in this intricate game, as it is susceptible to environmental factors and can be modulated and altered in different ways, including nutrition, pre- and probiotics administration, and antimicrobial treatments (Langdon *et al.*, 2016; Lee *et al.*, 2018; Daliri *et al.*, 2020). The association between bacterial exposure and future health was postulated for the first time in the 'hygiene hypothesis of allergic disease' (Strachan, 1989), when bacteria were recognized as environmental factors influencing the development of the immune system. As research on the microbiome progressed, this theory evolved into the 'microflora hypothesis' (Noverr and Huffnagle, 2005; Shreiner *et al.*, 2008), sustaining that an altered microbiota contributes to the development of allergic disease. The immature immune system of the fetus and newborn is sensitive to microbial modulation, and this is not limited to immune tolerance (i.e., recognition of 'self' and 'non-self' elements), as the microbiome or its metabolites are involved in inflammation and immune cell differentiation, maturation, and function (Schluter *et al.*, 2020; Zheng *et al.*, 2020; Kim, 2021). For this reason, appropriate development of the neonatal microbiota (Marchesi and Ravel, 2015) is generally recognized as a key feature for immunological and physiological maturation (Stinson *et al.*, 2017; Cheng *et al.*, 2019). However, in the light of the DOHaD concept, the exact timing and dynamics of the pioneer microbial colonization may elucidate which window of exposure can be targeted to reduce risk factors for future disease and improve the general health of newborns and adults. Although the maturing gut microbiome during the neonatal and pediatric phases has been shown to play a key role in future health outcomes in humans, dogs, and cats (Deusch *et al.*, 2014; Garrigues *et al.*, 2022; Khan *et al.*, 2023), bacteria may interact with the fetus during intrauterine life, potentially priming the immune system (Stinson *et al.*, 2017).

The spark that fired the debate about the existence of a characteristic feto-maternal microbiome was the detection of bacteria in the basal plate of the healthy human placenta, reported by Aagaard *et al.* (2014). This, together with the advent and widespread use of non-culture-based methods to identify bacteria, set the stage for the manifold literature that has been published during the last decade on this

topic in humans (Funkhouser and Bordenstein, 2013; Romano-Keeler and Weitkamp, 2015; Perez-Muñoz *et al.*, 2017; Willyard, 2018; Senn *et al.*, 2020; Fricke and Ravel, 2021; Walter and Hornef, 2021; Zakis *et al.*, 2022). Similar research on animals has been performed only recently and is still fraught with multiple challenges and opportunities. Animal studies have proven to be fundamental for comparative research, and studies in laboratory species elegantly proved that immunity of the fetus starts in the uterus under the influence of the maternal gut microbiome (Gomez de Agüero *et al.*, 2016). However, this topic has never been researched in small animals, leaving an open question on whether the gestational microbiome is a factor that can be modulated in the view of developmental programming.

2. Anatomical and physiological differences do not allow drawing common conclusions among mammals

Unravelling the pathways of bacterial colonization of the uterine environment is a prerequisite to confirm the existence of a pre-natal microbiome. It has been hypothesized that bacterial presence in fetal tissues during gestation is related to a characteristic uterine microbiome (Coscia *et al.*, 2021). Interestingly, the presence of bacteria in the non-pregnant uterus is a further topic of dispute. In women, microbial communities have been identified all along the reproductive tract and throughout the different stages of the menstrual cycle by molecular techniques, with site-specific characteristics, while the viability of some of these bacteria was confirmed by culture (Chen *et al.*, 2017). Non-primate mammals display an estrous cycle, with estrus referring to the period of sexual receptivity and ovulation, diestrus to the time characterized by the presence of the corpus luteum, and anestrus to the period of sexual rest. During di- and anestrus, the cervix is a mucin-made seal, that prevents ascending contamination (Chen *et al.*, 2017). The cervix is patent in pro-estrus, estrus, and at parturition, allowing for the ascending migration of vaginal bacteria. Therefore, unsurprisingly, bacteria were found in post-partum cows (Huszenicza *et al.*, 1999; Santos *et al.*, 2011) and in virgin heifers following estrus induction (Moore *et al.*, 2017), as well as in healthy mares in estrus (Christoffersen *et al.*, 2015; Holyoak *et al.*, 2022). In small animals, the uterine environment has been investigated for the presence of microorganisms throughout the cycle (Lyman *et al.*, 2019; Praderio *et al.*, 2019). In dogs and cats, ovariectomy/ovariohysterectomy is commonly performed as a means of definitive surgical contraception, allowing to access the uterus without passing through the vagina nor the cervix. Interestingly, no bacteria were isolated by culture from the feline (Ström Holst *et al.*, 2003) or canine healthy uterus in diestrus (Praderio *et al.*, 2019). However, using molecular techniques, Lyman *et al.* (2019) described a uterine microbiome in bitches throughout the estrous cycle.

Whether sterile or not, the journey of a bacterium from the maternal environment to the fetus would include overcoming the placental barrier (Perez-Muñoz *et al.*, 2017). In eutherian mammals, the fetus develops within a unique and complex environment and the placenta works both as a physical barrier and as a connection with the mother. This organ has species-specific anatomy and features (Furukawa *et al.*, 2014), and research on feto-maternal membranes and fluids in humans is much more prevalent than in animals (Miller *et al.*, 2020). The extent and depth of the feto-maternal connection is highly variable among different animal species and is responsible for a varying degree of defense and exchange in nutrients, waste products, and oxygen. Specifically, the placenta of primates (i.e., hemomonochorial) has a discoidal morphology and the highest level of invasion, building a deep connection between the mother and the fetus, with a direct contact between the chorionic epithelium and the maternal blood and only one trophoblast layer. In laboratory animals, often considered as a model for human studies, the trophoblast consists of two layers in rabbits (i.e., hemodichorial) and three in mice and rats (i.e., hemotrichorial). In these types of placentae, chorionic villi are directly exposed to microbial products being present in the maternal circulation (Megli and Coyne, 2022), whereas in all domestic species, the number of layers interposing between the dam's blood and the fetus, is higher (*Figure 1*) (Hafez, 2017). This thicker mechanical separation is known to limit the passage of immunologically relevant molecules (Chucri *et al.*, 2010; Furukawa *et al.*, 2014), and likely of bacteria as well. Moreover, the immune function of the placenta is enhanced by the presence of white blood cells (e.g., Hofbauer cells, natural killer NK, B cells) and active substances (e.g., antimicrobial peptides, defensins, toll-like receptors) (Soto *et al.*, 2007; Stock *et al.*, 2007; Dall'Ara *et al.*, 2015; Para *et al.*, 2020), although species-specific features have not been fully unveiled yet.

If hypothetically bacteria could successfully pass through the placental barrier (Loughran and Tuomanen, 2016), the amniotic fluid would represent a further obstacle. Its composition varies among species (Canisso *et al.*, 2019)(Canisso et al., 2019) and besides mechanically protecting the fetus acting as a cushion against physical trauma, this fetal substrate contains substances with antimicrobial properties (e.g., defensins, calprotectin) (Espinoza *et al.*, 2003).

Because of species-specific anatomical and physiological characteristics of feto-maternal relationships, it is impossible to generalize any finding about the gestational microbiome that applies to humans to other mammals (Furukawa et al., 2014), including dogs and cats. For this reason, species-specific research is needed to clarify the matte of the feto-maternal microbiome in small animals.

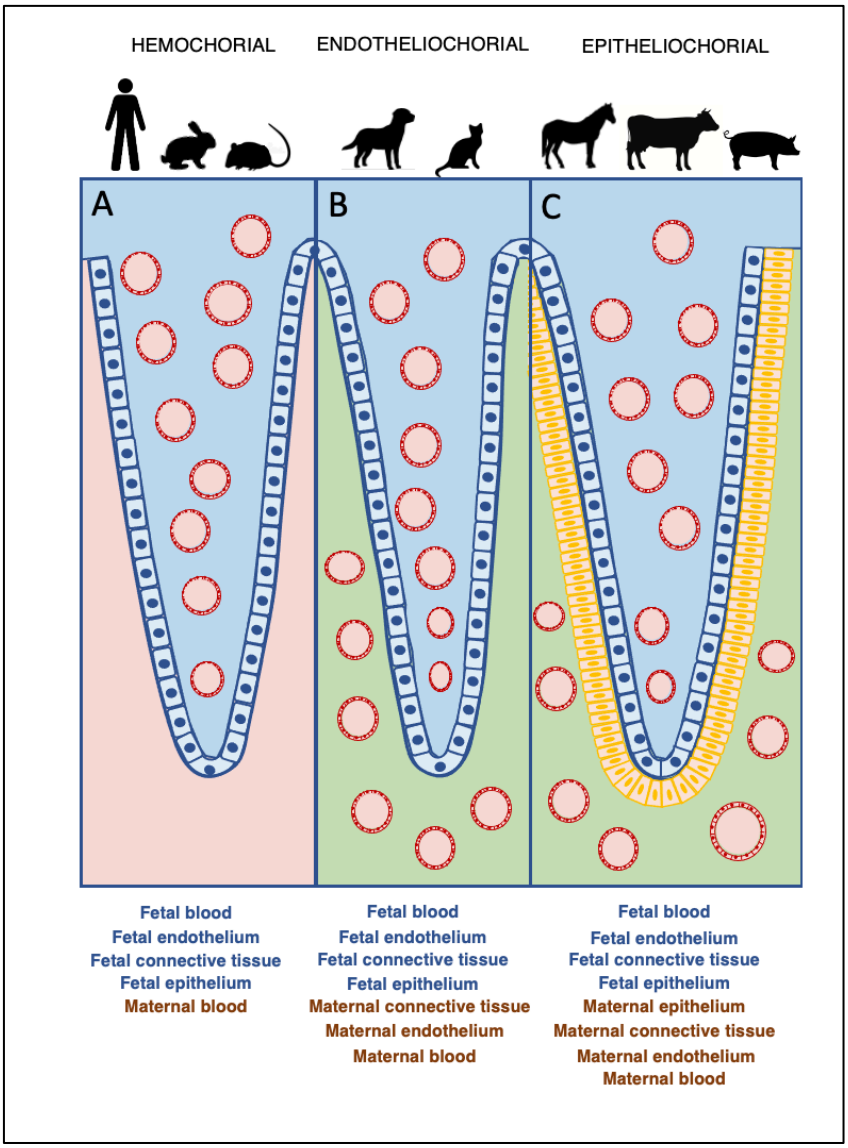

Figure 1. Schematic comparative representation of layers interposing between the fetal and maternal blood in different type of placentae.

2.1 The feto-maternal connection in small animals

A prerequisite for a successful feto-maternal connection is the maternal recognition of pregnancy, which will lead to deep modifications of the uterine environment, immune system, and endocrine and metabolic functions of the dam.

The process of pregnancy recognition starts long after the formation of the zygote, when the spermatozoon fertilizes the mature oocyte (i.e., fertilization). The zygote undergoes reductive cleavage to evolve into a blastocyst, in which different cell layers (i.e., endoderm, mesoderm, and ectoderm) will differentiate. These will give rise to the formation of the embryo and extraembryonic tissues, including fetal annexes (Paulson and Comizzoli, 2021). These pre-implantation processes are

common among mammals, although the timing of these events varies across species, including the domestic dog and cat (*Table 1*).

Table 1. Timing of canine and feline pregnancies from ovulation to placental formation.

	Dog	**Cat**
Gestation length	63 +/- 1 days after ovulation	61 days after the last mating
Oocyte maturation (secondary oocytes)	2-3 days after ovulation (spontaneous ovulation)	Mature at ovulation (induced ovulation)
Fertilization site	Oviduct (infundibulum)	Oviduct (infundibulum)
Development to blastocyst	Day 7	Day 7
Migration into the uterus	7-10 days post-fertilization	/
First contact with the uterine endometrium	12-14 days post-fertilization	/
Apposition and adhesion	Between day 17-20	Day 12
Implantation	Day 20	Day 14
Placental grindle	Complete	Incomplete
Marginal hematoma	Green and macroscopically visible on both sides of the grindle	Brown and only microscopically visible

Paulson and Comizzoli, 2021; Kowalewski *et al*., 2020; Miglino *et al.*, 2006

Although canine and feline pregnancies have similar duration, embryo implantation occurs earlier in cats compared to dogs but with the same centric pattern (Paulson and Comizzoli, 2021). The embryo adheres to the uterine wall and expands towards the lumen with an invasive process in which the trophoblast anchors the embryo to the endometrium. Specifically, this process involves the luminal layer of the endometrium (i.e., functional layer), which undergoes cyclic remodeling under the influence of estradiol and progesterone in preparation for eventual pregnancies. It includes epithelial cells of different shapes (cuboidal to columnar), stromal, immune, and endothelial cells (Van Cruchten *et al.*, 2004). Also, endometrial glands increase their secretory activity during the estrus and elongate into cylindric structure in parallel with embryo migration, adhesion, and implantation (Aralla *et al.*, 2013). Furthermore, some endometrial stromal cells undergo a process named decidualization (Kowalewski *et al.*, 2020) promoted by progesterone, estradiol, prostaglandin E2, and cAMP. Decidua cells have increased secretory activity and express decidualization-specific factors (Kautz *et al.*, 2015). These cells resist the invasion from the trophoblast and represent the maternal component of the placenta together with the maternal endothelium (Kautz *et al.,* 2015). The formation of the chorioallantoic placenta starts around day 20 of gestation in dogs and cats (Miglino *et al.*, 2006). At this early stage the fetal membranes are difficult to distinguish, the yolk sac is larger than the embryo, and the amnion and allantois do not present blood vessels. About four days later, the allantois doubles

the yolk sac in size, the allantochorion presents small blood vessels (Miglino *et al.,* 2006), and the placental grindle has expanded, representing the main feto-maternal contact region until the end of pregnancy.

The canine and feline placentae are defined as zonary, endotheliochorial, and *deciduatae* (Furukawa *et al*., 2014; Kowalewski *et al*., 2020). The first adjective refers to the shape of the area where the fetus adheres to the maternal endometrium, the second to the layers involved into the feto-maternal connection (i.e., endothelium of the maternal vessels and epithelial cells of the chorion), and the latter defines the intimate degree of invasion of the maternal endometrium by the fetal villi that causes the shed of some uterine material when fetal membranes are expelled (Kowalewski *et al*., 2020). From mid pregnancy to term, each fetus develops inside an amniotic sac filled with fluid surrounded by the thin chorioallantoic membrane (i.e., non-vascular zone of the placenta). Macroscopically, this presents a belt-shaped area with a central region (i.e., transfer zone of the placenta) encompassed by two green-pigmented hemophagous ones filled with maternal blood (i.e., marginal hematoma, pigmented zone of the placenta) (Enders and Carter, 2012; Senger, 2012) (*Figure 2*).

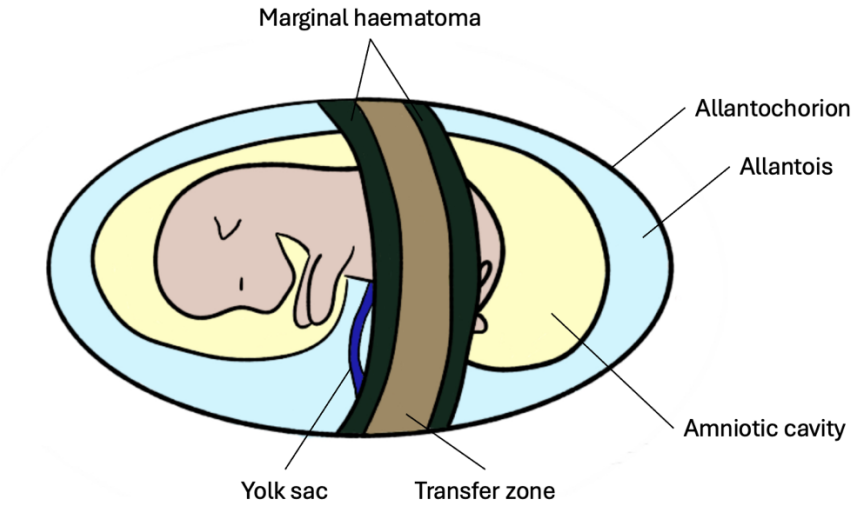

Figure 2. Schematic representation of the zonary endotheliochorial placenta of dogs and cats.

Microscopically, the three regions can be histologically defined within the transfer zone:

(i) Placental labyrinth: has a lamellar organization (Lavanya *et al.,* 2019), with fetal projections (i.e., fetal villi) interdigitating with maternal septa (Aralla *et al.*, 2013; Tesi *et al.*, 2021). This asset has the function of increasing the surface area available for cross-

current blood exchanges (Senger, 2012; Lavanya *et al*., 2019). The placental labyrinth is composed of several lamellae, in which the endothelium of maternal vessels is in direct contact with the cells of the chorionic villi (i.e., the external syncytiotrophoblast and internal cytotrophoblast cells).

(ii) Junctional zone: represents the transition between the labyrinth and the glandular zone. It is characterized by a sponge-like texture due to the wide intercellular spaces between the columnar cells infiltrating the endometrial glandular area (Lavanya *et al.,* 2019; Furukawa *et al.,* 2014).

(iii) Glandular zone: presents distended endometrial glands that retain their secretes due to trophoblast invasion of the maternal endometrium (Lavanya *et al.,* 2019; Furukawa *et al.,* 2014).

The placenta regulates the exchanges between the dam and the fetuses. Molecules of different sizes are transferred by simple or facilitate diffusion or by active transport (Senger, 2012). The interhemal distance between the dam and the fetus and the uterine blood flow represent two key factors in regulating placental exchange (Senger, 2012; Griffiths and Campbell, 2015). The latter increases about nine times in pregnant bitches compared to non-pregnant ones, reaching about 27 mL/min (Abitbol *et al.*, 1980).

The placenta of species with higher interhemal distance (e.g., the epitheliochorial placenta of ruminants) is permeable to molecules up to 400 Da, whereas the hemochorial placenta of humans allows the passage of solutes up to 1350-5200 Da (Thornburg *et al.*, 1988; Burton and Fowden, 2015). The interhemal distance between fetal and maternal blood in dogs and cats is higher compared to that of humans and smaller compared to that of other domestic species (Hafez, 2017). Hence, the permeability of endotheliochorial placentae is somewhere in between that of hemochorial and endotheliochorial ones. Some small molecules and minerals, such as sodium, chloride, gases, water, glucose, amino acids, are transferred by passive diffusion (Griffiths and Campbell, 2015). However, the passive transfer of most of these molecules (i.e., glucose, amino acids, water, sodium, chloride, calcium, iron) is insufficient and facilitated diffusion or active transfer by specific proteins is needed (Griffiths and Campbell, 2015; Illsley and Baumann, 2020). Triglycerides are too large to cross the placental barrier. However, these molecules are essential to build biological membranes and other compounds, including prostaglandins. Therefore, maternal triglycerides and lipoproteins are cleaved by a lipase enzyme located on the maternal surface of the placenta and fatty acids and glycerol are transferred by simple or facilitate diffusion (Griffiths and Campbell, 2015). In general, proteins are

too large to cross the placental barrier, therefore amino acids are transferred to the fetus and proteins are built.

2.2. Transfer of immunoglobulins and immunity

Among proteins, immunoglobulins (Ig) have a fundamental role in protecting the individual from pathogens, and a maternal-fetal transfer mediated by specific receptors has been confirmed in humans (Clements *et al.*, 2020). However, in dogs and cats the exchange of immunoglobulins by pinocytosis is limited to the last third of pregnancy, reaching up to 5-10% in dogs and 25% in cats (Borghesi *et al.*, 2014; Pereira *et al.*, 2023). The transfer is not sufficient to guarantee passive immunity to neonates, that are born with very low IgG concentrations compared to adult animals (0.3 g/L versus 8-25 g/L and values below detection limits versus 40-50 g/L in dogs and cats, respectively) (Claus *et al.*, 2006; Crawford *et al.*, 2006; Mila *et al.*, 2014).

Any potentially harmful event, including contacts with pathogens, is physiologically detected and contrasted by the immune system. This complex protective system starts developing during fetal life (Pereira *et al.,* 2023). Immune cells arise from the yolk sac as early as 17-25 days of gestation in dogs and cats. The liver, spleen, and bone marrow subsequently take over immune cells production. Neutrophils, eosinophils, basophils, monocytes, and B lymphocytes are matured in the bone marrow and then released in the blood stream. T lymphocytes mature in the thymus, that develops around days 27-34 and become actively lymphopoietic around days 35-45 in canine and feline fetuses (Pereira *et al.*, 2019, Pereira *et al.* 2023). Specifically, the thymus is the site of differentiation into TCD4+ and TCD8+ lymphocytes (40-45 days of gestation). Subsequently, these cells migrate from the thymus to secondary lymphoid organs between 40-52 days of gestation in cats and 45-55 days of gestation in dogs.

At birth, the immune system of puppies and kittens is functional yet immature, leading to a slow response to harmful events. Hence, protection from pathogens in canine and feline neonates relies on colostrum intake in the first hours of life (Pereira *et al.,* 2023).

3. Do bacteria cross the placental barrier?

The bacterial genome has a mass ranging from 5 $x10^8$ to 10 $x10^8$ Da (Bak *et al.*, 1969). Hence, simple diffusion through the placenta is not possible for bacterial cells. Nevertheless, some pathogens have developed strategies to colonize the fetus, and the presence of bacteria in the placenta has been associated with adverse pregnancy outcomes (Sahin *et al.*, 2014; Doyle *et al.*, 2017; Tomlinson *et al.*,

2019). Maternal blood can convey pathogens to the placenta, and some bacteria can cross this barrier by cell-to-cell transfer. For example, *Brucella canis* can colonize resident macrophages of the canine placenta (Santos *et al.*, 2021), whereas mechanisms related to the passage of *Listeria monocytogenes* through this barrier are yet to be fully understood (Hoelzer *et al.*, 2012). Other pathogens have no specific ability to cross the feto-maternal interface, although they could break into the fetal environment whenever the placental barrier is damaged (Robbins and Bakardjiev, 2012). This concept is known as the 'double-hit hypothesis', postulating that a first insult may be well tolerated by organs such the placenta, whereas a second may overcome the protection mechanisms (Robbins and Bakardjiev, 2012).

Besides these pathological events, what is still controversial is whether commensal non-pathogenic bacteria colonize and cross the placenta during healthy pregnancies. The reason for the dispute is that detection of bacteria within feto-maternal tissues, including the placenta, is fraught with multiple challenges.

3.1 Technical challenges in assessing bacterial presence and viability

The debate on the 'sterile womb paradigm' has blossomed in parallel with the evolution of molecular techniques. However, culture-dependent and independent methods often yield very different results. *Table 2* lists the main available techniques for bacterial detection in fetal samples, along with their main advantages and limitations.

Table 2. Main techniques available for bacterial detection are reported, along with their main advantages and limitations and their suitability for research assessing bacterial presence in the fetal environment during healthy pregnancies.

	Advantages	Limitations	Suitability to confirm or deny bacterial presence in fetal tissues
16S rRNA gene sequencing	• High sensitivity and specificity. • Identifies a wide range of bacteria, including non-culturable ones. • Provides taxonomic information. • Description of bacterial communities.	• Risk of contamination in low-biomass samples. • Bacterial viability is not assessed. • Results are highly dependent on the protocol.	Yes, although bacteria might be dead.
Bacterial culture	• Easily available	• Misses more than 90% of bacteria	Yes, as unculturable bacteria are missed.

	• Gold standard to detect the presence of live bacteria. • Can be coupled with biochemical tests and MALDI/TOF-MS for species identification.	(unculturable bacteria). • Results depend on culture media and culture conditions. • May miss fastidious bacteria.	
Electron microscopy	• Direct visualization of bacteria in samples. • Localizes bacteria within a tissue. • Information about bacterial morphology.	• Low sensitivity, especially for low-biomass samples. • Does not provide species identification.	No, as fetal tissues are zero-to-low-bacterial biomass samples.
Enzyme-Linked Immunosorbent Assay (ELISA)	• Indirect technique that detects bacterial antigens. • Relatively fast.	• Limited to specific bacteria, requires previous knowledge of target bacteria. • Lower sensitivity compared to molecular methods.	No, as prior knowledge of target bacterial is required.
Flow cytometry	• Detection and sorting of bacterial cells. • Can differentiate viable cells.	• Requires fluorescent tagging. • Limited sensitivity for low-biomass samples.	No, as fetal tissues are zero-to-low-bacterial biomass samples.
Fluorescence In Situ Hybridization (FISH)	• Detection and spatial localization of bacterial signal within a tissue. • Probes can be chosen based on the required specificity.	• Technically complex and time-consuming. • Difficult in some types of samples. • Limited availability.	Yes, only as confirmatory test for bacterial presence since sensitivity is limited in low-biomass samples.
Gram staining	• Quick and inexpensive. • Provides morphological data.	• Low sensitivity for low-biomass samples. • Does not provide species identification.	No, as fetal tissues are zero-to-low-bacterial biomass samples.

Immunohistochemistry (IHC)	• Detects specific bacterial proteins in tissue samples. • Localizes bacteria in tissue sections.	• Requires specific antibodies and previous knowledge of target bacteria. • Unknown bacteria cannot be detected.	No, as prior knowledge of target bacterial is required.
Metagenomic shotgun sequencing	• Comprehensive identification of bacteria and their genes. • Sequencing of bacterial genes allows functional analyses. • Description of bacterial communities.	• Requires higher bacterial DNA concentration (often not possible for low-biomass samples). • Risk of contamination. •	No, high DNA concentration is needed (not possible in low-biomass samples).
Quantitative PCR (qPCR)	• Useful for bacterial detection and quantification. • Propidium monoazide qPCR can be used to check bacterial viability.	• Requires prior knowledge of target bacteria. • Unknown bacteria cannot be detected.	No, as prior knowledge of target bacterial is required. Furthermore, confirming bacterial viability using propidium monoazide qPCR requires DNA concentration higher than that of low biomass samples.

Traditional culture is the main investigation tool in clinical settings and researchers applied culture to assess bacterial presence in cases of adverse pregnancy events for decades (Perez-Muñoz *et al.,* 2017). Conducting research using culture-dependent methods alone comes with great limitations. In fact, it is common knowledge that different microorganisms require different environmental conditions and nutrients to grow (Bonnet *et al.*, 2020). 'Non-fastidious' bacteria (e.g., Staphylococci, Streptococci) grow in less complex media compared to 'fastidious' ones (e.g., *Campylobacter spp.*, *Helicobacter spp.*, *Lactobacillus spp.*, *Mycoplasma spp.*) (Rishmawi *et al.*, 2007), the latter often requiring specific media and long incubation times to be isolated. In addition, there are bacteria defined as 'unculturable' (i.e., do not grow in laboratory conditions) that are missed when using traditional bacteriology techniques (Wade, 2002), and these represent more than 90% of bacterial species (Kaeberlein *et al.*, 2002). This undoubtedly leads to underestimate the bacterial density and richness of samples. However, sequencing alone does not allow the assessment of bacterial viability (Perez-Muñoz *et al.*,

2017), and comes with its own methodological issues and limitations. Among these are the problems related with the presence of unknown bacteria, the dependency on the DNA extraction kits, the possibility of amplification biases, and the influence of bioinformatic analyses (Hornung *et al.*, 2019). All these limitations, together with potential environmental contamination of samples during collection and/or processing, often prevent to formulate definitive conclusions.

Feto-maternal tissues are considered as potential 'zero-biomass' samples (Kennedy *et al.*, 2023), meaning that the existence of bacteria is not confirmed and possibly non-existent. This could lead to a false detection of bacteria, entirely originating from contamination. Therefore, research should be designed considering contamination as the main issue. To mitigate contamination some precautionary measures should be integrated in the study design.

- Inclusion of multiple controls
- Samples should be concentrated before processing to increase input biomass
- Consider any source of contamination during sampling
- Carry out the analyses in a clean environment (UV treated surfaces)
- Monitor the presence of contamination from the kit (i.e., the so called 'kitome') by inclusion of 'blank' laboratory negative controls
- Post-sequencing analyses, using programs like 'decontam', and analysis steps are helpful in detect sequences from contaminants (de Goffau *et al.*, 2018)
- Combine different techniques

Combining different techniques for microbiome studies has been suggested as a more complete approach to overcome limitations associated with each separate technique (Perez-Muñoz *et al.*, 2017; Kennedy *et al.*, 2023). However, it remains challenging to interpret the results of feto-maternal microbiome analyses in a comprehensive way and to conclude whether different methods are sufficiently complementary. The combination of culture and molecular techniques is often applied, although the implementation of more techniques assessing different aspects, such as microscopic visualization, has been suggested to increase results reliability.

Culture is often used to investigate bacterial viability, which might be overestimated when using Next Generation Sequencing (NGS), since not all microorganisms identified through sequencing are alive. Therefore, the viability of *strictu sensu* unculturable bacteria and of those that are viable but not culturable (i.e., those that fail to grow on media on which they should normally grow because they entered a dormant state) cannot be assessed by culture-NGS combinations (Kumar & Ghosh, 2019).

Although culture-independent techniques for bacterial viability have been developed (Kumar and Ghosh, 2019), there is currently no generally accepted method guaranteeing unbiased results.
Molecular methods on the other hand, allow for an in-depth description of microbial populations and offer the chance to unveil the presence of genetic material also originating from unculturable bacteria. However, these techniques are not free of controversies because studies based on 16s rRNA gene sequencing involve many steps. Among these are sample collection and storage, DNA extraction, amplification of the 16S rRNA gene, sequencing of the amplified nucleotide chains, identification and classification of the sequences referring to a taxonomic database (Maki *et al.*, 2019), and bioinformatic analyses. A description of how each step can influence the results of studies on feto-maternal microbiome is provided below.

3.1.1 Bacterial DNA extraction and amplification

Different extraction kits were chosen to conduct studies on the feto-maternal microbiome, with a potential impact on the absolute numbers, relative abundance, and richness of microbial populations (Henderson *et al.*, 2013). The existence of a 'kitome' refers to results being rather associated with the extraction materials than with the feto-maternal elements (Lauder *et al.*, 2016). Decontamination of PCR reagents from microbial DNA can partially solve this problem (Stinson *et al.*, 2018), however only few animal studies applied any kind of decontaminating procedure (Theis *et al.*, 2020; Zou *et al.*, 2020; Husso *et al.*, 2021; Winters *et al.*, 2022).
Furthermore, it has been demonstrated that the choice of PCR primers and selection of the 16S rRNA hypervariable region may influence the results in terms of bacterial richness and diversity (Bukin *et al.*, 2019). For instance, targeting the V1-V2 region may lead to underestimate some relevant bacteria of the genital tract (Graspeuntner *et al.*, 2018). Parnell *et al.* (2017) sequenced all the nine hypervariable regions in human placenta samples. The authors found that no or few sequences resulted from the amplification of certain regions (V1, V5, V7, V8, V9), whereas the regions V2 and V6 were amplified in most samples, but also in negative controls. The hypervariable region V4 was found to be the best choice (Parnell *et al.,* 2017) and it was targeted (alone or in combination with V3 or V5) in most of animal studies, whereas the hypervariable regions V1-V2 were amplified only in two studies, one in cows and one in mice (Karstrup *et al.*, 2017; Winters *et al.*, 2022). In human studies, this choice is characterized by a higher heterogenicity, although the hypervariable region V4 was most frequently amplified (Parnell *et al.*, 2017; Lim *et al.*, 2018; Liu *et al.*, 2019; Stinson *et al.*, 2019; Theis *et al.*, 2020; Turunen *et al.*, 2021; Wang *et al.*, 2022).

3.1.2 Bacterial DNA sequencing and taxonomic assignment

Additional influences on the results of microbiome studies can arise from the sequencing platform and from the taxonomic assignment database (Campos *et al.*, 2022). Different platforms are available for 16S rRNA bacterial gene sequencing, with read-length and error-rate being the two main characteristics to consider. Among sequencing platforms, the Illumina MiSeq is regarded as the best compromise due to its high throughput, low error rates, and cost-effectiveness. MiSeq offers paired-end sequencing (about 300 base pairs per pair-end read), which allows for the reconstruction of full-length reads, improving taxonomic resolution compared to platforms with shorter read lengths (e.g., Illumina MiniSeq). In contrast, platforms like Ion Torrent, Oxford Nanopore, and PacBio may offer different advantages, such as faster run times (Ion Torrent) or longer reads (Oxford Nanopore, PacBio), but often with higher error rates (Ion Torrent, Oxford Nanopore) or higher costs (PacBio, Oxford Nanopore), making them less suitable for exploratory sequencing studies targeting the 16S rRNA gene. The Illumina technology (Illumina, San Diego, USA) was used in all the studies assessing the feto-maternal microbiome in animals by sequencing of bacterial 16S rRNA genes and in some human studies. In humans, the Ion Torrent technology was used in two studies (Stinson *et al.*, 2019b; Turunen *et al.*, 2021). Among Illumina technologies, the Illumina MiSeq platform was used the most, except for one study in cows (Karstrup *et al.*, 2017) and one in humans (Liu *et al.*, 2019), in which the Illumina HiSeq platform was used. However, except for moderately abundant populations, similar results were retrieved for diversity and abundance (Na *et al.*, 2020). Interestingly, the production of the HiSeq system has recently been discontinued by the manufacturer and it is therefore unlikely that future microbiome studies will be conducted by means of this machinery.

Equally important is the choice of the reference database used for taxonomic classification. Popular databases such as SILVA (Yilmaz *et al.*, 2014), RDP (Ribosomal Database Project) (Wang *et al.*, 2007), and Greengenes (McDonald *et al.*, 2012) differ in size, taxonomic coverage, and update frequency. These are suitable for alignment of data generated by the Illumina MiSeq technology. SILVA stands out for its comprehensive coverage and regular updates, containing 411,933 curate sequences, and providing identification of a higher number of amplicon sequence variants (ASVs) compared to other databases.

SILVA (Yilmaz *et al.*, 2014), Greengenes (McDonald *et al.*, 2012), and RDP (Wang *et al.*, 2007) were the most reported databases in the papers focusing on the feto-maternal microbiome, except for few papers in humans (Lim *et al.*, 2018; Stinson *et al.*, 2019a, b). SILVA database was chosen in four animal studies (Malmuthuge and Griebel, 2018; Theis *et al.*, 2020; Husso *et al.*, 2021; Winters *et al.*, 2022), whereas Greengenes and RDP were chosen in three (Moore *et al.*, 2017; Martinez *et al.*, 2018;

Zou *et al.*, 2020), and two (Karstrup *et al.*, 2017; Guzman *et al.*, 2020) animal studies, respectively. The choice of the taxonomic database impacts the overall results of microbiome research (Campos *et al.*, 2022), being particularly relevant for investigations on low biomass samples.
Eventually, it is worth mentioning that analyses are often performed at different taxonomic levels, making in-between studies comparisons extremely hard. It is possible to convert results at species level to phylum, but not the other way around.

3.1.3 Zero-to-low biomass samples

A main issue of prenatal microbiome research is that feto-maternal samples are widely prone to contamination both during collection as well as during processing, making the sampling and laboratory protocols decisive to obtain reliable results (Stinson *et al.*, 2017; Eisenhofer *et al.*, 2019). The low microbial biomass of these samples does not only interfere with 16S rRNA gene sequencing, with the specific risk of amplification of contaminants, but also with other molecular techniques such as the fluorescence *in situ* hybridization (Jensen, 2014). The practical issue of the low biomass is commonly overcome by increasing the number of PCR cycles in the amplification process until bacterial sequences are identified. This procedure significantly increases the risk of amplifying contaminating DNA (Witzke *et al.*, 2020). Finally, the inclusion of positive and negative controls at every step of the process (i.e., sample collection, laboratory procedures) is a main point when conducting research on low-biomass samples (Eisenhofer *et al.*, 2019).

4. Target tissues to address the feto-maternal microbiome dilemma

The spark that started the "in-utero colonization" debate had its origin in human medicine and extended later to animal research. Using animal models, such as the murine one, might be tempting due to the fewer ethical implications and the shorter gestation time compared to humans. However, as mentioned, anatomical and physiological differences do not allow drawing common conclusions among different species. Nevertheless, a comparative perspective can help defining best practices and highlighting species-specific differences. The placenta, the amniotic fluid, and the meconium are considered representatives of the gestational environment and have been targeted by studies investigating the human (*Table 3*), murine, canine, equine, caprine, ovine, and bovine (*Table 4*) gestational microbiome.

Table 3. List of papers reporting results on the microbiome of the placenta, amniotic fluid, and meconium in healthy human pregnancies (1st author, year of publication, collected samples, population characteristics)

First author, year	Sample			Population[d]
	P[a]	AF[b]	M[c]	
Aagaard *et al.*, 2014	X			320 mother-neonate pairs (vaginal delivery)
Campisciano *et al.*, 2021	X	X		Pregnant women: 24 chorionic villi samples and 29 amniotic fluid samples (first and second trimester of pregnancy)
Collado *et al.*, 2016	X	X	X	15 mother-neonate pairs (C-section)
Del Chierico *et al.*, 2015			X	31 mother-neonate pairs (6 vaginal deliveries, 25 C-sections)
Hansen *et al.*, 2015			X	15 mother-neonate pairs (vaginal delivery)
He *et al.*, 2020		X	X	39 mother-neonate pairs (vaginal delivery)
Jiménez *et al.*, 2008			X	21 vaginally delivered neonates
Kennedy *et al.*, 2021			X	20 term fetuses at elective caesarean section
Lauder *et al.*, 2016	X			6 mother-neonate pairs (vaginal delivery)
Lim *et al.*, 2018		X		24 mother-neonate pairs undergoing elective C-section
Liu *et al.*, 2019	X	X	X	78 mother-neonate pairs (36 vaginal deliveries, 42 C-sections)
Liu *et al.*, 2020		X		Pregnant women: 42 amniotic fluid samples from (37 pregnancies, mid-gestation)
Parnell *et al.*, 2017	X			57 mother-neonate pairs (23 vaginal deliveries, 34 C-sections)
Rehbinder *et al.*, 2018		X		24 mother-neonate pairs (10 elective C-sections and 14 at term pairs following membrane rupture as a positive control)
Rowlands *et al.*, 2017		X		Pregnant women: 344 amniotic fluid samples (mid-gestation)
Sterpu *et al.*, 2021	X	X		Mother-neonate pairs undergoing elective C-section (N=50) or vaginal delivery (N=26)
Stinson *et al.*, 2019			X	5 neonates (vaginal delivery)
Stinson *et al.*, 2019		X	X	50 mother-neonate pairs (elective C-section)
Turunen *et al.*, 2021	X	X	X	Mother-neonate pairs (23 vaginal deliveries, 21 C-sections; first passing meconium of all 44 neonates; amniotic fluid and placenta of C-section delivered neonates)
Theis *et al.*, 2019	X			29 mother-neonate pairs (placenta) undergoing C-section
Wang *et al.*, 2022		X		Pregnant women: amniotic fluid samples (mid-gestation)

[a]P: placenta; [b]AF: amniotic fluid; [c]M: meconium; [d]sample collection was performed at term, unless stated otherwise.

Table 4. List of papers reporting results on the microbiome of the placenta, amniotic fluid, and meconium in healthy animal pregnancies (1st author, year of publication, species, collected samples, population size, gestational age, and applied techniques for bacterial detection).

	Species	Sample			Population, gestational age	Techniques
		P-U[a]	AF[b]	M[c]		
Martinez *et al.*, 2018	Mouse	X		X	Dams (n=4) and their fetuses; Day 17 of gestation and day 1 post-partum.	16s rRNA gene qPCR; 16s rRNA sequencing.
Theis *et al.*, 2020	Mouse	X		X	Dams (n=11) and two fetuses per dam; Day 17.5 of gestation.	16s rRNA gene qPCR; 16s rRNA sequencing; Culture.
Winters *et al.*, 2022	Mouse		X		21 pregnant dams; Days of gestation 13.5-18.5.	16s rRNA gene qPCR; 16s rRNA sequencing; Culture.
Younge *et al.*, 2019	Human and mouse	X	X	X	Two dams and their fetuses; Mid to late gestation (days 12-20 of gestation).	16s rRNA sequencing; Culture; FISH[d].
Rota *et al.*, 2021	Dog	X	X	X	Bitches (n=15) and their first extracted fetus; At term (emergency and elective caesarean section).	Culture.
Zakošek Pipan *et al.*, 2020	Dog	X		X	Dams (n=17) and their puppies (n=91); At term (natural birth).	Culture.
Hemberg *et al.*, 2015	Horse		X		Foaling mares (n=50); At term (natural birth).	Culture.
Hemberg *et al.*, 2023	Horse	X	X		Foaling mares (n=24); At term (natural birth).	16s rRNA sequencing; Culture.
Sones and Heil, 2018	Horse	X			Foaling mares (n=15); At term (natural birth).	16s rRNA sequencing.
Guzman *et al.*, 2020	Cow		X	X	Pregnant cows (n=12); 5, 6, 7 months pregnant.	16s rRNA gene qPCR; 16s rRNA sequencing; Culture.
Husso *et al.*, 2021	Cow		X	X	Pregnant cows (n=25); At term.	16s rRNA gene qPCR; 16s rRNA sequencing; Culture.
Karstrup *et al.*, 2017	Cow	X			Pregnant bovine uteri (n=43); 28-265 DG[e].	FISH[d]; 16s rRNA sequencing.
Malmuthuge and Griebel, 2018	Sheep	X	X	X	Pregnant ewes (n=16); Third trimester (from 118 of 148 DG[e]).	16s rRNA gene qPCR; 16s rRNA sequencing.
Moore *et al.*, 2017	Cow	X	X		Pregnant Holstein cows (n=10); 187-216 DG[e].	16s rRNA sequencing.
Zou *et al.*, 2020	Goat	X	X		Pregnant does (n=9) and their fetuses (n=22); 90-100-120 DG[e].	16s rRNA sequencing.

[a]P-U: placenta and/or uterus; [b]AF: amniotic fluid; [c]M: meconium. [d]FISH: Fluorescence In-Situ Hybridization. [e]DG: days of gestation.

4.1 Placenta

A recent systematic review (Zakis *et al.*, 2022) aimed to assess the available literature on the placental microbiome in healthy human pregnancies. The included papers were assessed for the risk of bias, being the lack of quantification of microorganisms (74% of the studies) and the absence of negative controls (72%) as most reported issues. Furthermore, many papers lacked a detailed description of the sampling procedure, making the risk of contamination impossible to assess. The current literature (*Table 3*) does not allow disproving the existence of a low biomass placental microbiome in healthy human pregnancies (Gil *et al.*, 2020; Zakis *et al.*, 2022).

Because of the structural similarities between human and murine placentae, rodents may serve as a model for the human feto-maternal microbiome (Winters *et al.*, 2022). However, some important differences still exist, since mice and rats have two more trophoblast layers compared to humans (i.e., hemotrichorial) and are typically polytocous (i.e., litter size higher than one). Two studies reported the presence of bacteria in the murine placenta using 16s rRNA sequencing. Specifically, Martinez *et al.* (2018) unveiled a resemblance between the placenta and the fetal intestine in murine fetuses at 17 days of gestation, although the placental samples had a lower relative bacterial biomass compared to the intestinal ones. Younge *et al.* (2019) recorded the presence of bacteria in the murine placenta at 12-20 days of gestation using both culture and 16S rRNA gene sequencing. Once again, the fetal gut microbiome resembled the placental one in mid and late gestation. Conversely, subsequent research performed by Theis *et al*. (2020) showed that the microbial load of placenta samples was not higher than that of DNA extraction kit controls. A comparison between the bacterial profile of technical controls and fetal samples was not possible because only one placental sample yielded results within the criteria set by the authors.

Sones *et al.* (2018), investigated the microbiome of the fetal membranes at natural birth in horses using 16s rRNA sequencing. This research suggested an association between placental and extra-placental (maternal oral and vaginal) microbiomes. More recently, Hemberg *et al.* (2023) sampled the placenta after expulsion through the vaginal canal, targeting three different areas of the organ (i.e., the cervical star, the umbilical cord at the attachment of the amniotic sac, the mid-part of the pregnant horn). Sequencing revealed a different bacterial composition in the three placental regions (Hemberg *et al*., 2023), suggesting that the equine placenta might harbor a characteristic microbiome. However, during natural birth the risk of contamination is too high to deliver definitive conclusions on the presence of bacteria in the equine placenta.

In ruminants, the presence of bacteria in the placenta or pregnant uterus was investigated in three articles (n = 3/6, 50%) (Moore *et al.,* 2017, Malmuthuge & Griebel, 2018, Zou *et al.,* 2019), mainly

by sequencing of the 16s rRNA gene. The abundance and composition of microbial communities did not vary among placentomes/intercotyledonary areas of the placenta and the amniotic fluid, according to Moore *et al.* (2017). Furthermore, feto-maternal samples significantly differed from positive controls (cervical sample of the dam). Nevertheless, no negative control was processed, leaving an open question on whether the samples would have differed in abundance and composition. In contrast, Zou *et al.* (2020) did not include any positive control in their research on the caprine feto-maternal microbiome. The authors found that 78.77% (SD = 4.11%) of sequence reads were shared between fetal and maternal samples, whereas 38.5% (SD = 1.49%) were common to fetal samples and negative controls. Finally, Malmuthuge and Griebel (2018) investigated the presence of bacteria in ovine feto-maternal units in the third trimester of pregnancy using both positive (pure culture of *Mannheimia haemolitica*) and negative controls (no-template controls containing only PCR reagents). The authors concluded that the fetus develops in a sterile environment and that bacterial presence could arise from contamination. In this study, no amplification of bacterial DNA through qPCR was obtained for any feto-maternal nor negative control sample.

In dogs, Zakošek Pipan *et al.* (2020) observed an 57% prevalence of bacteria in placental samples. However, the risk of contamination from both the vagina as well as from the environment was extremely high because the authors included bitches that had natural parturition (i.e., the placenta was sampled after expulsion through the vaginal canal). Nevertheless, similar results in terms of prevalence (60%) were reported by Rota *et al.* (2021). These studies only applied culture to investigate the feto-maternal microbiome of the canine placenta.

4.2 Amniotic fluid

The amniotic fluid surrounds the fetus and contributes to the formation of the meconium. In humans, it is routinely sampled as a biological medium to diagnose prenatal diseases and at-risk pregnancies during gestation (Geer *et al.*, 2015); consequently, some of the studies assessing the microbiome of this medium were conducted before term (Rowlands *et al.*, 2017; Campisciano *et al.*, 2021; Wang *et al.*, 2022). Amniotic fluid samples were also collected at term during caesarean section (Collado *et al.*, 2016; Lim *et al.*, 2018; Liu *et al.*, 2019, 2020; Sterpu *et al.*, 2021) or after vaginal delivery (He *et al*., 2020, Sterpu et al., 2021). Overall, 58% of the 12 studies assessing the microbiome of the amniotic fluid in healthy human pregnancies suggested the absence of bacteria (Rowlands *et al.,* 2017, Lim *et al.,* 2018, Rehbinder *et al.*, 2018, Liu *et al.,* 2020, Sterpu *et al.,* 2021, Turunen *et al.,* 2021, Wang *et al.,* 2022), whereas others (42%) reported the presence of a characteristic microbiome (Collado *et al.,* 2016, Liu *et al.,* 2019, Stinson *et al.*, 2019a , He *et al.,* 2020, Campisciano *et al.,* 2021), although only

14% of the amniotic fluid samples were positive for bacterial DNA in the study of Campisciano *et al.* (2021). A difference in the microbial composition of the amniotic fluid based on the mode of delivery was reported by Liu *et al.* (2019), suggesting the presence of bacteria that matched those of the vaginal environment in cases of natural birth.

In mice, bacterial sequences were identified in homogenized murine samples, although amniotic fluid presented low bacterial load (Younge *et al.,* 2019). A higher bacterial signal and a different profile resulted from the 16s rRNA gene qPCR and sequencing of murine amniotic fluid when compared with controls in the study by Winters *et al.* (2022). The most relatively abundant genera were *Corynebacterium*, *Pseudomonas*, *Sphingobium*, and *Streptococcus*. Interestingly, some of these have been recognized as contaminants that are typically present on human skin (Grice and Segre, 2011). Furthermore, none of the bacteria identified through molecular analyses were isolated using culture, that yielded only one positive isolate of *Lactobacillus murinus*. Since this bacterium was not detected in the 16S rRNA gene profile of any amniotic fluid sample, the authors suggested a cross-contamination from other murine body sites. This led to believe that murine amniotic fluid does not harbor any viable – culturable – bacteria.

Hemberg *et al.* used culture to detect bacteria in the amniotic fluid of naturally delivered foals in two studies (Hemberg *et al.,* 2015, Hemberg *et al*., 2023). Bacteria were detected in more than 50% of the amniotic fluid samples in the first study, although immediate microbial colonization is unavoidable when the foal is passing through the birth canal. The latter study had an improved sampling protocol, although collection happened in the birth canal. Specifically, the surface of the amniotic sac was swabbed with 70% ethanol before fluid collection into a 20 mL syringe. Bacteria did not grow in 75% of cultures from equine amniotic fluid samples. The positive cultures included common contaminants, such as *Acinetobacter lwoffii*, *Streptococcus*, *Enterococcus faecalis* or coagulase-negative staphylococci (Hemberg *et al*., 2023).

In ruminants, a few studies assessed bacterial presence in the amniotic fluid (Malmuthuge and Griebel, 2018, Zou *et al.*, 2020, Guzman *et al.,* 2020, Husso *et al.,* 2021). As mentioned for the placenta, caprine feto-maternal samples shared almost 40% of the sequences with negative control samples (Zou *et al.*, 2020). Ovine feto-maternal samples, including amniotic fluid ones, showed no amplification of bacterial DNA through qPCR in the study of Malmuthuge and Griebel (2018).

The microbiome of the bovine amniotic fluid was assessed by Husso *et al.* (2021) and Guzman *et al.* (2020). Both studies are characterized by the inclusion of multiple technical controls. The first study also included investigation by culture, whereas in the latter, culture was used just for controls (negative and positive) and for the fetal gastrointestinal tract (ruminal fluid). Husso *et al.* (2021)

found that the absolute 16S rRNA gene copy numbers were not different between negative field controls and amniotic fluid samples. Also, when cultured, amniotic fluid samples and negative field controls showed no bacterial growth. Interestingly, when compared to meconium samples alpha-diversity (i.e., within-sample diversity) did not differ significantly between meconium and amniotic fluid, suggesting similar intra-sample microbial diversity. Nonetheless, meconium and amniotic fluid samples clustered separately when the beta-diversity (i.e., inter-sample diversity) was assessed at genus level. This finding, together with the lack of correlation between samples from the same animal, suggests a different microbial composition based on sample-type.

Amniotic fluid of canine feto-maternal units at term was investigated by culture during emergency and elective caesarean sections (Rota *et al.*, 2021).

4.3 Meconium

Being made of ingested amniotic fluid, other than cells and secretions from the liver, pancreas, and gastrointestinal tract of the fetus, meconium is the ideal proxy for in-utero microbiome (Jiménez *et al.,* 2008, Del Chierico *et al.,* 2015, Hansen *et al.,* 2015, Collado *et al.,* 2016, Liu *et al.,* 2019, Stinson *et al.,* 2019c, He *et al.,* 2020, Turunen *et al.,* 2021). While amniotic fluid changes dynamically over time, meconium microbiome may reflect the accumulation of substances throughout gestation. Human studies (*Table 3*) investigated bacterial presence in meconium samples collected from diapers in the first few hours of life, and it is not possible to exclude bacterial contamination. Potentially, the immediate contamination in the vaginal canal and in the environment could mask the real microbial setting at term, as “en caul” birth is a rare event in humans. Collection by rectal swab immediately after the extraction of the fetus and the rupture of the amniotic sac is the optimal sampling procedure. In human neonates, this procedure has only been performed in one study that reported no differences in sequencing results between meconium and negative controls, ascribing the positivity of some isolates to contamination from the skin (Kennedy *et al.*, 2021). The authors suggest that a characteristic gut microbiota is absent in neonates born by caesarean-section.

Martinez *et al.* (2018) assessed the presence of bacteria in the murine fetal intestine and reported a low bacterial load and suggested that the fetal intestinal microbiota has a placental origin. The fetal intestine microbiota at 17 days of gestation presented higher diversity compared to that of the newborn (Martinez *et al.,* 2018). However, culture was always negative for bacterial growth. Younge *et al.* (2019) confirmed the presence of bacteria in the intestine of murine fetuses (days 12-20 of gestation) using fluorescence in-situ hybridization (FISH). Bacterial growth in culture was observed more frequently in mid gestation compared to late gestation samples. Subsequent research at 17.5

days of gestation showed that the bacterial signal in fetal intestine samples was not higher compared to that of DNA extraction kit controls (Theis *et al.,* 2020). Interestingly, this study used maternal samples as positive (i.e., lungs, cervix, and skin) and negative (i.e., liver) controls.

Meconium samples were collected at elective caesarean section also in newborn calves by Husso *et al.* (2021). Results showed that the absolute 16S rRNA gene copy numbers were different between negative field controls and meconium samples, whereas 20% of cultures was positive for bacterial growth. The authors suggested that meconium might accumulate the bacteria that reach the fetus throughout the pregnancy, although concluding that the in-utero colonization of the bovine fetus is likely not significant compared to that occurring at parturition.

Finally, in dogs, Zakošek Pipan *et al.* (2020) observed an 86.5% prevalence of bacteria in meconium. However, the collection of the meconium was performed following natural parturition and after stimulation of the perineal area and after colostrum intake. Hence, such study design does not allow to support nor reject the theory of in-utero colonization. In the study by Rota *et al.* (2021), 80% of canine feto-maternal units that were sampled during elective caesarean section were positive. Since culture precludes any possibility to detect unculturable bacteria, further research applying molecular methods (e.g., sequencing of 16S rRNA) is needed.

5. Time dynamics of feto-maternal microbiota

Most of the studies on the feto-maternal microbiota were conducted at term, either at natural birth or caesarean section. Others were conducted before term, by amniotic fluid collection during gestation, following euthanasia, or at the slaughterhouse (*Table 3*). Interestingly, some studies included samples collected at different stages of gestation, investigating time-dependent dynamics in feto-maternal microbiota (*Table 3*). Specifically, Younge *et al.* (2019) reported a temporal shift in the murine fetal microbiome, with mid gestation samples (12-16 days of pregnancy) showing a more abundant and variable microbiome compared to late gestation ones (17-20 days of pregnancy). All samples differed from controls in composition, suggesting the existence of a characteristic feto-maternal microbiome from early gestation to term. Furthermore, culture was used to assess the viability of the retrieved bacteria, detecting more viable bacteria (i.e., mainly *Lactobacillus*, *Escherichia*, *Enterococcus*, *Bacteroides*, and *Bacillus*) in mid-gestation samples compared to later ones. Therefore, the authors postulated that the bacterial population in late gestation might be dominated by unculturable microorganisms.

Differences were also found longitudinally at different gestational stages in bovine fetuses by Guzman *et al.* (2020). An increase in the bacterial 16S rRNA gene copy numbers and a turnover in

microbial communities throughout gestation was detected. Finally, even though in the study by Karstrup *et al.* (2017) the gestational stage among the included cows ranged from 28 to 263 days, covering more than 80% of the pregnancy duration in this species, changes in microbial composition of the placenta over time were not assessed.

From a practical point of view, it is important to define whether bacterial colonization of the fetus occurs during pregnancy, but above all it is important to identify at what point the fetus has first contact with the bacteria or their components. Therefore, further research is needed to confirm or deny a dynamic temporal shift in the fetal microbiota.

6. Conclusions

Any information derived from human studies should be critically considered when debating the *sterile womb paradigm* in animals and *vice versa*. Hypothetically, the pioneer microbiome might colonize the fetus *in utero* in some species while at birth in others.

Extreme differences in the applied methods seriously impede the possibility to compare results carried out in one single species, let alone comparing results from studies in different species.

What we know now is that the physiology of the feto-maternal connection is different among mammals and that the passage of immunoglobulins is limited or even absent in many domestic species as opposed to humans. We also know that certain pathogens carry specific factors and characteristics that may help them to circumvent the placental barrier (Loughran and Tuomanen, 2016; Perez-Muñoz *et al.*, 2017). However, the efficiency of the immunological, chemical, and physical features of the placenta have only been tested for a limited number of culturable bacteria (e.g., *Brucella abortus*, *Listeria monocytogenes*, *Streptococcus pneumoniae*) (Zare-Bidaki *et al.*, 2017). Therefore, this does not exclude the existence of unculturable bacteria that might be able to pass through the healthy placenta without necessarily causing disease. Moreover, some microorganisms might be able to elude most detection methods by hiding inside the trophoblast cells (Zakis *et al.*, 2022).

For these reasons, species-specific research using a multi-technique approach is needed to draw conclusions on the pioneer colonization in dogs and cats and to later explore it in the context of the DOHaD theory.

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Chapter 2
General objectives

The feto-maternal interface is a critical environment where maternal and fetal tissues interact during gestation. Recent studies in humans and some animals support the establishment of a fetal microbiota, although results remain controversial. Moreover, research on the feto-maternal microbiome in dogs and cats is extremely limited. Specifically, no studies have ever been conducted on cats, while two studies have been conducted on dogs, both using culture-dependent techniques (Zakošek Pipan *et al.*, 2020; Rota *et al.*, 2021).

The present research aimed to contribute, and possibly settle, the debate on the feto-maternal microbiome in small animals, unveiling the timing of pioneer bacterial colonization. The overall objective of such research was to draw a critical window that could be targeted by future research to prevent future disease and improve neonatal and adult health in dogs and cats.

More specifically, the objectives of the present thesis were:

- To confirm or deny the presence of a resident feto-maternal microbiome.
  The placenta, amniotic fluid, and meconium of healthy canine and feline fetuses were sampled during elective procedures within the surgical room. A multimodal approach was applied for sample processing. This allowed gaining information on both bacterial presence and viability. Samples underwent processing by culture-dependent methods exploiting Matrix-Assisted Laser Desorption/Ionization Mass Spectrometry (MALDI-TOF MS), a high-throughput technique such as 16S rRNA bacterial gene sequencing, and a cytogenetic technique providing visual feedback on bacterial location (i.e., fluorescence *in situ* hybridization, FISH).

- To characterize the microbial communities within canine and feline feto-maternal tissues.
  If canine and/or feline feto-maternal samples harbor a detectable microbiome, one of the aims of the present thesis was to characterize it using 16S rRNA bacterial gene sequencing in parallel with bacterial culture.

- To determine the timing of pioneer colonization in dogs and cats.
  To draw a critical window for bacterial exposure, information on the first contact between the fetus or the neonate with bacteria is needed. Hence, the present research targeted different time-points. Samples were collected between 30 and 40 days of gestation (i.e., mid-gestation in dogs and cats) during elective ovariohysterectomy due to unwanted pregnancies, and at the end of the pregnancy. Specifically, samples were collected either at term during elective

caesarean section or following natural birth, immediately after the passage through the vaginal canal. The first method allowed detecting eventual bacteria within the fetal environment, whereas the latter provided information on how birth shapes the neonatal microbiome.

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Chapter 3
Methods

1. 16S rRNA bacterial gene sequencing

Bacterial genomic DNA from animal samples and negative environmental and laboratory controls was extracted using the RNeasy Power Microbiome KIT (Qiagen, Hilden, Germany) according to the manufacturer's instructions in chapters 4 and 6, while the QiAmp DNA Microbiome Kit (Qiagen, Hilden, Germany) was used for the study presented in chapter 5 following protocol optimization. Specifically, 200 µL of sterile ice-cold Phosphate Buffered Saline (PBS) were added to each frozen swab and centrifuged for 1 minute at 6,000 g. After swab removal from the Eppendorf tube, swabs and amniotic fluid samples followed the same extraction protocol. One microliter of RNaseA (Thermo Fisher Scientific, Waltham, MA, USA) was added to digest RNA, with an incubation of 1 h at 37 °C. DNA was quantified with a fluorimetric method using the Qubit High Sensitive dsDNA kit (Life Technologies, Carlsbad, CA, USA) and standardized at 5 ng/µL.

Sequencing of the 16S rRNA bacterial gene were performed at the laboratory of the Department of Veterinary Sciences (University of Turin) in the studies presented in Chapters 4 and 6, whereas samples were shipped to an external laboratory (Novogene, Cambridge, UK) following DNA extraction in the study presented in Chapter 5. Further steps of the sequencing and bioinformatic analysis are described in the respective chapters.

2. Bacterial Culture

Bacterial isolation was at the Istituto Zooprofilattico Sperimentale delle Venezie (IZSVe) in Legnaro (Italy), and performed following standard laboratory procedures in all the presented studies (Chapters 4-6). Briefly, swabs were diluted in 1 mL of nutrient broth (Heart Infusion Broth, HIB, Conda, Madrid, Spain). Afterwards, 10 µL and 100 µL aliquots of the suspension were inoculated into solid and liquid media, respectively. Specifically, the presence of aerobic microorganisms was assessed using nutrient Blood agar with 5% sterile defibrinated sheep blood, Heart Infusion Broth, McConkey agar for Enterobacteriaceae, and Bile-Esculin Azide agar for Enterococci. These media were then incubated at 37°C ± 1°C under aerobic conditions for 24 hours.

To assess the presence of anaerobic microorganisms, inoculations were performed on nutrient Blood agar, a selective medium for *Clostridium perfringens* (Agar Base with specific *C. perfringens* selective supplement with 5% sterile defibrinated sheep blood), and fluid Thioglycolate medium. These media were incubated for 48 hours at 37°C ± 1°C in anaerobic jars equipped with an anaerobic generation system and indicator (AnaeroGen 2.5 L/3.5 L/Compact—Atmosphere Generation System and Anaerobic Indicator BR0055b, Thermo Fisher Oxoid Ltd., Basingstoke, UK). Non-inoculated plates were incubated along the inoculated ones as negative controls for bacterial growth in culture.

Solid and liquid media were provided by Biolife (Milan, Italy), whereas the defibrinated sheep blood was provided by Allevamento Blood (Teramo, Italy).
Bacterial growth was evaluated after 24 hours for aerobe microorganisms and 48 hours for anaerobe ones. If no bacterial growth was observed on agar media but turbidity was noted in the nutrient broth, the plates were incubated for an additional 24 or 48 hours and broth seeding was performed.

The number of colony-forming units (CFUs) on the initial isolation plates was counted to estimate bacterial growth, which was classified as low (1–10 CFU/10 μL), moderate (11–30 CFU/10 μL), or high (≥31 CFU/10 μL), according to standard laboratory operating procedures of the Istituto Zooprofilattico delle Venezie. Bacterial identification was conducted using MALDI-TOF MS (Microflex LT instrument, MALDI Biotyper, Bruker Daltonics, Billerica, MA, USA) with FlexControl software (version 3.3, Bruker Daltonics, Billerica, MA, USA).

Macroscopic observation of colonies, Gram stain, cellular morphology, growth on selective medium, catalase, oxidase, mobility tests, and coagulase tube test were used for the identification of the bacterial genera. Bacterial species identification was carried out via MALDI-TOF MS: Microflex LT instrument (MALDI Biotyper, Bruker Daltonics, Billerica, MA, USA) using the FlexControl software (version 3.3, Bruker Daltonics, Billerica, MA, USA).

3. Fluorescence In-Situ Hybridization

Fluorescence In-Situ Hybridization (FISH) was used only in the study presented in Chapter 5, to detect bacteria within canine and feline placentae. Briefly, at the moment of sample collection during elective ovariohysterectomies due to unwanted pregnancies, placentae from two fetuses were separately collected and placed in two jars with 10% formalin. Formalin-fixed canine and feline placentae were processed for histological assessment and fluorescence in situ hybridization (FISH). Briefly, five cylindric sections were collected from each formalin-fixed placenta using 6-mm sterile punches onto a sterile sampling table. Each section included all placental layers from the endometrial to the foetal side of the organ. Sections were placed into a sterile cassette following a scheme allowing to obtain slides capturing both the maternal and foetal sides of the placenta and an intermediate part of the organ. The cassettes were embedded into paraffin and cut using a microtome to obtain seven 5-μm slices. Two slices were placed onto regular glass slides and stained using haematoxylin-eosin to histologically assess the presence of necrotic, calcified, haemorrhagic areas, and inflammation (Sarli *et al.*, 2021; Tesi *et al.*, 2021). Five slides per placenta were placed onto polarized glass-slides and shipped to the Simpson's Laboratory, at the Department of Clinical Sciences of Cornell

University College of Veterinary Medicine (Ithaca, NY, USA). FISH was performed according to the standard operative protocols of the laboratory (Janeczko *et al.*, 2008; Twedt *et al.*, 2014). Specifically, paraffin-embedded biopsy specimens were de-paraffinized by sequential immersion in xylene (three times for 10 minutes each), 100% ethanol (twice for 5 minutes each), 95% ethanol (for 5 minutes), and 70% ethanol (for 5 minutes). The slides were then dry-aired, and FISH probes labelled at the 5' end, were reconstituted in sterile water and diluted to a working concentration of 5 ng/μL using the appropriate hybridization buffer. The probe combination of EUB-338-cy3 (5cy3/GCTGCCTCCCGTAGGAGT) and non-EUB-338-6FAM with a DAPI counterstain to control for non-specific hybridization was used to detect and quantify bacteria within placental tissue specimens. Sections were hybridized with 30 μL of the DNA probe mix in a hybridization chamber for 12-14 hours, followed by a wash with post-hybridization buffer and sterile water. Finally, slides were air-dried and mounted with ProLong Antifade Gold (Thermo Fisher Scientific, Waltham, MA, USA) to preserve the fluorescent dyes. Sections were examined on an Olympus BX51 epifluorescence microscope evaluating 10 fields at 60x for each section and capturing images using an Olympus DP-7 camera (Olympus America, USA).

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Chapter 4
Investigating bacterial presence in canine and feline feto-maternal units at term

Adapted from:

Challenging the Hypothesis of *in Utero* Microbiota Acquisition in Healthy Canine and Feline Pregnancies at Term: Preliminary Data

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Abstract

At present, there are no data on the presence of bacteria in healthy canine and feline pregnancies at term. Here, we investigated the uterine microbiome in bitches (n = 5) and queens (n = 3) undergoing elective cesarean section in two facilities. Samples included swabs from the endometrium, amniotic fluid, and meconium, and environmental swabs of the surgical tray as controls. Culture and 16S rRNA gene sequencing were used to investigate the presence of bacteria. Culture was positive for 34.3% of samples (uterus n = 3, amniotic fluid n = 2, meconium n = 4, controls n = 0), mostly with low growth of common contaminant bacteria. With sequencing techniques, the bacterial abundance was significantly lower than in environmental controls ($p < 0.05$). Sequencing results showed a species-specific pattern, and significant differences between canine and feline bacterial populations were found at order, family, and genus level. No differences were found in alpha and beta diversities between feto-maternal tissues and controls ($p > 0.05$). Dominant phyla were Bacteroidetes, Firmicutes, and Proteobacteria in different proportions based on tissue and species. Culture and sequencing results suggest that the bacterial biomass is very low in healthy canine and feline pregnancies at term, that bacteria likely originate from contamination from the dam's skin, and that the presence of viable bacteria could not be confirmed most of the time.

1. Introduction

It is a long-standing dogma that a sterile uterine environment is necessary to carry a healthy pregnancy to term. This theory is known as the 'sterile womb paradigm' (Perez-Muñoz *et al.*, 2017) and it has been questioned in recent years, since bacterial communities were identified in samples from feto-maternal units (placenta, amniotic fluid, and/or meconium) of humans (Aagaard *et al.*, 2014; Collado *et al.*, 2016; Younge *et al.*, 2019; He *et al.*, 2020; Campisciano *et al.*, 2021) and animals (mice, ruminants, dogs, and horses) (Hemberg *et al.*, 2015; Younge *et al.*, 2019; Campisciano *et al.*, 2021). This supports the hypothesis that the neonatal microbial colonization starts prior to birth. However, other studies failed to detect consistent evidence of bacterial presence both in healthy human (Liu *et al.*, 2020; Kennedy *et al.*, 2021; Turunen *et al.*, 2021; Wang *et al.*, 2022) and animal (Malmuthuge and Griebel, 2018; Theis *et al.*, 2020; Winters *et al.*, 2022) pregnancies, using molecular-based culture-independent and culture-dependent techniques alone or in combination. A recent perspective (Kennedy *et al.*, 2023) led to reject the *in utero* colonization hypothesis in humans, showing the pitfalls of DNA sequencing on 'low-' and 'zero-biomass' samples, as feto-maternal samples are often classified. Nevertheless, the species specificity of the feto-maternal connection and the high heterogenicity of studies assessing the feto-maternal microbiome in animals do not allow us to draw

definitive conclusions on non-human models, and this topic remains controversial. Adequate sampling procedures and processing are fundamental when dealing with low-biomass samples with the primary aim of avoiding contamination (Eisenhofer *et al.*, 2019).

To the authors' knowledge, no data are available on feline pregnancies and only two studies were carried out in dogs (Zakošek Pipan *et al.*, 2020; Rota *et al.*, 2021), both including puppies born either through natural birth or emergency cesarean (C) section. During natural birth, the fetus is colonized by the dam's vaginal flora, whereas in case of emergency C-section, the cervix is normally open, allowing for in-uterus migration of bacteria. Hence, only planned elective C-sections in the presence of a closed cervix can guarantee the microbiological isolation of the pregnant uterine environment at term. Furthermore, previous results in dogs were only based on culture-dependent investigation (Zakošek Pipan *et al.*, 2020; Rota *et al.*, 2021). Although traditional culture allows for the assessment of the viability of the isolated bacteria, it fails to detect those microorganisms that cannot grow in culture (Kaeberlein *et al.*, 2002) and false negatives are not uncommon when the bacterial load is extremely low, as can be expected for feto-maternal samples (Stinson *et al.*, 2019; Kennedy *et al.*, 2023).

The objective of the present research was to test the *in utero* colonization hypothesis in healthy canine and feline pregnancies at term, during elective C-sections, using both culture-dependent and culture-independent techniques.

2. Materials and Methods

2.1 Animals

Five bitches and three queens that underwent elective C-section at the Veterinary Teaching Hospital of the University of Turin (Italy) or at a private practice (Iunovet-Clinique Vetérinaire Saint Hubert, Beau-soleil, France) were enrolled in this research. Each dam and her fetuses were considered as one feto-maternal unit. No restrictions were placed on the breed, sex, age, parity, or weight of the animals. A C-section was planned due to breed predisposition to dystocia (e.g., brachycephalic breeds) (Ekenstedt *et al.*, 2020) or previous C-sections (Schrank *et al.*, 2022).

Healthy bitches and queens having uneventful pregnancies were included after a full clinical examination and ultrasound evaluation to assess fetal viability. Specifically, the biparietal diameters were measured, and the presence of intestinal peristalsis was verified to assess gestational age and to verify the maturity of the fetuses, respectively (Milani *et al.*, 2020). Blood progesterone concentration was measured in bitches to confirm cervical closure before the onset of the first stage of parturition, since a serum progesterone concentration lower than 2 ng/mL was found to be associated with an

open cervix (Nöthling and De Cramer, 2018). Drug administration (i.e., antimicrobial agents and corticosteroids) in the past six months and the presence of pregnancy complications (e.g., dead fetuses, dystocia), were considered as exclusion criteria.

This research was performed in agreement with the ARRIVE guidelines (Animal Research: Reporting In Vivo Experiments) (Kilkenny *et al.*, 2010; Percie Du Sert *et al.*, 2020), which were specifically adjusted for this type of observational study. This research has been approved by the Ethical Committee of the Department of Veterinary Sciences of the University of Turin (Italy) (n. 66/10/01/2020 and n. 310/9/2/2021). All the owners provided informed written consent and the procedures were carried out in accordance with the EU Directive 86/609/CEE and with the guidelines of the Italian Ministry of Health for the care and use of animals (D.L. 4 March 2014 n. 26 and D.L. 27 January 1992 n. 116).

2.2 Sample Collection

Animals were prepared for surgery according to standard procedures, in the pre-operative room. General anesthesia was induced inside the surgical room with propofol i.v. (Proposure, Boehringer Ingelheim Animal Health, Milano, Italy), and maintained with isoflurane (Iso-Vet 1000 mg/mL, Piramidal Critical Care Italia, San Giovanni Lupatoto, Italy) after intubation. The surgical area was scrubbed with three passages of two different antiseptic solutions (70% ethanol and 2% povidone-iodine) and sterile surgical drapes were placed around the surgical site.

C-sections were always performed following standard procedures (Rota *et al.*, 2021) and samples were collected concurrently by an operator other than the surgeon. Only the first extracted fetus was sampled to (1) limit possible environmental contamination related to the time of exposure of the uterine content, (2) avoid prolonging surgical time, and (3) immediately extract all the other fetuses and resuscitate them.

Briefly, after opening the abdomen, the uterus was slowly exposed, and a single incision was performed at the base of a uterine horn. Two sterile nylon regular swabs (ESwab® 480CE, Copan Italia Spa, Brescia, Italy) were used to sample the site where the placenta of the first extracted fetus was attached to the endometrium. One swab was placed into a 5 mL tube containing 1 mL of modified Liquid Amies Medium (ESwab® Copan Italia Spa, Brescia, Italy) for bacterial culture. The other swab was cut with sterile scissors and stored in a sterile Eppendorf tube (Eppendorf Tubes® 3810X, Eppendorf s.r.l., Hamburg, Germany) for molecular analyses. Samples of amniotic fluid were also collected from the closed amniotic sac of the first extracted fetus using a sterile syringe and a sterile

20 G needle. The amniotic fluid was dropped onto a sterile nylon regular swab (ESwab® 480CE, Copan Italia Spa, Brescia, Italy) for culture and 1 mL was poured in an Eppendorf tube for molecular analyses. The fetus was passed to an assistant wearing sterile gloves and placed on a surgical tray covered with sterile surgical drapes, fetal membranes were opened, and resuscitation of the fetus was started by rubbing it with sterile drapes. Once the puppy was resuscitated, two samples of meconium from the rectal ampulla were collected using two 'mini' swabs (ESwab® 484CE, Copan Italia Spa, Brescia, Italy), one for culture and one for molecular analyses. Meanwhile, the C-section was completed. Cefazolin (20 mg/kg i.v., Teva, Italy) was administered to the dam after the extraction of the first fetus. Two regular swabs of the same type used for sampling (one for culture and one for molecular analyses) were left open on the surgical tray throughout the surgical procedure as environmental controls.

All the samples collected for bacterial culture were immediately sent to the Istituto Zooprofilattico Sperimentale delle Venezie (Legnaro, Italy) and processed within 48 h, whereas all the samples for metagenomic analysis were frozen at −80 °C and processed at the same time.

2.3 16S rRNA bacterial gene sequencing

Bacterial DNA extraction was performed on placental (n = 8), amniotic fluid (n = 8), meconium samples (n = 8), respective controls from the surgical tray (n = 8), and laboratory controls from the DNA extraction kit. Specifically, the RNeasy Power Microbiome KIT (Qiagen, Hilden, Germany) was used, as described in chapter 3. Subsequently, the 16S rRNA gene was amplified following the Illumina 16S Metagenomic Sequencing Library Preparation Protocol (Illumina Inc. San Diego, CA, USA), with some modifications. Briefly, the V3-V4 region of the 16S gene was amplified with unique barcoded PCR primers containing the Illumina adapter overhang nucleotide sequences:

16S Forward Primer:
5′- TCGTCG-GCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG;
16S Reverse Primer:
5′-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTA-ATCC.

PCR amplicons were cleaned up using NucleoMag® NGS Clean-up and Size Select (Macherey-Nagel, Allentown, PA, USA) following the double size selection protocol. The resulting products were tagged by using the Nextera XT Index Kit (Illumina Inc., San Diego, CA, USA). After the

second purification step, amplicon products were quantified using Qubit High Sensitive dsDNA kit (Life Technologies, Carlsbad, CA, USA). Purified and normalized libraries were then pooled and diluted to a 4 nM concentration. The pooled library was then denatured with 0.2 N NaOH, diluted to 10 pM, and combined with 20% (vol/vol) denatured 10 pM PhiX, prepared according to Illumina guidelines. The sequencing was performed on the MiSeq Illumina platform (Illumina Inc., San Diego, CA, USA) with V3-600 cycles chemistry.

2.4 Bioinformatic analysis

The analysis, including classification of the sequences in OTUs (Operational Taxonomic Unit) with 97% similarity and taxonomy assignment, was performed in QIIME2 (Bolyen *et al.*, 2019), following the QIIME2 standard operating procedure for MiSeq data.

Specifically, paired-end reads were assembled and assigned to their original sample based on the barcode. Primer sequences and barcodes were then removed. For the denoising procedure, the Deblur method implemented in QIIME2 was used. Alpha diversity and Beta diversity were measured using QIIME2, as well as all the core metrics. Taxonomy was retrieved using BLASTn rRNA typestrains; a 16S ribosomal RNA database and sample bacterial population was assessed using hand-made R procedures. Data analyses were further conducted using R ver. 4.2.2 (Vienna, Austria). Differences among samples were calculated using alpha (Shannon and Faith indexes) and beta diversity estimation, based on Jaccard, Bray–Curtis and Unweighted UniFrac distance matrices, with a QIIME pipeline. Finally, we considered the presence/absence of bacterial strains. In more detail, we evaluated Pearson's correlation index between paired samples using the quantitative presence/absence patterns of bacterial strains at all the taxonomical levels (order, family, genus, species; dist.binary function in R). Cluster analysis was conducted drawing complete-linkage dendrograms and reconstructing population structure space using two-dimensions scaling plots (cmdscale function in R). Population subdivisions and clusters were evaluated using the k-means clustering approach (Hartigan and Wong, 1979). The association between the population structure and the samples features was evaluated using the Chi-squared test and Fisher's exact test. In particular, the association (k means function in R) was evaluated between the evaluated clusters membership and animal species (K = 2 for k-means clustering evaluation), surgical facility (K = 2), feto-maternal units (K = 5 for dogs, K = 3 for cats), feto-maternal tissues (K = 4, for dogs and cats separately). Moreover, we evaluated the differences between feto-maternal tissues and controls (K = 2).

2.5 Bacterial culture

Bacterial culture was performed on placental (n = 8), amniotic fluid (n = 8), meconium samples (n = 8), and respective controls from the surgical tray (n = 8) as described in chapter 3.

3. Results

The mean and standard deviation (SD) for age, weight, and litter size along with the list of breeds of the bitches and queens included in the study are reported in *Table 1*. One bitch was primiparous, whereas four bitches were pluriparous. All the queens were primiparous.

Table 1. Age, weight, litter size, and list of breeds of the bitches and queens included in the study.

	Age (Years) Mean ±SD	Weight (kg) Median (IQR)	Litter Size Mean ±SD	Breed
Bitches	3.8 ±2.2	38 (3.2–63)	5 ±1.4	Boston Terrier, Chihuahua, Dogue de Bordeaux, English Staffordshire Bull Terrier, French Bulldog
Queens	4.5 ±2	3.9 (3–3–4.3)	2.5 ± 0.5	Russian blue (n = 2), Scottish fold

SD: standard deviation; IQR: interquartile range

A total of 25 puppies and 10 kittens were born through cesarean section. Twenty-four puppies were alive and one stillborn puppy was extracted from the uterus of the bitch with the largest litter size (n = 7). All kittens were alive. This study therefore includes five canine feto-maternal units (FMU) and three feline FMU.

3.1 16S rRNA bacterial gene sequencing

A total of 31 samples were sequenced using Illumina MiSeq platform. Specifically, all animal samples (except for one feline amniotic fluid sample from F-FMU-1) and all environmental samples were sequenced. Laboratory controls and the amniotic fluid from F- FMU-1 did not produce sufficient amplification for further processing. The QIIME protocol identified a total of 239 different features (i.e., unique 16S rRNA sequences). Among the 31 available samples, the median number of features was 912 (range: 2–91,866). The features abundance was significantly higher in controls compared to animal ones (controls median = 16,866.5, IQR = 20,951; feto-maternal samples median = 587, IQR = 909; Wilcoxon test $p < 0.05$). The rarefaction curve evaluated on both Shannon and Faith indexes showed a plateau for all grouping methods (samples, species, group, and tissue).

The dominant phyla in the amniotic fluid of both species were *Bacteroidetes* and *Firmicutes*. A match was present in dominant phyla between dogs and cats, with *Firmicutes*, *Proteobacteria*, and *Bacteroidetes* being the most prevalent. *Proteobacteria* and *Bacteroidetes* were the most represented phyla in canine meconium. The latter was the most dominant in feline meconium, followed by

Firmicutes. As for the controls, *Firmicutes*, *Proteobacteria*, and *Bacteroidetes* were the prevalent phyla during the sampling of both species. Within- and among-group diversities (alpha and beta diversity) were evaluated using the QIIME standard pipeline. No differences were found in Shannon indexes when animal species or tissues were compared (Wilcox test and Kruskal–Wallis test $p > 0.05$, respectively). When among-groups differences were evaluated, beta diversity did not reveal any statistically supported differences among samples for all the used methods (Jaccard, Bray–Curtis and unweighted UniFrac distance matrices; PERMANOVA $q > 0.05$).

Patterns of presence/absence of each bacterial strain were calculated for the 31 sequenced samples and Pearson's correlation index was calculated to construct a distance matrix and to depict two-dimensional scaling plot for the four taxonomic levels (order, family, genus, species). The K means approach was used to independently identify the clusters within the bacterial population structure obtained with the two-dimensional scaling reconstruction at each taxonomical level. The association between cluster membership and grouping methods was reported in *Table 2*. The only statistically significant association is obtained when animal species are considered for the grouping method (at order, family, and species levels). As shown in *Figure 1*, clusters always discriminate between feline and canine samples. Interestingly, association analysis revealed no differences between animal samples and controls. Finally, a slight difference among dog tissues was recorded, only at the bacterial family taxonomical level.

Table 2. Association in bacterial strain at order, family, genus, and species levels between canine and feline feto-maternal units, feto-maternal tissues and controls, surgical facility, and laboratory run procedure. *p* values of a) Chi-square test and b) Fisher's exact test indicates the associations between the cluster membership and the grouping method.

Grouping Method	K [1]	Order *p*-Value	Family *p*-Value	Genus *p*-Value	Species *p*-Value
Animal species	(K = 2)	0.017 [a] **	0.033 [a] **	0.062 [a]	0.033 [a] **
		0.008 [b] ***	0.020 [b] **	0.057 [b]	0.020 [b] **
Feto-maternal unit	Cats	0.45 [a]	0.73 [a]	0.38 [a]	0.34 [a]
	(K = 3)	0.68 [b]	1 [b]	0.63 [b]	0.59 [b]
	Dogs	0.59 [a]	0.52 [a]	0.58 [a]	0.61 [a]
	(K = 5)	0.68 [b]	0.60 [b]	0.71 [b]	0.82 [b]
Feto-maternal tissues vs. controls	Cats	0.82 [a]	1 [a]	1 [a]	1 [a]
	(K = 2)	0.54 [b]	1 [b]	1 [b]	1 [b]
	Dogs	0.51 [a]	0.51 [a]	0.51 [a]	0.43 [a]
	(K = 2)	0.53 [b]	0.53 [b]	0.53 [b]	0.31 [b]
Tissues (uterus, amniotic fluid, meconium, controls)	Cats	0.46 [a]	0.47 [a]	0.34 [a]	0.22 [a]
	(K = 4)	0.77 [b]	0.78 [b]	0.58 [b]	0.18 [b]
	Dogs	0.11 [a]	0.035 [a] **	0.07 [a]	0.35 [a]
	(K = 4)	0.25 [b]	0.09 [b]	0.08 [b]	0.34 [b]
Surgical facility	(K = 2)	0.90 [a]	0.74 [a]	1 [a]	0.73 [a]
		0.70 [b]	0.67 [b]	1 [b]	0.67 [b]

[1] K indicates the value of k clusters recognized during k-means analysis; [a] Chi-squared test p; [b] Fisher's Exact test p; ** and *** show $p < 0.05$ and $p < 0.01$, respectively.

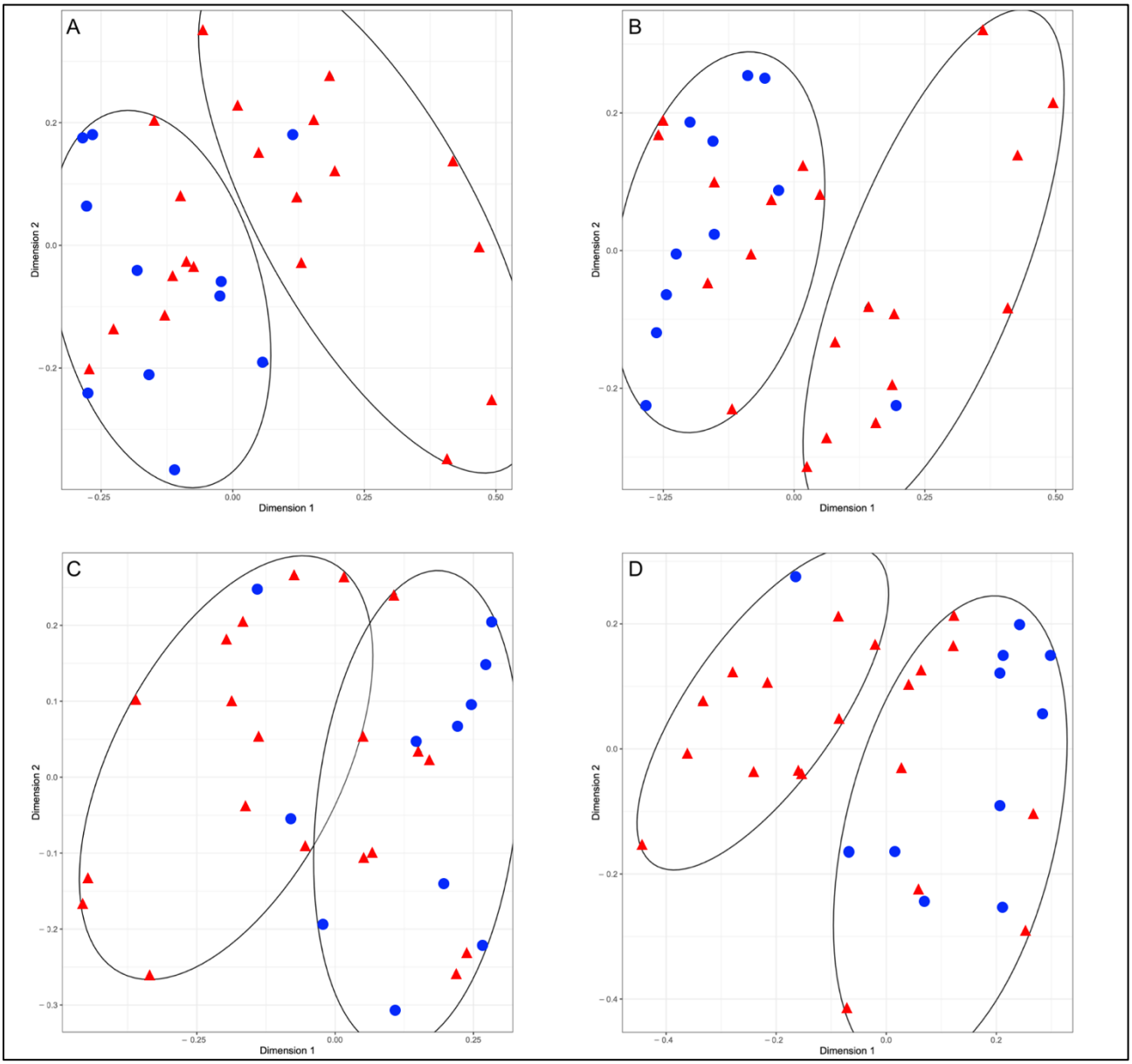

Figure 1. Two-dimension plot based on Pearson's correlation index evaluated based on the bacterial presence/absence patterns of each sample. Ellipses represent the clusters evaluated using k-means function with K = 2 for animal species comparison. Blue circles represent canine samples; red triangles represent feline samples. The four panels show the clustering of samples at all the considered taxonomical levels ((A): order; (B): family; (C): genus; (D): species).

3.2 Bacterial Culture

3.2.1 Bacterial culture of dog samples

The results of the bacterial culture from canine samples are reported in *Table 3*. Two canine feto-maternal units (C-FMU-1 and C-FMU-4) were negative. Three out of five meconium samples resulted in bacterial growth. Additionally, two uterine samples and one amniotic fluid sample showed

positive culture results. C-FMU-2 was the only one to show bacterial growth for all samples, although bacterial species were different in-between samples (*Bacillus* spp. in the uterus and amniotic fluid; *Staphylococcus hominis* and *Acinetobacter baumannii* in the meconium).

Pure cultures were obtained for all the positive samples, except for the meconium of the C-FMU-2, presenting substantial growth of *Staphylococcus hominis* and *Acinetobacter baumannii*. The same FMU (C-FMU-2) was the only one in which positivity was recorded for all the samples and growth was reported as high; differently from meconium, the amniotic fluid and the uterine sample were positive for *Bacillus* spp. *Pseudomonas fluorescens* was isolated from the uterine sample of C-FMU-5 (low growth), whereas *Acinetobacter baumannii* presented high growth when the meconium belonging to the same C-FMU was investigated. Microorganisms belonging to the genus *Clostridium* or other strict anaerobic bacteria were never isolated. Controls were always negative.

Table 3. Bacteria isolation from five canine feto-maternal units (C-FMU): uterus (placental site), amniotic fluid, and meconium of the first extracted fetus: results of isolation are reported, together with the isolated bacterial species and the growth* based on the number of colony-forming units (CFUs) in the first isolation plates.

	Uterus[a]			Amniotic Fluid[b]			Meconium[c]		
FMU	**Result**	**Bacteria**	**Growth***	**Result**	**Bacteria**	**Growth***	**Result**	**Bacteria**	**Growth***
C-FMU-1	neg			neg			neg		
C-FMU-2	+	*Bacillus* spp. [X]	High	+	*Bacillus* spp.	High	+	Coagulase-negative Staphylococcus (*S. hominis*) [X]	High
								Acinetobacter baumannii [X]	High
C-FMU-3	neg			neg			+	Coagulase-negative Staphylococci (*S. epidermidis*)	Low
C-FMU-4	neg			neg			neg		
C-FMU-5	+	*Pseudomonas* spp. (*p. fluorescens*)	Low	neg			+	*Acinetobacter lwoffii* [X]	High

[a] Site of attachment of the placenta to the endometrium of the first extracted fetus; [b] amniotic fluid of the first extracted fetus; [c] meconium sampled from the rectal ampulla of the first extracted fetus; [X] the presence at genus level was confirmed by sequencing. * Low (1–10 CFU/10 µL), Moderate (11–30 CFU/10 µL), or High (≥31 CFU/10 µL).

3.2.1 Bacterial culture of cat samples

The results of the bacterial culture from feline samples are reported in *Table 4*. The culture of one feline feto-maternal unit (F-FMU-1) was negative. In the two feto-maternal units presenting positive results, only pure cultures were obtained. Specifically, two uterine samples, one amniotic fluid sample, and two meconium samples showed bacteria growth. High growth (≥31 CFU/10 µL) was recorded only for one uterine sample (F-FMU-3), whereas all the other positive samples showed very low bacterial growth.

Pseudomonas aeruginosa was the bacterium isolated from the uterine sample that showed high growth (F-FMU-3). The other positive uterine culture showed a few colonies of a coagulase-negative Staphylococcus (C-FMU-2). *Pseudomonas aeruginosa* was the only bacterium isolated from the amniotic fluid of F-FMU-2, although in very low numbers. Finally, few colonies of *Psychrobacter sanguinis* were isolated from the meconium of F-FMU-3. Microorganisms belonging to the genus *Clostridium* or other strict anaerobic bacteria were never isolated. Controls were always negative.

Table 4. Bacteria isolation from three feline feto-maternal units (F-FMU): uterus (placental site), amniotic fluid, and meconium of the first extracted fetus: results of isolation are reported, together with the isolated bacterial species and the growth* based on the number of colony-forming units (CFUs) in the first isolation plates.

	Uterus[a]			Amniotic Fluid[b]			Meconium[c]		
FMU	**Results**	**Bacteria**	**Growth***	**Result**	**Bacteria**	**Growth***	**Result**	**Bacteria**	**Growth***
F-FMU-1	neg			neg			neg		
F-FMU-2	+	Coagulase-negative Staphylococcus (S. epidermidis)	** Very low *	+	Pseudomonas aeruginosaX	** Very low	neg		
F-FMU-3	+	Pseudomonas aeruginosaX	High	neg			+	Psychrobacter sanguinis	** Very low

[a] Site of attachment of the placenta to the endometrium of the first extracted fetus; [b] amniotic fluid of the first extracted fetus; [c] meconium sampled from the rectal ampulla of the first extracted fetus; [X] the presence at genus level was confirmed by sequencing. * Low: (1–10 CFU/10 µL), Moderate (11–30 CFU/10 µL), or High (≥31 CFU/10 µL); ** Very low: bacterial cultures obtained from seeding of broth cultures (growth only from enrichment broth HIB, not on first isolation media).

4. Discussion

This preliminary study investigated the feto-maternal microbiota of two domestic animal species (i.e., dogs and cats) using culture-dependent and culture-independent methods, with the objective of testing the *in utero* colonization hypothesis and setting the stage for future research.

Strict asepsis measures were adopted to minimize the chances of contamination, and only elective C-sections were included before the onset of the first stage of parturition, when the cervix was still closed, sealing the uterus from the vaginal lumen. Elective C-sections are rather infrequent in cats, and this led to the inclusion of only three feline feto-maternal units; however, this study represents pioneering research in this field in cats. In a previous study in dogs (Rota *et al*., 2021), the presence of bacteria was investigated solely using culture; microorganisms were detected in the placenta, amniotic fluid, and meconium, although both elective and emergency C-sections were included. Furthermore, some of the bitches selected for the elective procedure had serum progesterone levels lower than 2 ng/mL. In the light of the association between serum progesterone concentration and cervical opening (Nöthling *et al*., 2018), the present research included only bitches with a serum progesterone concentration higher than 2 ng/mL and queens that had not shown any sign of impending parturition, to avoid possible ascending contamination via the vagina. Compared to previous

investigations (Zakošek Pipan *et al.*, 2020; Rota *et al*., 2021), this research had stricter inclusion criteria and negative laboratory and environmental controls were added: all pregnancies were healthy, C-sections were elective, and more samples were included (i.e., swabs from the surgical tray) to assess environmental contamination.

Both culture and sequencing resulted in detection of bacteria but, despite the strict procedures that were adopted, a critical analysis of both culture and sequencing data suggests that contamination is still the most likely source. All the bacteria species isolated via culture in the present research are ubiquitous and commonly found in the environment. In fact, both coagulase negative staphylococci and *Bacillus* spp. are known to be common environmental contaminants in microbiology laboratories (Dargère *et al.*, 2018). Bacteria belonging to the genus *Acinetobacter* are ubiquitous (Tega *et al.*, 2007); specifically, *Acinetobacter baumannii* is renowned for its presence in hospitals, where it can be responsible for nosocomial infections (Urban *et al.*, 2003) as well as *Pseudomonas aeruginosa* (Crone *et al.*, 2020). Finally, *Pseudomonas fluorescens* optimally grows in substrates such as disinfectants (Wong *et al.*, 2011). The presence of *S. epidermidis* and *S. hominis* was detected either in the placenta or in the meconium of newborn puppies by Zakošek Pipan *et al.* (2020), in a study that included natural births and emergency C-sections (Zakošek Pipan *et al.,* 2020). Coagulase-negative staphylococci were isolated from the amniotic fluid of horses (Mols *et al.*, 2020), along with *Acinetobacter* spp., which was also reported in bovine feto-maternal elements (Husso *et al.*, 2021). However, contamination cannot be ruled out due to the inclusion of emergency C-sections or natural birth (Hemberg *et al.*, 2015; Zakošek Pipan *et al.*, 2020) and due to the high abundance of some of these genera in laboratory reagents (e.g., DNA extraction kit) (Husso *et al*., 2021).

The genetic material of the bacteria that were isolated in culture was not always identified using molecular techniques, leading us to hypothesize that contamination could have occurred during culture seeding or incubation. Bacteria that were found through both culture and sequencing (54.5%) were not abundant (absolute reads count ranging from 4 to 189 reads and relative abundance ranging from 0.8 to 40%) and, as mentioned, were ubiquitous microorganisms. The bacterial abundance detected using molecular analyses in all animal samples (from uterus, amniotic fluid, and meconium) was lower than the abundance found in environmental swabs from the surgical tray, which served as a negative control for the sampling procedures, being sterile by definition (Dreikausen *et al.*, 2023), and always yielding negative cultures in the present study. This means that the pregnant uterus of small animals has a very low bacterial load, approaching the sterile womb hypothesis. Interestingly, sequencing results showed a species-specific pattern. In fact, bacterial populations resulting from feline and canine samples grouped into two clusters, matching the species of origin. This cannot be

linked to cross-contamination during laboratory processing because canine and feline samples were sequenced in the same run. We may hypothesize that this in-between species difference is related to the characteristic mucosal and skin microbiota of dogs and cats (Cuscó *et al.*, 2017; Older *et al.*, 2019) that enters the surgical room via its host. Similarly, in humans, C-section-born neonates harbor bacterial communities that resemble those of the skin of the mother (Dominguez-Bello *et al.*, 2010). However, we acknowledge that the limited sample size of the present preliminary study does not allow us to draw definitive conclusions, and that further research is needed.

The microbial profile of canine and feline amniotic fluid, uterus, and meconium resulting from 16S rRNA sequencing was dominated by Bacteroidetes, Firmicutes, and Proteobacteria in different proportions. However, no relevant difference was found among tissues at lower taxonomic levels and the same phyla were also prevalent in the environmental controls. Overall, these results were consistent with those of previous studies in other species (Husso *et al.*, 2021; Winters *et al.*, 2022). Interestingly, some sequences belonging to anaerobic bacteria (i.e., belonging to the Phylum Fusobacteria) were identified using molecular techniques in very low abundance. These were never isolated in culture, although the samples were collected and transported using specific swabs and media intended to preserve the viability of aerobes, anaerobes, and fastidious bacteria (Copan eSwab with Amies medium, Copan, Italy), and anaerobic culture was performed. However, the presence of these bacterial sequences in multiple samples can hardly be associated with contamination from the animal skin of from the environment, as these bacteria are strict anaerobes. If the feto-maternal samples are the source of this genetic material, it probably resulted from dead microorganisms, as no isolation in culture occurred. This observation may be consistent with the protective role of the placental barrier and amniotic fluid against possible pathogens (Perez-Muñoz *et al.*, 2017). If this hypothesis were confirmed, it could explain the presence of genetic material belonging to other non-viable bacteria. Next-Generation Sequencing (NGS) techniques have the advantage of detecting all bacteria, including the unculturable ones (Wade, 2002), or those that require very specific media and long incubation times to grow (Naidu *et al.*, 2021). Still, molecular techniques act as a double-edged sword, and the presence and significance of bacteria can be overestimated. NGS techniques detect bacterial DNA without discriminating between living and dead microorganisms and between sequences deriving from cross-contamination (during sampling and/or DNA extraction) and biologically relevant ones. Furthermore, low biomass samples often contain the same amount of DNA and similar alpha diversity (within-sample diversity) when compared to controls, not allowing us to discriminate whether the genetic material originates from the animal samples or from the environment. Contamination is a major challenge when investigating feto-maternal microbiome and

low-biomass samples in general (Perez-Muñoz *et al.*, 2017; Eisenhofer *et al.*, 2019; Kennedy *et al.*, 2023), and the sampling techniques used in most studies in humans and veterinary species did not guarantee sterility (Mijten *et al.*, 1997; Jiménez *et al.*, 2008; Hemberg *et al.*, 2015; Zakošek Pipan *et al.*, 2020). In the present study, we tend to exclude the environment of the surgical room as a source of contamination because samples were collected in two different facilities, but this did not influence either the results obtained via culture nor via sequencing. The coat, skin, and mucosae of the animals undergoing C-sections may represent the source of contamination. The hypothesis that contamination originated from the animal itself and not from the surgical room is also supported by the negative culture results for all environmental controls. This, along with bacteria selection related to culture media (Bonnet *et al.*, 2020), could explain the presence of bacteria. Future research should include more controls, including swabs of the skin of the mother, the abdominal serosa, and the gloves of the surgeon, to better define the possible source of contamination.

5. Conclusions

This is the first study investigating feto-maternal microbiota in domestic carnivores using a combination of techniques and implementing measures for strict asepsis, from animal selection, sampling procedures, and inclusion of controls. Furthermore, to the best of the authors' knowledge, this study represents pioneering research on feline feto-maternal microbiota.

In the framework of the debate concerning the *in utero* colonization of newborns, the results of the present study add more knowledge about the microbial presence within the fetal environment of domestic carnivores. Regardless of the preliminary nature of this research and the small number of animals included, we suggest that a very low load of bacterial genetic material of unknown viability can be found during healthy pregnancies at term.

6. Declarations

Declaration of competing interest: there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Funding: This research did not receive any specific grant from any funding agency in the public, commercial or not-for-profit sector.

Ethical approval and consent to participate: The authors confirm that all methods were performed in accordance with relevant guidelines and regulations. The study was carried out in

compliance with the ARRIVE guidelines and it was approved by the Ethical Committee of the Department of Veterinary Sciences of the University of Turin (Approval number n. 66, 10/01/2020 and n. 310 09/02/2021) and was performed in accordance with the EU Directive 86/609/CEE and with the guidelines of the Italian Ministry of Health for the care and use of animals (D.L. 4 March 2014 n. 26 and D.L. 27 January 1992 n. 116). Previous informed consent was obtained from the owners of the animal included in the study.

Author contribution statement: PB: Conceptualization, Methodology, Investigation, Data Curation, Writing – Original Draft, Visualization.; BC: Investigation, Writing – Review & Editing; ADC: Investigation; MC: Investigation, Writing – Review & Editing; AB: Investigation, Writing – Review & Editing. UA; Software; ADC: Investigation; AVS: Resources, Supervision, Writing – Review & Editing, Supervision, Project administration; LB: Supervision, Writing – Review & Editing, Supervision; Software; AR: Conceptualization, Resources, Writing – Review & Editing, Supervision, Project administration, Funding acquisition.

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Chapter 5
Improved protocol: bacterial presence in canine and feline fetuses using a multi-technique approach

Adapted from:

Approaching the sterile womb theory in dogs and cats: a multi-technique investigation.

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Abstract

The study investigated whether bacterial seeding occurs in utero in dogs and cats using a multi-technique approach, including 16S rRNA gene sequencing, culture, and fluorescence in situ hybridization (FISH). Healthy pregnant bitches and queens (n=8) undergoing ovariohysterectomy between 30 and 45 days of gestation were included. Placenta and amniotic fluid samples from two fetuses per dam, along with multiple controls (uterine serosa, sampling table, and surgeon's gloves), were collected and analysed.

Bacterial sequences were detected in all fetal samples, with no significant differences in intra- and inter-sample diversity (i.e., alpha and beta diversities, respectively) based on sample type or species. However, the surgeon's gloves showed species-specific differences in bacterial composition. After removing control sequences, significant differences based on feto-maternal units emerged. *Moraxella* spp. was cultured from the canine placenta with the highest bacterial load, and *Burkholderia cepacia* was isolated from two feline placentae. FISH showed low bacterial presence in 50% of placentae without histological signs of inflammation. No bacterial growth was observed in amniotic fluid or control samples.

A multi-technique approach, including multiple controls, is essential in studies involving low-biomass samples, as the results of the present study indicated that contamination could mask real bacterial composition of fetal samples. After decontamination, differences were observed based on the feto-maternal unit.

This study confirmed the presence of bacterial DNA in placental and amniotic fluid samples during mid-term, healthy pregnancies in dogs and cats. Bacterial DNA is not equivalent to living bacteria, hence bacterial DNA fragments in the uterus probably originate from the dam but are unlikely to indicate the presence of a resident fetal microbiome.

1. Introduction

The existence of microorganisms in the fetal environment during healthy pregnancies is still debated, and is closely associated to the unique characteristics of the feto-maternal connection in each species (Banchi *et al.*, 2024). Some scientists endorse the "*in utero* colonization theory" (Funkhouser and Bordenstein, 2013; Aagaard *et al.*, 2014; Turunen *et al.*, 2021), suggesting that this process begins before birth, while others support the "sterile womb paradigm", which proposes that colonization starts during birth and early postnatal stages (Perez-Muñoz *et al.*, 2017; Leiby *et al.*, 2018; Theis *et al.*, 2020; Kennedy *et al.*, 2021). In human babies, the seeding and development of microbial ecosystems is critical in shaping the immune system and is associated with future health (Stiemsma

and Michels, 2018). In this context, early bacterial colonization is a key aspect of the debate on the "developmental origins of health and disease" (DOHaD), which proposes an association between prenatal and perinatal exposures and future health outcomes (Stiemsma and Michels, 2018; Lacagnina, 2020; Gaillard *et al.*, 2022). The importance of this concept for companion animals like dogs and cats has been recently highlighted, suggesting that early development and future health can be influenced by several factors, including birth weight, early growth, neonatal nutrition, maternal care, social interactions, and exposure to bacteria (Gaillard *et al.*, 2022). Early life microbiota has been confirmed to play a key role in shaping the immune system in humans (Noverr and Huffnagle, 2005; Schreiner *et al.*, 2008), while research on naturally delivered puppies has demonstrated an association between the dam's vaginal microbiota and that of her puppies at birth (Del Carro *et al.*, 2022). However, whether microbial imprinting from the mother occurs already *in utero* remains questionable.

Previous studies on canine feto-maternal units at term have reported bacterial presence in placental, meconium, and amniotic fluid samples from puppies delivered by both natural birth and caesarean section, based on bacterial culture methods (Zakošek Pipan *et al.*, 2020; Rota *et al.*, 2021). However, natural birth through the vaginal canal, as well as a patent cervix during caesarean sections (Nöthling and De Cramer, 2018), increase significantly the risk of contamination from maternal vaginal bacteria. This raises concerns about accurately assessing the fetal environment during pregnancy. Additionally, traditional culture methods fail to isolate over 90% of bacterial species (Kaeberlein *et al.*, 2002). This limitation has been addressed by molecular techniques, such as 16S rRNA gene sequencing (Banchi *et al.*, 2023), which allow for the identification of bacterial DNA, thus providing a more comprehensive understanding of the feto-maternal microbiome. Specifically, the existence of minimal amounts of bacterial genetic material of unknown viability was suggested in fetal and placental samples collected from canine and feline fetuses at elective caesarean section in the presence of a closed cervix (Banchi *et al.*, 2023). These results were consistent with the majority of human and animal studies (Banchi *et al.*, 2024) but did not allow to draw definitive conclusions. The possibility of contamination is a common issue when dealing with zero-to-low biomass samples, although the risk can be mitigated by strict inclusion criteria, sampling protocols, inclusion of multiple controls, and application of different techniques (Eisenhofer *et al.*, 2019; Kennedy *et al.*, 2023).

Various techniques are available for bacterial detection and the ideal protocol for low-biomass samples should include complementary techniques (Kennedy *et al.*, 2023). As culture and 16S rRNA

gene sequencing have been applied in several studies (Theis *et al.*, 2020; Husso *et al.*, 2021; Banchi *et al.*, 2023; Hemberg *et al.*, 2023; Kennedy *et al.*, 2023), fluorescence *in situ* hybridization (FISH) may be implemented as a microscopic technique providing a visual feedback. FISH is a fluorescence cytogenetic method that uses oligonucleotide probes with high complementarity to specific DNA sequences. These probes are labelled with a fluorescent dye, and sequences can be designed to target the 16S ribosomal bacterial RNA (Sekiguchi *et al.*, 1999; Pernthaler *et al.*, 2001), thus representing a culture-independent molecular technique. Unlike 16S rRNA sequencing or qPCR, FISH provides visual information on bacterial presence and distribution within a specific tissue when slides are observed under a fluorescent microscope. Therefore, including this technique could potentially lead to bacterial detection within the endotheliochorial placenta of dogs and cats.

The present study aimed to investigate bacterial presence within canine and feline fetuses between days 30 and 45 of gestation using an improved sampling protocol and a multi-technique approach, including culture, 16S rRNA bacterial gene sequencing and FISH.

2. Materials and methods

2.1 Animals

The present study included healthy pregnant bitches (N=4) and queens (N=4) that underwent elective mid-term ovariohysterectomy due to unwanted pregnancies at the Veterinary Teaching Hospital of the University of Turin, Italy. All the included animals were healthy, with no sign of clinically detectable disease, and had normal blood count and biochemical analyses results. Additionally, all queens tested negative for Feline Immunodeficiency Virus (FIV) and Feline Leukemia Virus (FeLV). The administration of antimicrobial agents or corticosteroids in the previous two months or the presence of pregnancy complications (e.g., dead fetuses, resorption) were considered exclusion criteria. The gestational age, estimated by history and/or ultrasound fetal measurements (Beccaglia *et al.*, 2016). was between 30 and 45 days. No restrictions were placed on breed, age, parity, and body weight of the dams.

This observational study was performed in agreement with adapted ARRIVE guidelines (Animal Research: Reporting In Vivo Experiments) (Kilkenny *et al.*, 2010; Percie Du Sert *et al.*, 2020). The Ethical Committee of the Department of Veterinary Sciences of the University of Turin (Italy) approved the present research (n. 310/9/2/2021). Written informed consent was obtained from each owner or person responsible of the shelter or cat colony.

2.2 Sample collection

Ovariohysterectomy was performed according to the standard procedures of the Veterinary Teaching Hospital, on patients under general anaesthesia with isoflurane (Iso-Vet 1000 mg/mL, Piramidal Critical Care Italia, San Giovanni Lupatoto, Italy). After carefully clipping and scrubbing the ventral abdomen, the abdominal wall was incised, and the uterine serosa was visualized and swabbed using two sterile nylon regular swabs (ESwab 480CE, Copan Italia Spa, Brescia, Italy). The first was immediately placed into a 5 mL tube containing 1 mL of modified Liquid Amies Medium (ESwab® Copan Italia Spa, Brescia, Italy) for bacterial culture. The second swab was cut with sterile scissors and stored in a sterile Eppendorf tube (Eppendorf Tubes® 3810X, Eppendorf s.r.l., Hamburg, Germany) for molecular analyses. All samples were collected by an operator wearing a sterile gown and surgical gloves, different from the surgeon. The surgeon removed the uterus *en bloc* and passed it to the operator, who placed the organ on an aseptically prepared sampling table placed within the surgical room covered with sterile drapes. Two fetuses were sampled, one from the right uterine horn and one from the left uterine horn of each uterus; fetuses located within the uterine body and near the cervix were not sampled. Briefly, after incising the uterine wall, the first foetus was extracted within the intact amniotic sac, and the placenta was gently detached from the endometrium to minimize blood contamination. The endometrial side of the placenta was double-swabbed. The placenta was then placed into 10% formalin for further processing. The amniotic fluid was aspirated from the intact amniotic sac using a sterile 20G needle connected to a 2.5 mL sterile syringe. Subsequently, 1 mL of fluid was poured into an Eppendorf tube for molecular analyses, whereas the remaining fluid was dropped onto a sterile nylon regular swab (ESwab 480CE, Copan Italia Spa, Brescia, Italy) for culture. At the conclusion of the surgery, the operator swabbed the surgeon's gloves twice. Two regular swabs, one for culture and one for 16S sequencing, were placed on the sampling table throughout the procedure. *Table 1* summarizes the samples collected from each feto-maternal unit. Samples for bacterial culture were immediately sent to the Istituto Zooprofilattico Sperimentale delle Venezie (Legnaro, Italy) and processed within 48 h, samples for 16S sequencing were frozen at −80 °C until simultaneously processed, placentas were maintained in 10% formalin until further processing for histology and FISH.

Table 1. List of samples collected for each feto-maternal unit for further processing by culture, 16S sequencing, and fluorescence in situ hybridization (FISH).

		Culture	16S sequencing	FISH
Placenta	*Fetus 1*	Swab[a]	Swab[b]	Whole organ
	Fetus 2	Swab[a]	Swab[b]	Whole organ
Amniotic fluid	*Fetus 1*	Fluid dropped onto a swab[a]	Fluid dropped into an Eppendorf tube[b]	
	Fetus 2	Fluid dropped onto a swab[a]	Fluid dropped into an Eppendorf tube[b]	
Sampling controls	*Uterine serosa*	Swab (after incision of the abdominal wall)[a]	Swab (after incision of the abdominal wall)[b]	
	Sampling table	Swab (throughout the sampling procedure)[a]	Swab (throughout the sampling procedure)[b]	
	Gloves of the surgeon	Swab (at the end of the surgery)[a]	Swab (at the end of the surgery)[b]	

[a]Immediately placed into a 5 mL tube containing 1 mL of modified Liquid Amies Medium and processed within 48h for bacterial culture. [b]Immediately placed into an Eppendorf tube and stored at -80°C until bacterial DNA extraction.

2.3 16S rRNA bacterial gene sequencing

Bacterial DNA was extracted as previously described (page 55, chapter 3) was extracted from two placental swabs, two amniotic fluid samples, and three sampling controls for each feto-maternal unit (N = 56) using the using the QiAmp DNA Microbiome Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Purified DNA was then transferred to new 2.0 mL collection tubes and stored at −20°C until shipment to an external laboratory (Novogene, Cambridge, United Kingdom) for further processing. As very low bacterial DNA abundance was expected based on our previous experiments (Banchi *et al.*, 2023), nested PCR was used for amplification of the V3-V4 hypervariable regions of the 16S bacterial gene to increase sensitivity (Green and Sambrook, 2019). Nested PCR uses two pairs of primers (*Table 2*) for amplification and two rounds of PCR cycles (Shen, 2019). Initial denaturation was achieved at 98℃ for 1 min and followed by 30 cycles of denaturation at 98℃ for 10 s, annealing at 50℃ for 30 s, and elongation at 72℃ for 30 s and 72℃ for 5 min. Afterwards, the PCR products underwent magnetic bead purification, followed by mixing and recovering of target bands. Sequencing libraries were generated, and indexes were added. The library was quantified using Qubit fluorometer (Thermo Fisher Scientific, USA) and real-time PCR,

and the size distribution was assessed using the Agilent bioanalyzer. Quantified libraries were pooled and sequenced using the Illumina MiSeq platform (Illumina Inc. San Diego, CA, USA).

Table 2. Nested polymerase chain reaction (PCR) primers used for amplification of the V3-V4 hypervariable regions of the 16S bacterial gene.

16SV34	Forward primer	CCTAYGGGRBGCASCAG
	Reverse primer	GGACTACNNGGGTATCTAAT
16S full length	Forward primer	AGAGTTTGATCCTGGCTCAG
	Reverse primer	GNTACCTTGTTACGACTT

2.4 Bioinformatic analysis

Paired-end reads were assigned to samples based on their unique barcode and truncated by cutting off the barcode and primer sequence. These were then merged using FLASH (Version 1.2.1 1) (Magoč and Salzberg, 2011), and raw tags were filtered using the fastp software (Version 0.23.1) to obtain high-quality clean sequences (Bokulich *et al.*, 2013). Chimera sequences were detected using the vsearch package (Rognes *et al.*, 2016) and removed, then dada2 pipeline (Callahan *et al.*, 2016) implemented in QIIME2 (Bolyen *et al.*, 2019) was used for denoising to obtain initial Amplicon Sequence Variants (ASVs). Taxonomical identification was then obtained using the Basic Local Alignment Search Tool (BLAST) (Altschul *et al.*, 1990) against both SILVA RNA gene database (Quast *et al.*, 2013) and NCBI RefSeq database (O'Leary *et al.*, 2016). Sequences belonging to laboratory controls and sampling controls (i.e., sampling table and gloves of the surgeon) were removed using the "IsNotContaminant" method, achieving an extreme decontamination. This choice allowed determining whether some bacterial sequences were still detected in fetal samples. Uterine serosa samples were not used for decontamination, since they presented similar, though fewer, sequences compared to samples from the gloves of the surgeon.

Data were analyzed both before and after decontamination. In the first case, the aim was to compare placenta and amniotic fluid samples with different sampling controls in terms of bacterial diversity. Then, after decontamination by removal of sequences belonging to sampling and laboratory controls, only amniotic fluid and placental samples were compared.

Sequencing data were analyzed using R ver. 4.2.2 (R project, Vienna, Austria) and Miniconda3 (Anaconda Software Distribution, Austing, TX, USA) to run QIIME2 for alpha- and beta-diversity analysis. Prior to statistical analysis, reads were rarefied to 34,872. Bacterial absolute and relative abundances were retrieved. Significance was considered for $P < 0.05$.

Alpha and beta diversities were analyzed using R phyloseq (McMurdie and Holmes, 2013), vegan (Oksanen *et al.*, 2001), and ggplot2 (Wickham, 2009) packages. Alpha diversity was calculated based on observed richness, Shannon, and Simpson indexes, whereas in-between samples or groups differences were estimated using Mann-Whitney, Kruskal-Wallis and pairwise Wilcoxon test. Beta diversity was assessed using Bray-Curtis distances and visualized drawing Principal Coordinates Analyses using QIIME2, differences were calculated using the Adonis function in the vegan R package as a method of nonparametric multivariate variance test according to the distance matrix.

2.5 Bacterial culture

Methods for bacterial culture are described in chapter 3.

2.6 Histology and fluorescence in situ hybridization (FISH)

Formalin-fixed canine and feline placentae were processed for histological assessment and fluorescence in situ hybridization (FISH). Briefly, five cylindric full thickness sections were collected from each formalin-fixed placenta using 6-mm sterile punches onto a sterile sampling table. Each section included all placental layers from the endometrial to the fetal side of the organ. Sections were placed into a sterile cassette following a scheme allowing to obtain slides capturing both the maternal and fetal sides of the placenta and an intermediate part of the organ. The cassettes were embedded into paraffin and cut using a microtome to obtain seven 5-µm slices. Two slices were placed onto regular glass slides and stained using hematoxylin-eosin to histologically assess the presence of necrotic, calcified, hemorrhagic areas, and inflammation (Sarli *et al.*, 2021; Tesi *et al.*, 2021). Five slides per placenta were placed onto polarized glass-slides and shipped to the Simpson's Laboratory, at the Department of Clinical Sciences of Cornell University College of Veterinary Medicine (Ithaca, NY, USA). FISH was performed according to the standard operative protocols of the laboratory (Janeczko *et al.*, 2008; Twedt *et al.*, 2014). Specifically, formalin fixed-paraffin-embedded biopsy specimens were de-paraffinized by sequential immersion in xylene (three times for 10 minutes each), 100% ethanol (twice for 5 minutes each), 95% ethanol (for 5 minutes), and 70% ethanol (for 5 minutes). The slides were then air-dried, and FISH probes labelled at the 5' end, were reconstituted in sterile water and diluted to a working concentration of 5 ng/µL using the appropriate hybridization buffer. The probe combination of EUB-338-cy3 (5cy3/GCTGCCTCCCGTAGGAGT) and non-EUB-338-6FAM with a DAPI counterstain to control for non-specific hybridization was used to detect and quantify bacteria within placental tissue specimens. Sections were hybridized with 30 µL of the DNA

probe mix in a hybridization chamber for 12-14 hours, followed by a wash with post-hybridization buffer and sterile water. Finally, slides were air-dried and mounted with ProLong Antifade Gold (Thermo Fisher Scientific, Waltham, MA, USA) to preserve the fluorescent dyes. Sections were examined on an Olympus BX51 epifluorescence microscope evaluating 10 fields at 60x for each section and capturing images using an Olympus DP-7 camera (Olympus America, USA).

3. Results

Data for age, body weight, and gestational age along with the list of breeds of the bitches and queens included in the study are reported in *Table 3*.

Table 3. Characteristics of the dams included in the study. Age, body weight, estimated gestational age, and breeds of the bitches and queens are reported.

		Age (months)	Body weight (kg)	Estimated gestational age (days)	Breed
Bitches	1C	10	16.2	40	Lagotto romagnolo
	4C	48	16.8	36	Mixed breed
	5C	59	36	45	Cane Corso
	7C	n/a	19	45	Mixed breed
Queens	2F	24	4	42	European Shorthair
	3F	9	3.5	45	European Shorthair
	6F	8	3	29	European Shorthair
	8F	8	3.2	35	European Shorthair

n/a: not available because the bitch was a shelter dog.

3.1 16S rRNA bacterial gene sequencing

The total read count of the processed samples (n = 58) was 2,647,720 (median 45,512, min = 34,872, max = 58,258). Detected amplicon sequence variants (ASVs) in placental samples were 743,831 (median 47,357, min = 37,208, max = 54,723) before removal of sequences detected in sampling and laboratory controls, after which the total number was reduced to 30,706 (median 1,082, min = 19, max = 9,416). Similarly, total ASVs that were detected in amniotic fluid samples were 711,163 (median 44,737, min = 34,872, max = 57,846), and reduced to 48,506 (median 2,176, min = 0, max = 13,012) after removal of sequences from controls. Laboratory controls yielded a total number of 82,647 ASVs (median 41,323, min = 38,741, max = 43,906). *Table 4* shows ASVs based on sample type and species, before and after removal of sequences belonging to controls. The number of bacterial ASVs did not differ between placenta and amniotic fluid samples, as determined by the

Wilcoxon signed-rank test for paired samples (i.e., comparison between each placenta and the amniotic fluid from the same fetus) either before or after decontamination (P = 0.21 and P = 0.37, respectively).

Figure 1 shows the relative abundance of bacterial genera detected in feto-maternal samples and controls. The three most abundant phyla in placental samples were Actinobacteria (33.6%), Proteobacteria (30.3%), and Firmicutes (24.18%). However, after excluding sequences from sampling and laboratory controls, the proportions of the most abundant phyla shifted to Actinobacteria (35.72%), Firmicutes (26.76%), and Proteobacteria (13.17%). In amniotic fluid samples**,** Proteobacteria (33.1%), Actinobacteria (27.48%), and Firmicutes (22.9%) were the most abundant phyla before decontamination, whereas Actinobacteria (25.41%), Proteobacteria (23.67%) and Firmicutes (20.5%) were the most abundant phyla after decontamination. Accordingly, Proteobacteria (32.9%) and Actinobacteria (32.14%) were the most abundant phyla in sampling and laboratory controls.

The three most abundant genera in placental and amniotic fluid samples were *Sphingomonas*, *Klebsiella*, and *Cutibacterium*, and their abundances changed following removal of sequences belonging to sampling and laboratory controls. Specifically, *Sphingomonas*, *Clostridium*, and *Steptococcus* became the most abundant bacterial genera in placental samples, whereas *Cutibacterium*, *Sphingomonas*, and *Klebsiella* were most prevalent in amniotic fluid samples. After decontamination, bacterial sequences were found in every placental and amniotic fluid sample, except for two amniotic fluid samples from two different feline feto-maternal units (6F and 8F), in which no bacterial sequences were detected.

Table 4. Amplicon Sequence Variants (ASVs) based on sample type (placenta and amniotic fluid) and species (dog and cat), before and after removal of sequences belonging to sampling and laboratory controls. Differences in ASVs based on sample type between canine and feline fetus were assessed and found not statistically significant ($p > 0.05$).

		Dog			Cat			
		Total	**Median (IQR)**	**Min-Max**	**Total**	**Median (IQR)**	**Min-Max**	**P-Value[a]**
Placenta	Before	372,596	47,357 (48,028.5 – 45,729.75)	37,208-54,723	371,235	47,027 (48,522.75 – 48,815)	41,720 – 50,133	*p*=0.06
	After	19,867	1,898.5 (2,738.5 – 541.75)	19-9,416	10,839	616 (2,186.5 – 388)	333-3,952	*p*=0.51
Amniotic fluid	Before	335,582	40,536.5	34,872-49,624	375,581	45,704.5	40,991-57,846	*p*=0.15

			(46,540.5 – 38,562.75)			(48,190.75 – 44,496.5)		
	After	16,799	2,309 (2,800.75 – 1,358.5)	249-3,690	31,707	2,149 (6,220 – 170.5)	0-13,012	p=0.78

[a]Mann-Whitney test based on the species.

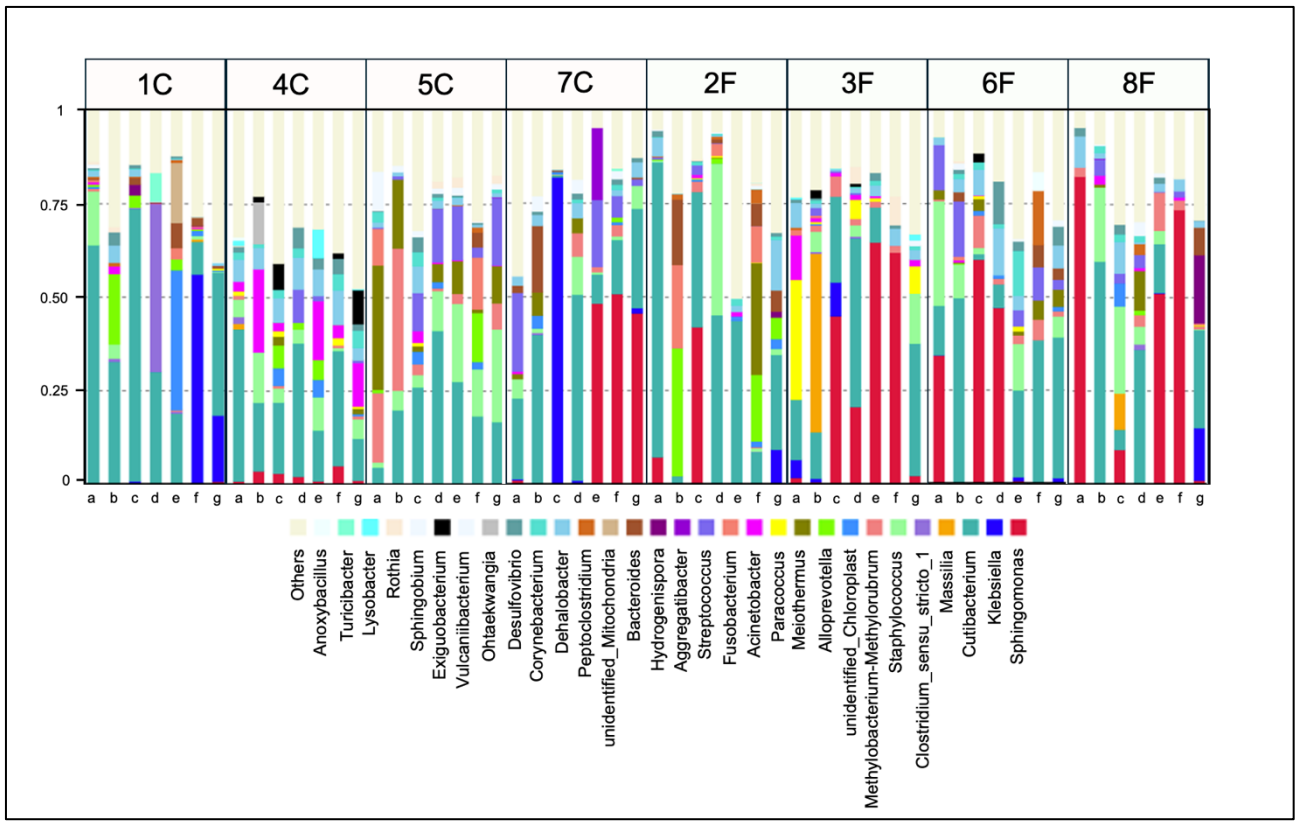

Figure 1. Relative abundance at genus level of the 30 most prevalent bacteria detected by 16S rRNA bacterial gene sequencing from healthy pregnancies (n = 8). These included four canine (1C, 4C, 5C, and 7C)) and four feline (2F, 3F, 6F, and 8F) feto-maternal units. Each included two placenta (d, e) and two amniotic fluid samples (f, g), along with sampling controls collected during each procedure. Specifically, swabs from the uterine serosa (a), the sampling table (b), and the gloves of the surgeon at the end of the procedure (c) were collected as sampling controls.

Alpha diversity (i.e., within-sample diversity) based on observed features (i.e., bacterial richness), Shannon, and Simpson indexes was not different between sample groups (Kruskal-Wallis $p > 0.05$ for all comparisons) and between sample groups based on the species (Kruskal-Wallis $p > 0.05$ for all comparisons). Therefore, neither placenta nor amniotic fluid samples significantly differed from sampling and laboratory controls before decontamination (*Figure 2*). There was no significant

difference in alpha-diversity between placenta and amniotic fluid samples even after removal of sequences belonging to sampling and laboratory controls based on Shannon and Simpson indexes (Wilcoxon test P = 0.52 and P = 0.35, respectively). However, when canine and feline samples were separately analyzed, a difference in within-sample diversity based on Shannon index was detected between canine placenta and amniotic fluid, with the latter presenting higher diversity (Wilcoxon test P = 0.02). No significant difference was detected in-between feline samples (Wilcoxon test based on Shannon index P = 0.24).

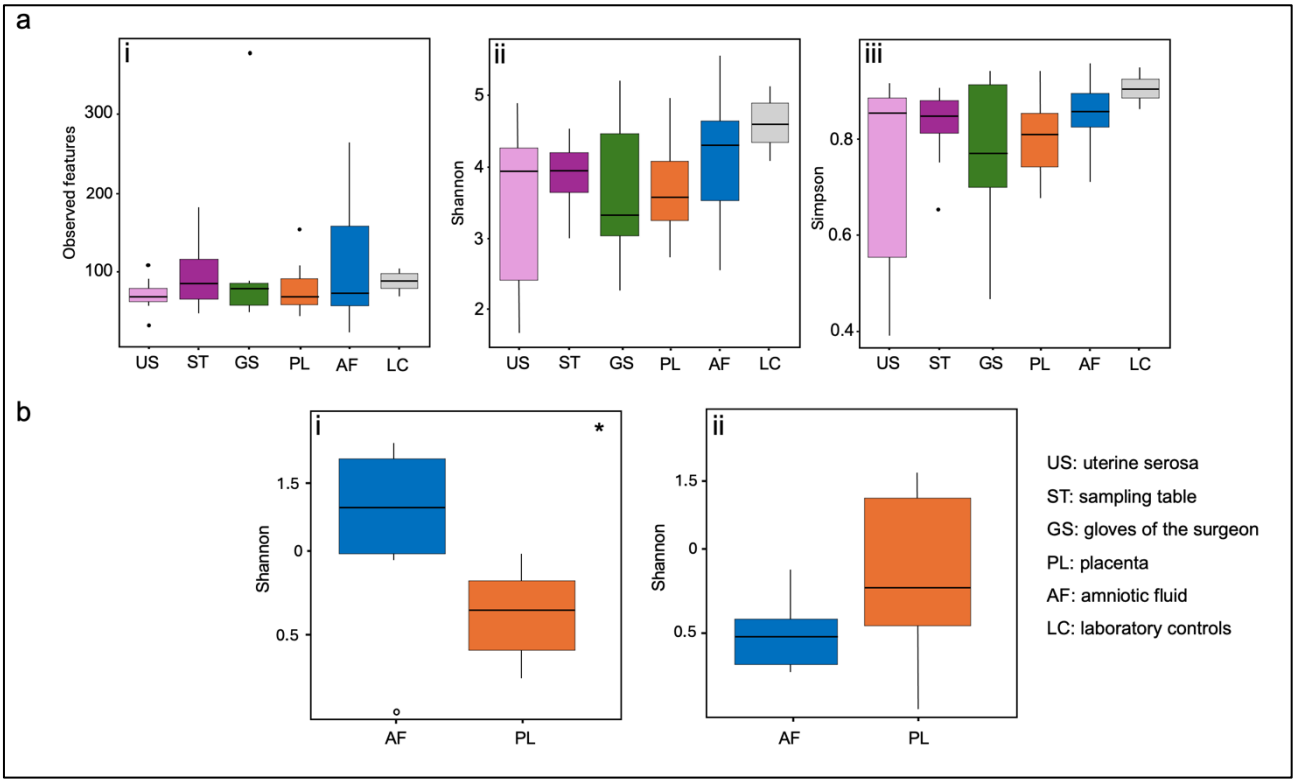

Figure 2. Box plots of alpha diversity measures based on sample type. a. Bacterial richness (i), Shannon (ii), and Simpson (iii) indexes variation are shown for canine and feline samples together. No difference was found ($p > 0.05$) between sampling controls (uterine serosa n = 8, sampling table n = 8, and gloves of the surgeon n = 8), fetal samples (placenta n = 16, amniotic fluid n = 16), and laboratory controls (n = 2). b. Alpha diversity was also assessed after removal of sequences detected in sampling and laboratory controls. (i) Canine placenta had significantly lower bacterial diversity compared to amniotic fluid ($p = 0.02$), (ii) although no difference was found in-between fetal samples in cats ($p > 0.05$).

Beta diversity (i.e., in-between group diversity) was not different between sample groups (Adonis based on Bray-Curtis distance matrix $p > 0.05$ for all comparisons, Figure 3.a). Hence, neither placenta nor amniotic fluid samples significantly differed from sampling and laboratory controls before decontamination. There was a statistical difference in bacterial composition of samples of the gloves of the surgeon based on the species ($p = 0.036$).

After decontamination, there was no difference neither in bacterial composition between samples based on the species ($p = 0.31$), nor based on the sample type ($p = 0.45$). Moreover, bacterial composition of placental samples and amniotic fluid samples did not differ neither in dogs ($p = 0.65$) nor in cats ($p = 0.77$). However, samples belonging to the same feto-maternal unit showed a tendency to group together, with some significant differences in both dogs and cats (*Figure 3.b*, *Table 5*).

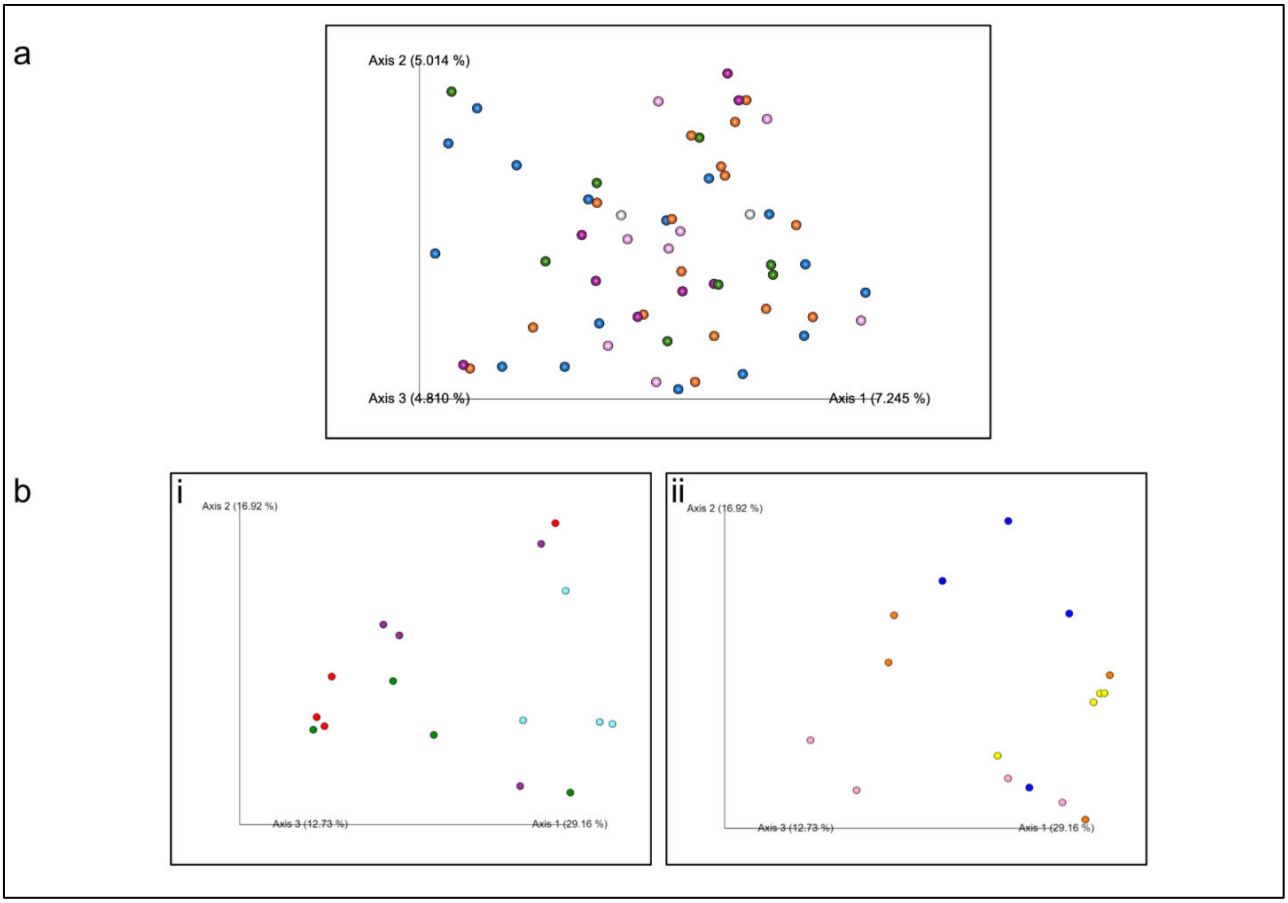

Figure 3. a) Principal Coordinate Analysis (PCoA) based on Bray-Curtis distance-matrix including all animal samples and controls. Color code based on sample type is the following: placenta = orange, amniotic fluid = blue, uterine serosa = pink, sampling table = purple, gloves of the surgeon = green, and laboratory controls = grey); b) Principal Coordinate Analysis (PCoA) based on Bray-Curtis distance-matrix including placenta and amniotic fluid samples after removal of sequences detected in

sampling and laboratory controls. Canine (i) and feline (ii) samples are color-grouped by feto-maternal unit.

Table 5. Differences in beta-diversity (Adonis) based on Bray-Curtis distance-matrix for canine (1C, 4C, 5C, 7C) and feline (2F, 3F, 6F, 8F) feto-maternal units, including two placenta and two amniotic fluid samples each.

	Comparisons between feto-maternal units		P-values
Dogs	1C	4C	0.04*
	1C	5C	0.05
	1C	7C	0.04*
	4C	5C	0.02*
	4C	7C	0.02*
	5C	7C	0.07
Cats	2F	3F	0.11
	2F	6F	0.15
	2F	8F	0.08
	3F	6F	0.04*
	3F	8F	0.03*
	6F	8F	0.007*

*Significance for $p < 0.05$

3.2 Bacterial culture

Results for bacterial aerobic and anaerobic culture in canine and feline feto-maternal units and sampling controls are reported in *Table 6*. No bacterial growth was observed in any amniotic fluid samples, sampling controls (i.e., uterine serosa, sampling table, and gloves of the surgeon at the end of the procedure), or laboratory controls under aerobic and anaerobic conditions. One canine placenta (n = 1/8, 12.5%) exhibited growth of *Moraxella* spp. in aerobic culture, although the other placenta from the same feto-maternal unit presented no bacterial growth. Among feline placentae, two samples from the same feto-maternal unit presented high growth of *Burkholderia cepacia* (n = 2/8, 25%). *Moraxella* spp. is a common inhabitant of human oral cavity and oropharynx in humans and dogs (Padanilam *et al.*, 2022), whereas *Burkholderia cepacia* is a common contaminant in the environment, including disinfectants (Saeed *et al.*, 2024). Therefore, we attempted to confirm positive bacterial growth using sequencing data. However, both isolated species were not detected at genus or species level in matching samples by 16S rRNA sequencing. The canine placenta that showed growth of *Moraxella* spp. in culture, had the highest bacterial load among placenta samples by sequencing.

Table 6. Results for bacteria isolation from eight canine (n = 4) and feline (n = 4) feto-maternal units (FMU): placenta (endometrial side) and amniotic fluid of two fetuses per FMU and sampling controls (uterine serosa, sampling table, and gloves of the surgeon). When no bacterial growth was observed the result is reported as "neg".

		Canine feto-maternal units				Feline feto-maternal units			
		1C	4C	5C	7C	2F	3F	6F	8F
Placenta	Fetus 1	neg	neg	neg	neg	neg	*Burkholderia cepacia* (> 31 CFU/10 µL)	neg	neg
	Fetus 2	Moraxella spp. (> 30 CFU/10 µL)	neg	neg	neg	neg	*Burkholderia cepacia* (> 30 CFU/10 µL)	neg	neg
Amniotic fluid	Fetus 1	neg	neg	neg	neg	neg	neg	neg	neg
	Fetus 2	neg	neg	neg	neg	neg	neg	neg	neg
Controls	Uterine serosa	neg	neg	neg	neg	neg	neg	neg	neg
	Sampling table	neg	neg	neg	neg	neg	neg	neg	neg
	Gloves of the surgeon	neg	neg	neg	neg	neg	neg	neg	neg

[a]Site of attachment of the placenta to the endometrium of the first extracted fetus; [b]amniotic fluid of the first extracted foetus; [c]meconium sampled from the rectal ampulla of the first extracted foetus; *Low (1–10 CFU/10 µL), Moderate (11–30 CFU/10 µL), or High (≥31 CFU/10 µL).
neg: Negative

3.3 Histology and fluorescence in situ hybridization (FISH)

Histological evaluation (*Table 7*) aimed to detect and quantify (%) necrotic, calcified, hemorrhagic areas, and overall inflammation to exclude signs of ongoing placentitis. Most canine placentae (n = 7/8, 87.5%) presented necrotic areas occupying up to 20% of the evaluated tissue. The canine placenta presenting *Moraxella* spp. was also the one presenting the most extensive necrosis. Necrosis was less frequent (n = 4/8, 50%) and less extended (up to 10%) in feline placentae. Also, hemorrhagic areas were more frequent in canine (n = 5, 62.5%, up to 40% of the observed area) compared to feline placentae (n = 1/8, 12.5%, less than 0.5% of the observed area). On the contrary, calcification areas were more frequent in cats (n = 7/8, 87.5%, up to 20% of the observed area) compared to dogs (n = 2/8, 25%, less than 0.5% of the observed area). No signs of inflammation were detected in any of the analysed placentae. No intra- or extracellular bacteria were detected in any of the examined histological sections.

Bacterial signals were detected by FISH in 50% of placental samples (n = 8 out of 16), and they were equally distributed among dogs (n = 4) and cats (n = 4). Overall, the presumptive microbial communities were focal and of low abundance (one rod in 37.5% of positive samples), mainly localized within the placental tissue (*Table 7*, *Figure 4*).

Table 7. Histological observations (necrosis, hemorrhage, and calcification) and Fluorescence in situ hybridization (FISH) results for canine (n = 8) and feline (n = 8) placentae included in the present study.

Species	FMU*	Placenta	Histology			FISH
			Necrosis (%)	Hemorrhage (%)	Calcification (%)	
Dogs	1C	Fetus 1	10	40	0.5	Negative[a]
		Fetus 2	20	20	0.5	Positive (occasional rods)[b]
	4C	Fetus 1	0.5	0	0	Negative[a]
		Fetus 2	0	0	0	Positive (one rod)[b]
	5C	Fetus 1	10	30	0	Positive (occasional rods)[b]
		Fetus 2	20	10	0	Negative[a]
	7C	Fetus 1	10	0.5	0	Negative[a]
		Fetus 2	10	0	0	Positive (one rod)
Cats	2F	Fetus 1	0.5	0.5	20	Negative[a]
		Fetus 2	10	0	0.5	Positive (occasional rods)[b]
	3F	Fetus 1	10	0	10	Negative[a]
		Fetus 2	5	0	10	Positive (occasional rods)[b]
	6F	Fetus 1	0	0	0	Positive (occasional rods)[b]
		Fetus 2	0	0	0.5	Positive (one rod)
	8F	Fetus 1	0	0	5	Negative[a]
		Fetus 2	0	0	10	Negative[a]

*Feto-Maternal Unit; [a]Negative: no bacteria were visualized in 25 sections. [b]Positive: one or occasional rods within the placental tissue.

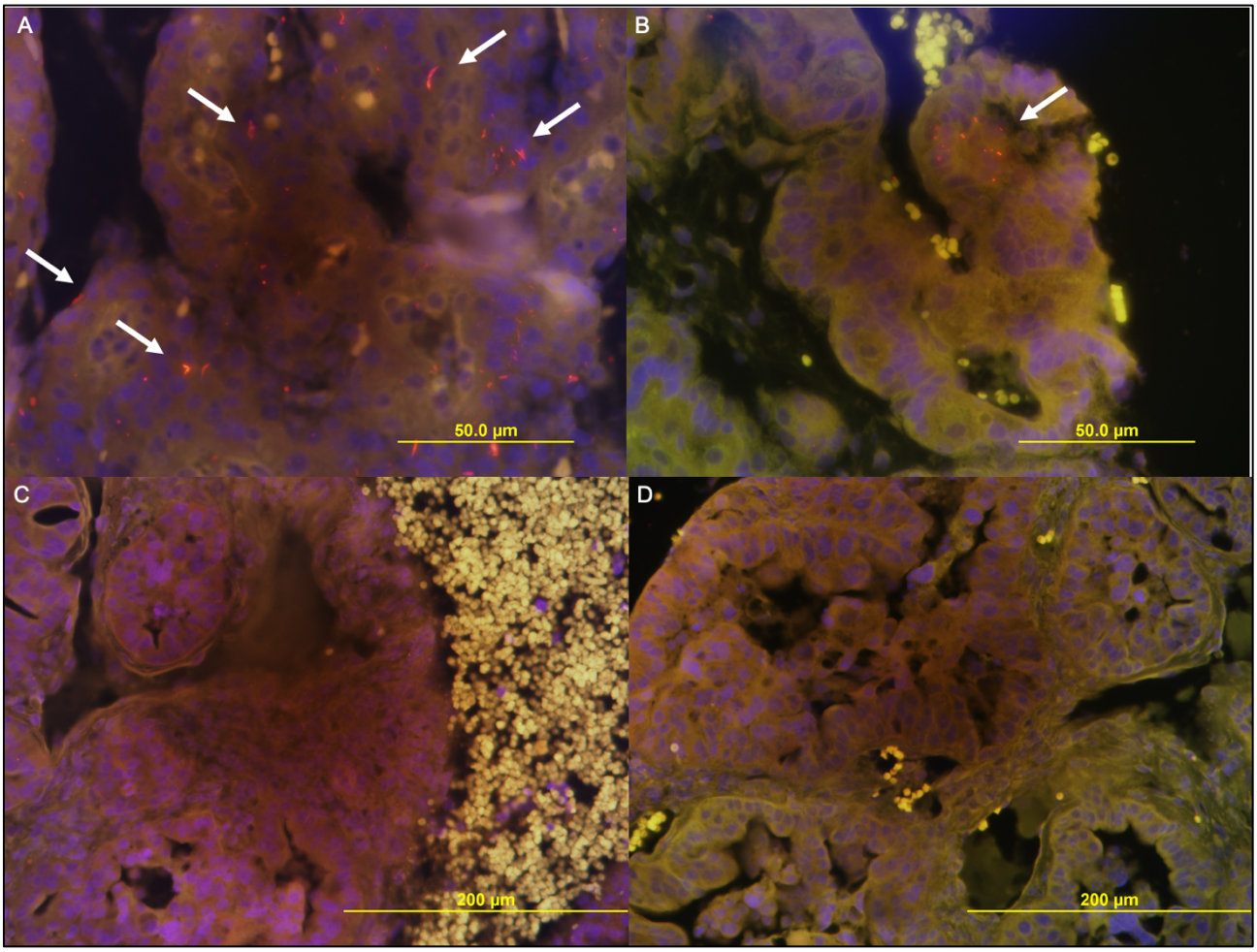

Figure 4. Fluorescence microscope visualization of placental samples using fluorescence in situ hybridization (FISH): epithelial cells of the placental labyrinth (purple nuclei) and red blood cells (yellow) are observed. A) Canine placenta with positive bacterial signal (white arrows indicating red bacteria); B) Feline placenta with positive bacterial signal (white arrow indicating red bacteria); C) Canine placental sample: no detection of bacteria; D) Feline placental sample: no detection of bacteria.

4. Discussion

The relationship between early bacterial colonization and future health (Stiemsma and Michels, 2018; Stinson, 2020) has fueled the debate about the existence of a fetal microbiota in humans, and raised questions about whether pioneer colonization could be modulated to reduce the risk of certain diseases later in life (Kennedy *et al.*, 2021).

Contamination is the main issue in studies involving low-bacterial-biomass samples (Eisenhofer *et al.*, 2019; Kennedy *et al.*, 2023), as reported in our preliminary research in dogs and cats and in

research in cows (Banchi *et al.*, 2023). For this reason, the present study was designed to control contamination at every step of the sampling and laboratory procedures.

Placenta and amniotic fluid samples did not differ from controls (uterine serosa, sampling table, gloves of the surgeon, and laboratory "blank" negative controls) in terms of alpha- and beta-diversity, in agreement with our previous study (Banchi *et al.*, 2023). However, after removing sequences belonging to controls, the bacterial load within placenta and amniotic fluid samples decreased by 24 and 14 times, respectively. This finding, along with the observed variation in the relative abundance of common phyla after decontamination, demonstrates that contaminants can mask the true bacterial composition.

Importantly, this does not imply that contact with harmful bacteria occurred during the surgical procedure, but that sensitive molecular techniques such as 16S rRNA sequencing can detect DNA fragments from non-viable bacteria even following sterilization procedures on surgical equipment (Yap *et al.*, 2013; Calderón-Franco *et al.*, 2020). Accordingly, no bacterial growth occurred in culture, although sequences from easily culturable bacteria were present in multiple samples. Therefore, this research confirmed the presence of bacterial genetic material in the uterine environment during healthy feline and canine pregnancies. However, the detection of this genetic material does not imply the presence of a viable resident microbiome. As "sterile" refers to the absence of living microorganism, the "sterile womb paradigm" may still hold true, despite the fact that sequences belonging to bacteria can be found in placenta and amniotic fluid samples, even after extreme decontamination.

Bacteria were detected in placental samples by sequencing, culture, and FISH in the absence of any histological signs of inflammation. Specifically, bacterial DNA was sequenced in all placental samples, whereas FISH detected bacterial signals in 50% of placentae, and only three samples yielded positive bacterial growth in culture. Therefore, bacterial isolation in culture occurred at a frequency of 18.75% (12.5% when considering only canine placentae): previous studies that investigated the presence of bacteria in canine placentae at term reported higher isolation rates, ranging from 40% to 57% (Zakošek Pipan *et al.*, 2020; Rota *et al.*, 2021; Banchi *et al.*, 2023). However, data are hardly comparable because of different timing and much stricter sampling procedures during elective mid-term "en-bloc" ovariohysterectomies. Interestingly, cultures of matching amniotic fluid samples were negative in the present study, possibly emphasizing the role of the placenta as a barrier protecting the fetus.

Moraxella spp. was isolated from the canine placenta that showed the highest bacterial load following sequencing and a positive bacterial signal according to FISH. This placenta also showed the most extensive necrosis in the histological evaluation, yet there were no abnormal findings when compared to previous literature (Sarli *et al.*, 2021; Tesi *et al.*, 2021). *Moraxella* is an aerobic Gram-negative bacterial genus commonly found in the respiratory tract and conjunctiva of humans and animals (Wang *et al.*, 2022; Yao and Liu, 2024). Although *Moraxella* spp. is not a common cause of pregnancy disturbance, experimental intraperitoneal injection of *Moraxella bovis* in pregnant mice was reported to cause abortion (Norman and Elissalde, 1979). Furthermore, no bacterial growth was observed in culture for the other placenta from the same pregnancy. Conversely, *Burkholderia cepacia* was isolated from two feline placentae from the same dam, although bacterial signals were detected by FISH in only one placenta. *Burkholderia cepacia* is a Gram-negative bacterium known for its multidrug resistance and ability to grow in antiseptic solutions. It has been associated with severe pulmonary infections, particularly in immunocompromised individuals, including pregnant women with underlying conditions (Saeed *et al.*, 2024). In cats, *B. cepacia* has been linked to subcutaneous abscesses and purulent cellulitis (Wong *et al.*, 2018). In this study, feline placentae that yielded positive isolation of *B. cepacia* in culture did not show any histological lesions. As samples were collected at midgestation, it is unclear whether *Moraxella spp.* or *B. cepacia* would have caused lesions later in pregnancy. However, the presence of *Moraxella spp.* in meconium, placenta, amniotic fluid, and colostrum has been reported in healthy term puppies and their dams (Zakošek Pipan *et al.*, 2020; Kajdič *et al.*, 2021; Tesi *et al.*, 2021)

The FISH results from our study indicated a low bacterial presence in the placenta of both dogs and cats during pregnancy. Although bacterial signals were identified in 50% of cases, each positive result was due to the detection of bacteria in only one of the 25 sections observed. Therefore, results were similar to observations in both pre-term and term human placentas from sterile caesarean sections performed in women that did not start labor at the time of the procedure (Seferovic *et al.*, 2019).

Contrary to previous research, which reported 20-40% positive bacterial isolates in culture (Rota *et al.*, 2021; Banchi *et al.*, 2023), no bacterial growth was detected in amniotic fluid samples, despite bacterial DNA being found in all samples by 16S rRNA sequencing. Interestingly, many sequences belonged to easily culturable bacteria, suggesting that DNA fragments found within the fetal environment are most likely to originate from non-viable bacteria. This can be explained by the function of the placenta as a filter and barrier against potential pathogens. Therefore, bacterial DNA fragments and possibly very limited numbers of living bacteria may reach the placental barrier, as

reported in humans (Aagaard *et al.*, 2014; Seferovic *et al.*, 2019). While the origin of such bacterial components was not investigated in the present study, a recent paper suggested the passage of bacterial DNA fragments from the gut of the mother to the fetal environment via extracellular vesicles (Kaisanlahti *et al.*, 2023).

The dam plays a key role in shaping the microbiota of the newborn at birth, with the vaginal microbiota of the dam contributing to meconium bacterial composition in newborn puppies (Zakošek Pipan *et al.*, 2020; Del Carro *et al.*, 2022; Bertero *et al.*, 2024). In the present study, samples from the same feto-maternal unit clustered together in most cases, suggesting a possible maternal imprint even before birth, although our small sample size limits broader generalizations about maternal influence on early colonization patterns. This phenomenon is worth investigating in future research, and interspecies differences should be assessed on larger cohorts. The effect of the dam may also justify the differences in alpha diversity between placenta and amniotic fluid samples that were found in dogs, but not in cats.

Sampling healthy bitches and queens with gestational ages between 30 and 45 days (i.e., mid to second third of gestation) was possible within the context of elective ovariohysterectomies due to unwanted pregnancies, which are only occasionally performed in dogs and cats (Eilts, 2002). This is not a common practice in other species, although it has been performed in laboratory animals, with sequencing results that were consistent with those of the present study in eleven pregnant mice euthanized at 17.5 days of gestation (Theis *et al.*, 2020).

The removal of the pregnant uterus “en bloc” offered stricter control against contamination compared to routine caesarean sections. Furthermore, the collection of multiple controls was key to assess the contribution of contaminants to the 16S sequencing results. Laboratory “blank” negative controls were processed along with fetal samples and sampling controls to remove possible contamination from the “kitome” (i.e., DNA from the reagents of the extraction kit) and extraction procedures (Paniagua Voirol *et al.*, 2021). Moreover, three sampling controls were collected during each surgical procedure. The uterine serosa was sampled immediately after the opening of the abdominal cavity to assess contamination during the extraction of the fetuses. Sampling was always performed on a sterile table with two control swabs placed to monitor possible contamination during placenta and amniotic fluid sampling. Additionally, the gloves of the surgeon were swabbed at the end of the procedure. This choice was prompted by findings from our preliminary study (Banchi *et al.*, 2023), which reported differences in feto-maternal bacterial profiles between dogs and cats. We proposed that these differences could be due to contamination from the dam’s skin, potentially introduced by the contact

with the scrubbed incision area. The difference in bacterial composition of samples from surgical gloves based on the species was significant, whereas no difference was found between feline and canine fetal samples after decontamination. This confirms that the source of interspecies differences in our previous study was due to contamination with bacterial sequences from the scrubbed skin of the dam and stresses the importance of sampling controls in this type of research (Eisenhofer *et al.*, 2019; Kennedy *et al.*, 2023; Banchi *et al.*, 2024).

The small sample size is a significant limitation of this study, which involved a very specific and rare material, such as healthy fetuses and annexes from pregnancies terminated at the request of those responsible for the animals. This imposed limitations on the timing of sampling, which was based on gestational age. Moreover, studies on the feto-maternal microbiome often involve small populations due to the extended time required for sample collection in clinical settings and the high associated costs (Lauder *et al.*, 2016; Martinez *et al.*, 2018; Stinson *et al.*, 2019; Younge *et al.*, 2019). Given these constraints, we aimed for a minimum of eight samples per group for each comparison. Additionally, we carefully selected non-parametric statistical tests, which do not assume normal data distribution and are well-suited for small and non-normally distributed datasets (Xia and Sun, 2017). These statistical approaches ensured the robustness of our analyses despite the sample size limitations.

As mentioned, the removal of sequences belonging to controls led to a significant decrease in ASVs. Contaminant identification and removal is suitable for low-biomass samples (Davis *et al.*, 2018), although we removed contaminants from multiple controls (i.e., gloves of the surgeon, sampling table, laboratory controls, thus with the risk of also removing some genuine sequences from target samples (Karstens *et al.*, 2019). This choice aligned with the objective of the present research, which was not to describe the fetal microbiome but confirm the presence of bacterial sequences within feto-maternal samples. Recently, new tools suitable for decontamination of low-biomass samples have been described (Hülpüsch *et al.*, 2023) and may be recommended in future studies on fetal samples for a more conservative approach.

The multi-technique approach used in the present study included three different complementary methods, therefore some unmatching results are not surprising, as previously reported in studies investigating low- and high-biomass samples (Lagier *et al.*, 2012; Zhou *et al.*, 2023). Sequencing of 16S rRNA is highly sensitive for comprehensive microbial profiling, but its specificity can be affected by the resolution method and contamination from non-target DNA from environmental contaminants or eukaryotic cells of the host. This method has typically limited taxonomical resolution compared to

other sequencing technologies such as Oxford Nanopore technology (Szoboszlay *et al.*, 2023), which is less efficient in detecting some bacterial genera (Heikema *et al.*, 2020). Culture has traditionally low sensitivity, as the majority of microorganisms do not grow in culture media (Kaeberlein *et al.*, 2002), but it is characterized by reasonable specificity depending on the media and culture conditions and provides information on bacterial viability (Kennedy *et al.*, 2023). Finally, FISH is a culture-independent technique targeting 16S rRNA with lower risk of contamination compared to 16S rRNA bacterial gene sequencing, as bacterial signal is considered positive and not artefactual when bacteria are detected within the tissue (Janeczko *et al.*, 2008). In this study, the FISH protocol was designed to have high sensitivity for detecting bacteria using the generic probe EUB-338-cy3, while specificity was enhanced with the non-EUB-338-6FAM probe to confirm positive fluorescent signals and exclude nonspecific binding (Schmiedel *et al.*, 2014).

Each method provided unique information about bacterial presence within different tissues, highlighting that such a complex topic cannot be adequately investigated using a single technique.

5. Conclusions

This multi-technique approach did not confirm the presence of a characteristic resident microbiome (i.e., the collection of microorganisms and their genetic material) (Hou *et al.*, 2022) in healthy canine and feline fetuses. However, bacterial components can reach the pregnant uterus and possibly interact with the fetus during its development, as hypothesized in other species (Gomez de Agüero *et al.*, 2016; Rakoff-Nahoum, 2016; Ruiz-Triviño *et al.*, 2023). Since the presence of bacterial DNA fragments within the fetal environment has been confirmed in dogs and cats, future research should aim to uncover its role within the framework of the Developmental Origins of Health and Disease (DOHaD) theory (Barker and Osmond, 1986; Gaillard *et al.*, 2022), possibly leading to new preventive strategies for future diseases in small animals.

6. Declarations

Declaration of competing interest: there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Ethical statement: This observational study was performed in agreement with adapted ARRIVE guidelines (Animal Research: Reporting In Vivo Experiments). The Ethical Committee of the Department of Veterinary Sciences of the University of Turin (Italy) approved the present research (n. 310/9/2/2021). Written informed consent was obtained from each owner or person responsible of the shelter or cat colony.

Author contribution statement: PB: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Resources, Data Curation, Writing – Original Draft, Visualization. AB: Investigation, Writing – Review & Editing. MC: Investigation, Writing – Review & Editing. BC: Validation, Investigation, Writing – Review & Editing. LM: Investigation, Writing – Review & Editing. AVS: Writing – Review & Editing, Supervision, Project administration, Funding acquisition. AR: Conceptualization, Resources, Writing – Review & Editing, Supervision, Project administration, Funding acquisition.

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Chapter 6
The microbiome of naturally delivered puppies is shaped by the passage through the vaginal canal

Adapted from:

Meconium microbiota in naturally delivered canine puppies

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Abstract

Microbial colonization during early life has a pivotal impact on the host health, shaping immune and metabolic functions, but little is known about timing and features of this process in dogs. The objectives of this study were to characterize the first step of intestinal microbiota development in naturally delivered canine puppies and to investigate its relationship with the maternal bacterial flora, using culture and 16S rRNA bacterial gene sequencing. Sixty puppies of two breeds, Appenzeller Cattle Dog (n = 3 dams) and Lagotto Romagnolo (n = 6), housed in the same breeding kennel, were included in the study. Swabs were collected in duplicate (for culture and 16S sequencing) from the dams' vagina and rectum at the end of parturition, from the puppies' rectum, before maternal care, and from the environment (floor of the nursery and parturition box). Most meconium (93.3%) samples showed bacterial growth, limited to a few colonies in 57.0% of cases. High bacterial growth was detected for *Enterococcus faecalis*, which was the most frequently isolated bacterium. The genus *Enterococcus* was one of the most represented in the dams' rectum and vagina (88.9% and 55.6%, respectively).

The genera *Staphylococcus*, *Enterococcus*, *Escherichia* and *Proteus* were also often isolated in meconium but belonged also to the maternal flora, together with ubiquitous bacteria (*Acinetobacter*, *Psychrobacter*). In the environmental samples, just a few bacterial species were found, all with low microbial load. Additionally, bacteria of the phyla Proteobacteria, Firmicutes, and Actinobacteria were identified in meconium through molecular analysis, confirming the culture results and the early colonization of the newborn gut. Maternal, meconium and environmental samples had similar alpha diversity, while beta-diversity showed differences among families (i.e. a dam and her litter), and association indexes revealed a significant correlation between family members and between sample origin, suggesting a strong contribution of the maternal flora to the initial seeding of the canine neonatal gut and a strong individual dam print. This study showed that the meconium of vaginally delivered puppies has its own microbiota immediately after birth, and that it is shaped by the dam, which gives a specific print to her litter.

1. Introduction

Microbial colonization during early life is a crucial developmental process that shapes host immunity and metabolism (Neu, 2015). Most microbial communities reside symbiotically in the intestine, where the establishment of the gut microbiome is fundamental for the development of the neonatal immune system, defense against enteric infections, and future health. Disruption of microbial communities has been linked to the occurrence of various chronic diseases in humans (Bittinger *et al.*, 2020) and

dogs (Pereira and Clemente, 2021). The potential to optimize or modulate the microbial ecosystem depends on understanding its origin and the timing of initial seeding; these aspects have been the focus of extensive research in both humans and animals. Traditionally, the human microbiota is thought to be acquired during and after birth, but the 'sterile womb' paradigm (i.e., the biological dogma that a human fetus is sterile) has been challenged, and the possibility of *in utero* colonization has been investigated with molecular approaches (Perez-Muñoz *et al.*, 2017; Kennedy *et al.*, 2023). Studies on dogs led to bacterial isolation from dog fetuses and placentae (Zakošek Pipan *et al.*, 2020; Rota *et al.*, 2021), but our research (chapters 4 and 5, Banchi *et al.*, 2023) showed the risk of contamination is high and that there is no resident fetal microbiota at birth. However, some viable bacteria can be found within the placenta, that works as a filter and a barrier against potential pathogens (Chapter 5).

Meconium forms before birth and is rapidly colonized by bacteria in human neonates (Kennedy *et al.*, 2021), with bacterial load and diversity increasing over time after birth (Bittinger *et al.*, 2020). In puppies, more direct contact with the environment, differences in maternal care, and the presence of littermates may influence initial microbial colonization compared to other species. However, knowledge about the microbial flora of meconium, as well as its bacterial colonization timing and pathways in newborn puppies, is limited (Guard *et al.*, 2017; Zakošek Pipan *et al.*, 2020).

The objectives of the present study were to characterize the initial stage of intestinal microbiota development in naturally delivered canine puppies and to investigate its relationship with the maternal bacterial flora, using bacterial culture and 16S rRNA bacterial gene sequencing.

2. Materials and methods

2.1 Animals

Litters (n = 9) of three Appenzeller Cattle Dog (ACD) and six Lagotto Romagnolo (LR) bitches were included in the study, with no restriction on age or parity. The characteristics of the bitches and their litters are reported in *Table 1*. All bitches were housed in the same breeding kennel and had uneventful pregnancies followed by natural vaginal delivery. Delivered puppies were 60, including 54 living puppies and six stillborn ones. Dogs did not receive any medication (including antibiotics) or supplements, except for standard deworming treatment, which was administered ten days before the expected parturition date (Milbactor® Krka, d.d. Novo Mesto, Slovenia). Animals were kept in indoor spaces with access to outdoor yards and fed with the same commercial food (MONGE Natural Superpremium Medium Adult Rich in Chicken®, Monasterolo di Savigliano, Cuneo, Italy), shifting towards a dry balanced diet for growing medium size dogs (MONGE Medium Puppy & Junior Rich

in Chicken®, Monasterolo di Savigliano, Cuneo, Italy) during the last two weeks of pregnancy. Food quantity was calculated based on FEDIAF Guidelines 2019 ('FEDIAF | Nutritional Guidelines', 2019).

Concurrently with the diet change, the bitches were transferred to the nursery area, with smaller kennels with tiled floor and walls and separate outdoor yards. Moreover, the indoor nursery kennels were cleaned twice per day using chlorine-based products.

The study was approved by the Ethical Committee of the Department of Veterinary Sciences of the University of Turin (Approval number 2200, 24/09/2019) and was performed in accordance with the EU Directive 86/609/CEE and with the guidelines of the Italian Ministry of Health for the care and use of animals (D.L. 4 March 2014 n. 26 and D.L. 27 January 1992 n. 116). Previous informed consent was obtained from the dog breeders.

Table 1. Purebred litters included in the study and breed, age, weight, and parity of their dams.

Litter	Breed	Puppies (n)		Dam ID	Age (years)	Weight (Kg)	Parity (n)
		Alive	Stillborn				
1	ACD	8	0	A01	6.5	25.6	4
2	ACD	6	3	A02[1]	4.2	22.8	3
3	ACD	4	0	A05[1]	6.0	26.4	4
				Mean ± SD	**5.6 ± 1.2**	**24.9 ± 1.9**	**3.7±0.6**
4	LR	8	1	A04[1]	5.6	12.5	5
5	LR	7	1	A07	1.5	13.6	1
6	LR	3	1	A08	7.6	12.4	6
7	LR	4	0	A09[1]	3.2	13.8	2
8	LR	6	0	A03	1.5	12.4	1
9	LR	8	0	A06	3.9	14.2	4
				Mean ± SD	**3.9 ± 2.4**	**13.2 ± 0.8**	**3.2 ± 2.1**

ACD=Appenzeller Cattle Dog; LR=Lagotto Romagnolo. Mean values ± standard deviation (SD) in bold. [1]Samples from these bitches and her puppies were analysed with both culture and 16S rRNA sequencing.

2.2 Sample collection

Rectal samples were taken from each puppy immediately at birth, after opening the amniotic sac, when intact. Respiration was stimulated by rubbing the puppy with a clean towel when deemed necessary, and the umbilical cord was clamped before presenting the puppy to the dam. Samples were collected by a single operator wearing gloves using sterile mini nylon flocked swabs (ESwab®, 484CE, Copan Italia Spa, Brescia, Italy). Both living puppies and stillborn ones were sampled.

Swabs were collected from the dams' vagina and rectum, by the same operator, at the end of parturition. Sterile nylon flocked swabs (ESwab®, 480CE, Copan Italia Spa, Brescia, Italy) were introduced into the rectum for fecal sampling and into the vagina. Specifically, a polypropylene tube (the plastic cover of the Heinz Herenz Dry Sample Collection Swab, Fisher Scientific Italia, Segrate, Milano, Italy) was inserted into the vagina as a sterile guide, after careful opening of the vulvar labia. Environmental samples were collected before parturition, rubbing the tip of the swabs (ESwab®, 480CE, Copan Italia Spa, Brescia, Italy) on the floor of the nursery and in the parturition box, before allowing the dam in. Another swab was exposed to ambient air as control.

Samples were always collected in duplicate, one for culture and another one for 16S rRNA sequencing. Swabs intended for culture were placed into modified liquid Amies medium (ESwab® Copan Italia Spa, Brescia, Italy) and immediately sent to the laboratory of the Istituto Zooprofilattico Sperimentale delle Venezie (Legnaro, Italy), in refrigerated boxes, and processed within 48 hours. Swabs intended for 16S sequencing were immediately stored at – 20°C in the breeding kennel, and subsequently transferred at – 80°C at the Department of Veterinary Sciences of the University of Turin (Grugliasco, Italy).

2.3 16S rRNA bacterial gene sequencing

Samples from dam-litter units (n = 4), including two ACD (A02 and A05, Table 1) and two LR (A04 and A09, Table 1) underwent bacterial DNA extraction and sequencing along with environmental swabs (n = 6). Specifically, a total of 38 samples was processed. Animal samples consisted of rectal 'mini' swabs collected from 24 puppies, four vaginal and four rectal swabs from their dams. DNA extraction was performed using the RNeasy Power Microbiome KIT (Qiagen, Hilden, Germany) and is described in chapter 3.

The 16S rRNA gene was amplified following the Illumina 16S Metagenomic Sequencing Library Preparation Protocol (Illumina Inc. San Diego, CA, USA), with minor modifications. Briefly, the V3-V4 region of the 16S gene was amplified with unique barcoded Polymerase Chain Reaction (PCR) primers containing the Illumina adapter overhang nucleotide sequences:

16S Forward Primer:
5'- TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG)
16S Reverse Primer:
5''-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC.

Polymerase Chain Reaction amplicons were cleaned up and size selected using NucleoMag® NGS Clean-up and Size Select (Macherey-Nagel, Allentown, PA, USA). The resulting products were tagged by using the Nextera XT Index Kit (Illumina Inc., San Diego, CA, USA). After the second purification step, amplicon products were quantified using Qubit High Sensitive dsDNA kit (Life Technologies, Carlsbad, CA, USA). Purified and normalized libraries were then pooled and diluted to a 4 nM concentration. The pooled library was then denatured with 0.2 N NaOH, diluted to 10 pM, and combined with 20% (vol/vol) denatured 10 pM PhiX and sequenced with the MiSeq Illumina platform (Illumina Inc., San Diego, CA, USA) with V3-600 cycles chemistry.

2.4 Bioinformatic analysis

Sequencing results were analyzed using Trimmomatic and QIIME 2-2019.10 standard pipelines and Green Gene (13-8-99-515-806) database for the taxonomical identification. Alpha (Shannon index) and beta (Jaccard distance matrix) diversities were calculated based on sample type (i.e., meconium, dam's rectum, dam's vagina, control) and dam-litter unit (i.e., one dam and her litter). Jaccard distance matrix was used to assess the bacterial population structure. Based on the obtained distance matrix by QIIME pipeline, cluster analysis with k groups was conducted using R statistical software: k parameter was equal to five for both the grouping methods (tissues type or family dam-litter unit, including controls); the association between cluster membership and biological features was evaluated by the X^2 association index.

2.5 Bacterial culture

Sixty rectal swabs from puppies, nine vaginal and nine rectal swabs from their dams were processed using bacterial culture along with nine environmental swabs from the nursery kennel and control swabs exposed to the environmental air. Methods for bacterial culture are described in chapter 3.

3. Results

The nine litters included in the study included nine dams and sixty puppies. Specifically, twenty-one Appenzeller Cattle Dog (ACD) and thirty-nine Lagotto Romagnolo (LR) puppies. The mean birth weight was considered normal by the breeder for the dams' lineage [(g)±SD: ACD 406±48; LR 251±76]. All the stillbirths (*Table 1*) were due to prolonged parturition, as suggested by the monitoring of the birthing progress and the immediate examination of the newborns.

3.1 Sequencing of 16S rRNA

Taxonomic composition at phylum level (*Figure 1*) showed that meconium samples primarily consisted of bacteria belonging to Proteobacteria (median percent of sequences = 33), Bacteroidetes (median percent of sequences = 16), Firmicutes (median percent of sequences = 13), Actinobacteria (median percent of sequences = 6), and Fusobacteria (median percent of sequences = 6).

Within the phylum Proteobacteria, 36.67% of the reads were attributed to the class Alphaproteobacteria, mainly represented by the family Phyllobacteriaceae (61.44%), 33.72% of the reads were assigned to the class Gammaproteobacteria, and 25.79% of the reads to the Betaproteobacteria, among which the order Burkholderiales was the most abundant (81.44%).

As for the phylum Bacteroidetes, 21.55% of the reads was assigned to the family Chitinophagaceae, followed by the family Flavobacteriaceae (7.94%).

Within the phylum Firmicutes, Bacilli (46.40%) and Clostridia (43.02%) accounted for most of the reads. Bacillales was the most abundant order (37.86%) in the class Bacilli, whereas Lachnospiraceae (37.75%), Clostridiaceae (31.92%), Peptostreptococcaceae (23.28%) were the most represented families in the class Clostridia.

Within the phylum Actinobacteria, most of the reads were attributed to the order Actinomycetales (94.72%), and particularly to the families Micrococcaceae (10.7%) and Intrasporangiaceae (10.4%)

Finally, Fusobacteriaceae was the most represented family (99.3% of the reads) belonging to the phylum Fusobacteria.

In maternal rectal samples the predominant phyla were Fusobacteria and, with lower frequency than in meconium, Actinobacteria and Proteobacteria. Vaginal samples were less homogeneous, displaying a more variable frequency of the listed phyla.

Alpha diversity was not different between meconium and maternal samples (i.e., rectum and vagina). Moreover, there were no differences based on the Shannon index between samples of the same type belonging to different dam-litter units (*Figure 2*) and between samples belonging to the same dam-litter unit (Kruskal Wallis rank sum test $p = 0.0989$ and $p = 0.3326$ respectively).

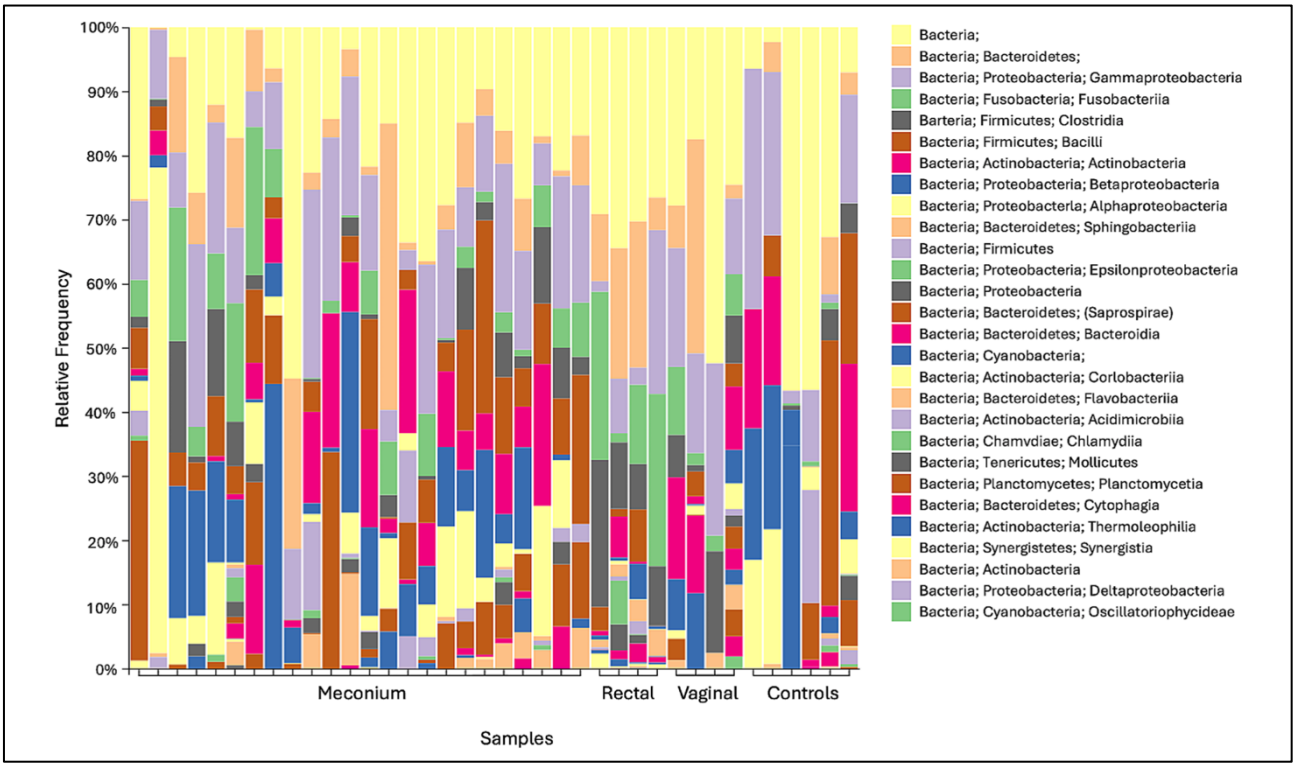

Figure 1. Relative abundance of bacterial phyla and classes detected by 16S rRNA bacterial gene sequencing in samples from dams and their puppies. Samples are grouped based on sample type (i.e., meconium, dam's rectum and vagina, environmental control).

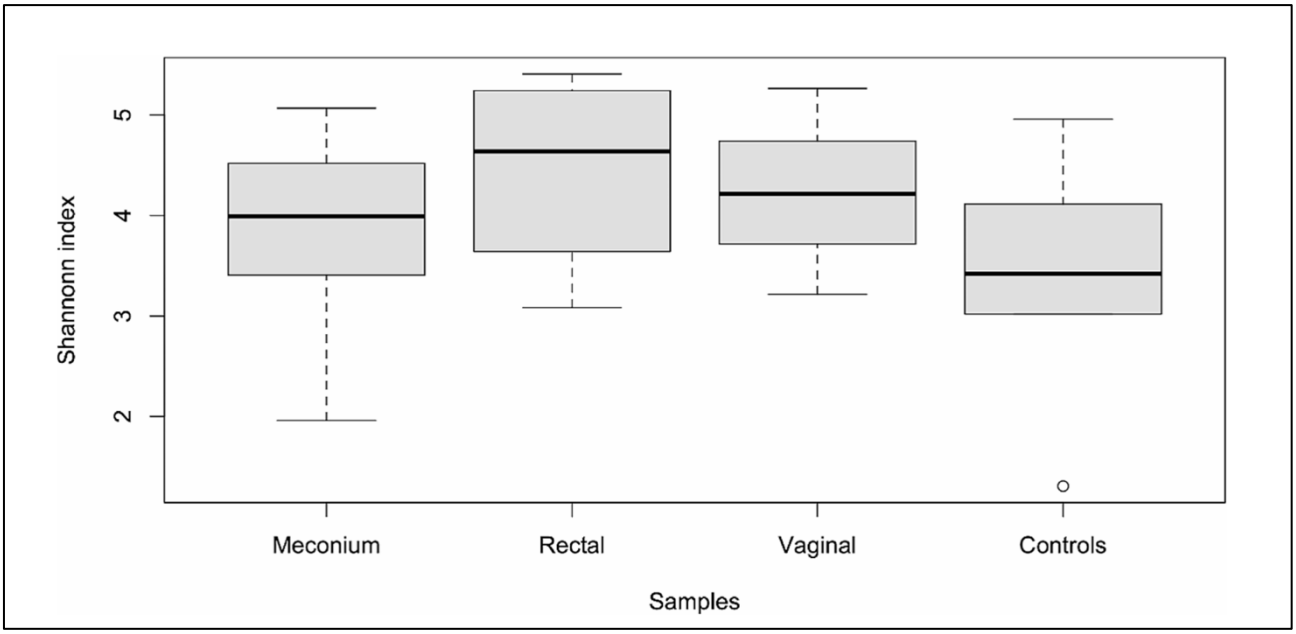

Figure 2. Box-plots for alpha diversity (i.e., within sample diversity) based on sample type (i.e., meconium, dam's rectum and vagina, environmental control). No significant differences were detected (Kruskal-Wallis test $p > 0.05$).

Beta diversity analysis revealed differences based on the sample type (PERMANOVA $q < 0.05$), except for meconium and vaginal samples (PERMANOVA $q = 0.323$) and vaginal and control samples (PERMANOVA $q = 0.1932$), that showed similar bacterial composition (*Figure 3A*).

Moreover, beta diversity analysis revealed differences in bacterial composition between dam-litter units (*Figure 3B*). Most of the comparisons showed significant outcomes, highlighting differences in bacterial composition based on the dam-litter unit and between canine and environmental samples.

Spatial distribution of samples based on their bacterial composition was drawn using a multidimensional scaling model based on the Jaccard distance matrix (*Figure 4*). Association indexes revealed a significant correlation between the clusters identified by k means algorithm and both the dam-litter unit (Pearson's Chi-squared test $p < 0.01$) and the sample type (meconium, rectum, vagina, or environment (Pearson's Chi-squared test $p < 0.01$).

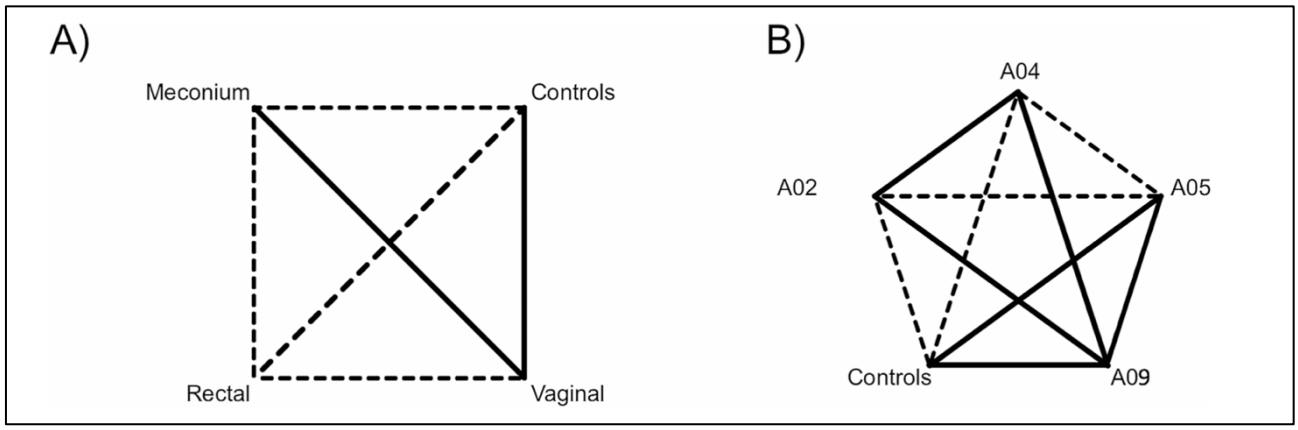

Figure 3. Differences in bacterial composition based on Jaccard distance matrix between different tissues (A) and dam-litter units (B). Statistically significant differences (PERMANOVA $q < 0.05$) are represented by dashed lines.

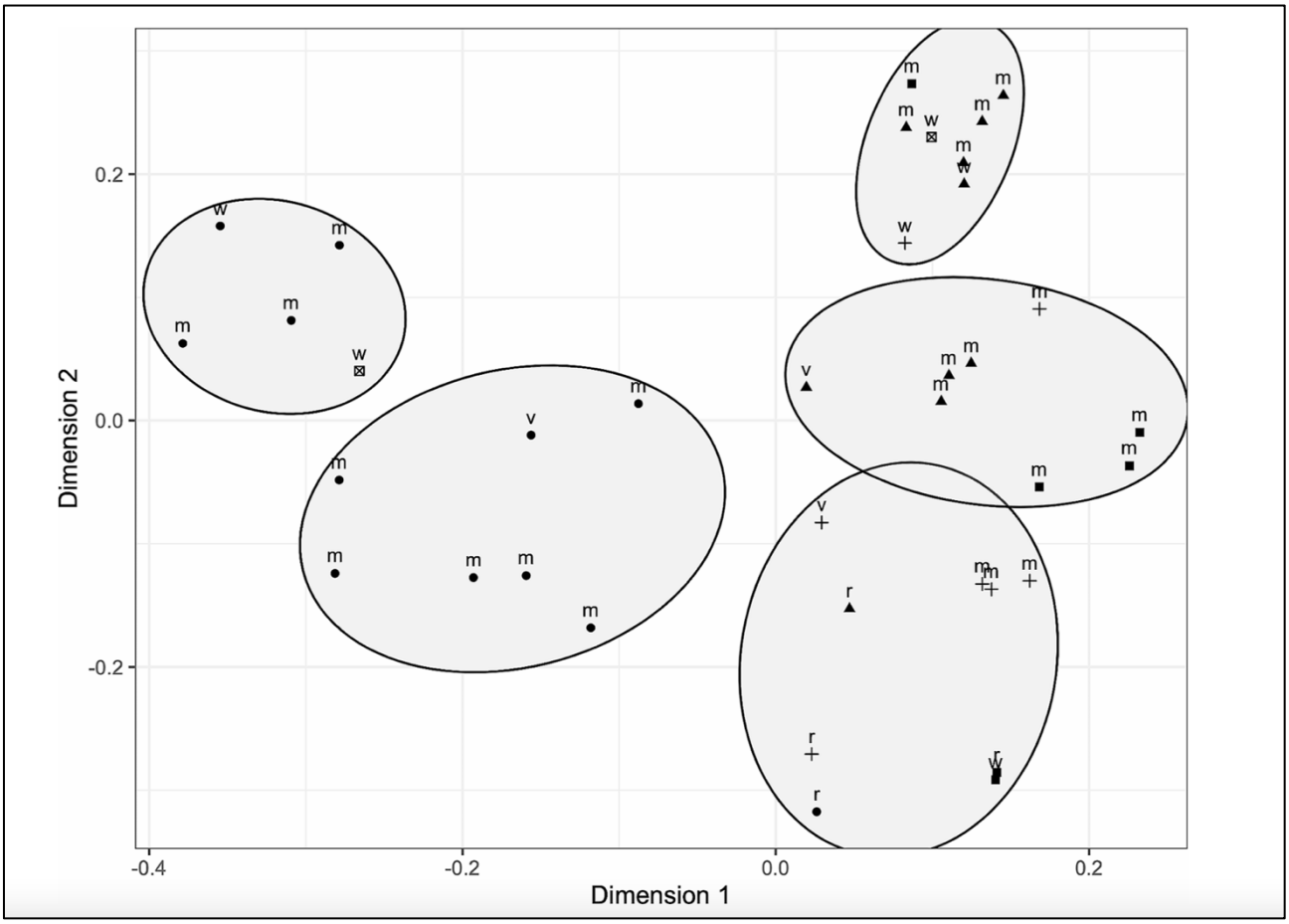

Figure 4. Multidimensional scaling representing Jaccard distances among sequenced samples. Clusters were identified by k-means algorithm with K=5. The shape of each sample indicates the family membership (circle: A02, triangle: A04, square: A05, cross: A09). Labels close to each sample indicate the sample type (m: meconium, r: rectal, v: vaginal, w: white/environment).

3.1 Bacterial culture

Only four meconium samples from two litters resulted negative in culture (6.7%) whereas 93.3% of the samples was positive for bacterial growth. The number of bacterial species detected in a single sample varied from none to six. Both Gram-positive and Gram-negative bacteria were identified and are presented in the Supplementary Material along with their frequency of isolation. Frequencies of the isolated bacteria is reported based on the genera and the phylum in *Figure 5*.

Growth in culture was limited to few colonies in 57.0% of cases, high growth was detected in 38.9% cases, especially for *Enterococcus faecalis*, while moderate growth was detected in 4.1% of samples. The number of bacterial species isolated from the nine vaginal swabs (V) was higher compared to that isolated from the nine rectal swabs (R) of the bitches (n = 13 and n = 17, respectively). A match

between puppies and their dam was observed for at least one bacterial species 48 times. Specifically, a match between meconium isolates and dam's vaginal ones was observed in 43 cases, whereas a match between meconium isolates and dam's rectal ones was observed in 28 cases. The bacterial species isolated from the meconium and from the rectal and vaginal swabs of the dams are listed in *Table 2* and detailed in the supplementary material (S1).

Some bacterial species were exclusively isolated from the meconium. These included: Staphylococcus spp. (*S. equorum*, *S. haemolyticus*, *S. lentus*, *S. napalensis*, *S. simulans*, *S. xylosus), Enterococcus faecium*, *Macrococcus canis*, *Micrococcus luteus*, *Bacillus pumilus*, *Aerococcus viridans*, *Klebsiella oxytoca*, *Leclercia adecarboxylata*, *Acinetobacter spp. (A. lwofii*, *A. radioresistens), Enterobacter cloacae*, *Psychrobacter pasteurii*, *Pantoea* spp., *Glutamicibacter* spp.

Bacteria that were isolated only from the dams included: *Streptococcus canis* (V), *Enterococcus canis* (R), *Lactobacillus* spp. (R), *Bacillus cereus* (V), *Kurthia* spp. (V), haemolytic *E. coli* (R, V), *Klebsiella variicola* (R), *Enterococcus hirae* (R) and *Citrobacter* spp*. (R).*

Enterococcus faecalis was the most frequently isolated bacterium from the puppies' meconium and the genus *Enterococcus* was generally one of the more represented in the dams' rectum and vagina (88.9% and 55.6%, respectively).

Clostridium perfringens was isolated only in two puppies of the same ACD litter, while five dams (55.6%) harbored the bacterium (three dams in the rectum, one in the vagina, and one in both sites).

Only few bacterial species were isolated from the environmental samples (*Enterococcus faecium, Bacillus cereus,* and *Psychrobacter* spp., all with low bacterial load). Only *Psychrobacter* spp. was isolated from swabs exposed to ambient air.

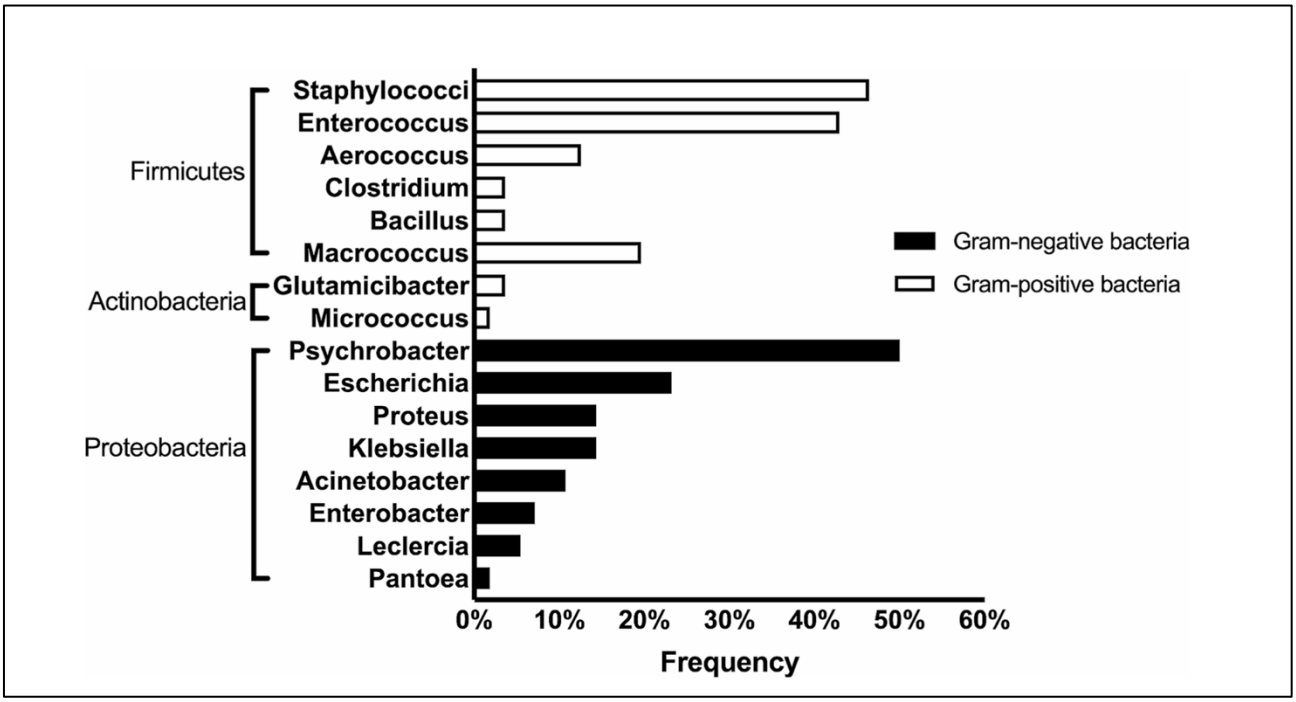

Figure 5. Frequency of bacterial genera (%) isolated from meconium samples in culture. Black bars represent Gram-negative bacteria, whereas white bars represent Gram-positive ones. For comparison with 16S rRNA sequencing results, bacterial phyla are also reported. Positive samples (N = 56) presented coagulase positive and negative Staphylococci (46.4%), *Enterococcus* (42.9%), *Macrococcus* (19.6%), *Aerococcus* (12.5%), *Clostridium* (3.6%), *Bacillus* (3.6%), *Micrococcus* (1.8%), *Psychrobacter* (50.0%), *Escherichia* (23.2%), *Proteus* (14.3%), *Klebsiella* (14.3%), *Acinetobacter* (10.7%), *Enterobacter* (7.1%), *Leclercia* (5.4%), *Glutamicibacter* (3.6%), and *Pantoea* (1.8%).

Table 2. Bacterial species identified in the samples were divided by sample origin (meconium, vagina of the dam, rectum of the dam).

	Gram-positive bacteria				Gram-negative bacteria		
	Meconium	**Vagina**	**Rectum**		**Meconium**	**Vagina**	**Rectum**
Staphylococcus	X	X	X	***Escherichia***	X	X	X
S. equorum	X			*E. coli*	X	X	X
S. haemolyticus	X			*E. coli (haemolytic)*		X	X
S. lentus	X			***Klebsiella***	X	X	X
S. napalensis	X			*K. oxytoca*	X		
S. saprophyticus	X	X		*K. pneumoniae*	X	X	
S. sciuri	X	X		*K. variicola*			X
S. simulans	X		X	***Proteus***	X	X	X
S. xylosus	X			*P. mirabilis*	X	X	X
S. aureus	X	X		***Leclercia***	X		
S. pseudintermedius	X	X		*L. adecarboxylata*	X		
Streptococcus		X		***Acinetobacter***	X		
S. canis		X		*A. lwofii*	X		
Enterococcus	X	X	X	*A. radioresistens*	X		
E. canintestini	X	X	X	***Enterobacter***	X		
E. canis			X	*E. cloacae*	X		
E. faecalis	X	X	X	***Citrobacter***			X
E faecium	X			*C. freundii*			X
E. hirae			X	***Psychrobacter***	X	X	X
Macrococcus	X	X		*P. pasteurii*	X		
M. canis	X			*P. sanguinis*	X	X	X
M. caseolyticus	X	X		***Pantoea***	X		
Lactobacillus			X	***Glutamicibacter***	X		
L. murinus			X				
Micrococcus	X						
M. luteus	X						
Clostridium	X	X	X				
C. perfringens	X	X	X				
Bacillus	X	X					
B. cereus		X					
B. pumilus	X						
Aerococcus	X						
A. viridans	X						
Kurthia		X					
K. zopfii		X					

X = positive sample.

4. Discussion

The present study investigated the seeding of the meconium microbiota in canine newborns on a population of 60 puppies belonging to nine litters. Both bacterial culture and sequencing were applied, although the latter was limited to 24 puppies belonging to four litters. Our observations confirmed the immediate colonization of the newborn gut, with a strong and characterizing maternal contribution.

The early development of the gut microbiota is believed to play a key role in perinatal life and in the future health of the individual, because the interactions between the newborn host and the commensal/symbiotic microorganisms shape the immune system and exert 'priming' effects on metabolic tracts (Neu, 2015). Investigating the association between meconium microbiota composition and puppy health requires the description of the initial microbial colonization and the later baseline intestinal microbiota profile.

'Priority effects' (i.e., the influence of microorganisms altering an ecological niche on further possible colonization by other microbial immigrants) (Sprockett *et al.*, 2018) have been hypothesized in humans, suggesting that the order of colonization by bacterial strains immediately after birth may influence the subsequent composition of the gut microbiota (Eggesbø *et al.*, 2011; Koenig *et al.*, 2011; Darcy *et al.*, 2020; Michel and Blottière, 2022). To the authors' knowledge, there is no previous studies describing the microbiota at birth, just after the exit of the newborn puppy from the vaginal canal, using molecular techniques as 16S sequencing. One study assessed the bacterial flora of the meconium of newborn puppies by culture; however, samples were not collected at birth, but after colostrum intake, adding time for bacterial colonization from multiple sources (e.g., the dam and the environment) and multiplication (Zakošek Pipan *et al.*, 2020). The sterility of meconium during fetal life has been challenged in humans and animals (Jiménez *et al.*, 2008; Del Chierico *et al.*, 2015; Collado *et al.*, 2016; Liu *et al.*, 2019; Stinson *et al.*, 2019; Guzman *et al.*, 2020; He *et al.*, 2020; Husso *et al.*, 2021; Kennedy *et al.*, 2021; Rota *et al.*, 2021; Turunen *et al.*, 2021). However, most of the studies that detected bacterial populations in human fetuses from healthy pregnancies have strong pitfalls (Kennedy *et al.*, 2023; Banchi *et al.*, 2024). Specifically, some studies in humans reported the existence of a fetal meconium microbiome, although these included naturally delivered infants (Jiménez *et al.*, 2008) or analyzed meconium samples collected from diapers within 24 hours from birth (He *et al.*, 2020; Turunen *et al.*, 2021). The time and mode of collection are critical factors to investigate meconium microbiome, because bacteria within the birth canal can colonize the newborn and collection long after delivery can alter the bacterial profile (Hansen *et al.*, 2015; Perez-Muñoz *et al.*, 2017). Moreover, the comparison between negative controls and meconium samples collected at

term during non-labored, elective caesarean deliveries using strict measures to reduce risk of contamination showed no microbiota before birth (Kennedy *et al.*, 2021). Similar preliminary observations were reported for canine and feline fetuses, both at term during elective caesarean sections (Banchi *et al.*, 2023, Chapter 4) and at mid-gestation during elective ovariohysterectomies (Chapter 5). Specifically, when strict sampling procedures are applied and multiple controls are included, the bacterial DNA detected in fetal samples is likely originating from contamination. Furthermore, the skin of the dam, even when the surgical area is rigorously scrubbed, may present fragments from dead bacteria, leading to bacterial DNA overestimation in fetal samples (Banchi et al., 2023). Detecting the exact timing of seeding is essential to deem the source of microbial populations in neonates. If the uterine environment is free from living bacteria, bacterial colonization begins during the birth process and the newborn gut is exposed to maternal flora and environmental sources.

Our results showed that the distal portion of the newborn puppy intestine is colonized by bacteria of maternal and environmental origin immediately after birth. Differently from humans, the birth canal may not be the major source of the neonatal flora in dogs, because the amniotic membrane is generally intact when puppies are born, and the bitch tears it with her teeth (Allaker *et al.*, 1992; Saijonmaa-Koulumies and Lloyd, 2002). All our observations were done on puppies that had not contact with the dam's mouth, allowing to assess the maternal (vaginal or rectal) and environmental contributions to the initial seeding. Previous observations on canine neonates showed that the entire gastrointestinal tract is colonized on the first day of life, and aerobic and anaerobic bacteria were isolated in the distal portion of the colon (Buddington, 2003). Sequencing of human meconium collected within 16 hours of delivery showed a high level of human DNA and low levels of microbial DNA (Bittinger *et al.*, 2020). In our investigation, culture revealed that bacteria of the genera *Staphylococcus*, *Enterococcus*, *Escherichia* and *Proteus* are commonly isolated; lactic bacteria (*Micrococcu*s and *Macrococcus*) were isolated less frequently. These seem to mirror the maternal microbiota, although ubiquitous/environmental bacteria (*Acinetobacter*, *Psychrobacter*) are also common. Hence, aerobic bacteria were isolated more frequently, and facultative anaerobes like *Enterobacter cloacae* and *Bacillus pumilus* and anaerobic bacteria like *Clostridium perfringens*, were only sporadically detected. In humans, Bittinger *et al.* (2020) observed an early shift in meconium composition, from facultative anaerobes (e.g., Bacilli and Enterobacteriaceae) to strict anaerobes (e.g., Bacteroides, Clostridium) 25 hours after birth. Accordingly, in dogs aerotolerant bacteria were dominant in the first day of life, but anaerobic bacteria increased in absolute and relative numbers during the following

days (Buddington, 2003). Since the present study captures the earliest moments after birth, the prevalence of aerobic bacteria is not surprising.

The role of the bacteria of the genus *Enterococcus*, one of the more represented in puppies' meconium and in dams' rectum and vagina, deserves further attention. Its prevalence in healthy newborns may suggest that bacteria belonging to this genus could be markers of a normal and balanced early gut flora.

We did not record whether puppies were born within intact amniotic sacs or if this was ruptured during parturition. When the amniotic sac tears during the passage through the birth canal, bacteria from the vagina and rectum of the dam can rapidly colonize the neonate. Conversely, when the puppy is born 'en caul' (i.e., within the intact amniotic sac), the initial colonization is likely to have stronger environmental components, also due to the assistance from the veterinarian, though wearing sterile gloves. In the present study, environmental samples yielded few bacterial species and low bacterial load, reflecting the strict hygienic conditions in which the deliveries occurred.

Even though 16S rRNA sequencing was performed only on four dam-litter units, due to costs and materials availability, our preliminary observations confirmed the early colonization of the newborn gut.

Results for beta-diversity (i.e., in-between samples diversity) suggested an relevant contribution of the maternal vaginal flora to the initial seeding of the canine neonate. Zakosek Pipan *et al.* (2020) drew similar conclusion, although puppies' meconium was sampled after the first colostrum intake and only culture-dependent techniques were used. In contrast, some studies in humans indicated that the infant pioneer microbiome resembles the fecal populations of the mother more than the vaginal ones (Mitchell *et al.*, 2020; Van Best and Hornef, 2021), albeit the colonization by vaginal microbial populations has also been reported (Enav *et al.*, 2022). Nevertheless, whatever the initial source, this is rapidly re-shaped one to three days postpartum (Enav *et al.*, 2022). The infant gut microbiome at birth shows low bacterial load, that increases during the first days of life (Yao *et al.*, 2021) along with an increase in alpha and a decrease in beta diversities (Vallès *et al.*, 2014; Laursen *et al.*, 2021). Also, Guard *et al.* (2017) reported a shift in the gut microbiota of puppies from 2 to 56 days after birth and showed modifications that included increased microbial diversity and species richness. Interestingly, in the present study alpha-diversities were not different based on the sample type. The limited number of maternal and environmental samples compared to the larger number of meconium ones, might have influenced this result.

As for the composition, meconium samples primarily consisted of bacteria belonging to the phyla Proteobacteria, Firmicutes, and Actinobacteria, confirming the results obtained through bacterial

culture, that led to the isolation of species belonging to the same phyla. Not surprisingly, sequencing revealed the presence of further bacteria, due to its higher sensitivity (Chen *et al.*, 2022). For instance, no bacteria belonging to the phyla Bacteroidetes and Fusobacteria were isolated in culture. This might be associated with the selection operated by culture media, as well as with the presence of DNA fragments from unviable bacteria.

In the study of Guard *et al.,* (2017) the phylum Firmicutes was dominant at day two, followed by Proteobacteria and Fusobacteria, with a median percent of sequences of 64.3, 12.5 and 4.5, respectively. Actinobacteria represented less than one percent of all sequences, similarly to Bacteroidetes. Our observations at birth show different proportions: Proteobacteria resulted as the most abundant phylum (n = 33), followed by Bacteroidetes (n =16) and Firmicutes (n =13). Actinobacteria and Fusobacteria were the least present (median percent of sequences = 6). Hence, we can hypothesize a very quick modification of the puppy's gut microbiota within the first 48 hours of life. However, different extraction kits, sequenced hypervariable regions, and sequencing platform may have affected the results.

A key finding of the present study is the strong similarity in microbiota among family members (i.e., within dam-litter units), more than between different families (i.e., between dam-litter units): since the dams shared the same living environment, this result suggests that the dam shapes first meconium microbiota of her litter. This strong role of the dam was recently recognized by similar result obtained using bacterial culture (Del Carro *et al.*, 2022). The dam appears to vertically transfer components of her microbiota and to give a signature to that of her litter: this finding could have clinical implications and is worth investigating in further studies and in subsequent puppies' ages and adulthood. Whether the dynamics of the microbial population throughout the pediatric period are associated with health conditions is a main area for future research.

Further investigations are also necessary to assess the effects of different factors on the microbiota of canine neonates, including delivery mode and intrapartum antimicrobial prophylaxis. Few studies were conducted on these topics only using bacterial culture (Zakošek Pipan *et al.*, 2020; Garrigues *et al.*, 2022). Furthermore, even though recent research failed to support in-utero bacterial colonization of the human (Kennedy *et al.*, 2021, 2023) and canine gut (Banchi *et al.*, 2023), it is still worth assessing whether bacterial fragments can pass from the dam to the fetus and impact future health, similarly to what was suggested in mice (Mancino *et al.*, 2019).

5. Conclusions

In conclusion, this study confirmed that the meconium of vaginally delivered puppies has its own microbiota immediately after the passage throughout the vaginal canal, and that such microbiota is shaped by the dam, even before any maternal care is provided to the puppies. Future research could investigate the role that oral maternal microbiota has on the puppies' microbiota composition. Since the dam seeds the initial bacterial populations of her litter, future research should also assess how modulating maternal microbiota affects that of the offspring, with the final aim of improving puppies' health.

Supplementary files

S1. Percentage of bacterial species identified in the samples, divided by dam and sample origin (meconium, vagina of the dam, rectum of the dam)

	A01 N=8			**A02**[1] N=9			**A05**[1] N=4			**A04**[1] N=9			**A07** N=8			**A08** N=4			**A09**[1] N=4			**A03** N=6			**A06** N=8		
	M	**V**	**R**	**M**	**V**	**R**	**M**	**V**	**R**	**M**	**V**	**R**	**M**	**V**	**R**	**M**	**V**	**R**	**M**	**V**	**R**	**M**	**V**	**R**	**M**	**V**	**R**
Staphylococcus	62.5	X		22.2	X		100	X		66.7	X	X	50.0	X		0		X	0			50.0	X		25.0		
S. equorum	0			0			0			11.1			0			0			0			0			0		
S. haemolyticus	25.0			0			0			0			0			0			0			0			0		
S. lentus	0			0			0			0			0			0			0			16.7			0		
S. napalensis	0			11.1			0			0			0			0			0			0			0		
S. saprophyticus	12.5			0	X		25.0			33.3			0			0			0			0			0		
S. sciuri	12.5			0			50.0	X		22.2			0			0			0			16.7	X		0		
S. simulans	12.5			0			0			0		X	0			0			0			0			0		
S. xylosus	0			0			50.0			11.1			25.0			0			0			16.7			25.0		
S. aureus	25.0	X		0			0			0			0			0			0			0			0		
S. pseudintermedius	0			11.1			0			0			12.5	X		0			0			0			0		
Streptococcus	0			0			0			0	X		0	X		0			0			0			0		
S. canis	0			0			0			0	X		0	X		0			0			0			0		
Enterococcus	0	X	X	33.3		X	25.0	X	X	66.7		X	87.5	X	X	25.0		X	0			33.3	X		50.0	X	X

E. canintestini	0	X	X	0			0			0			0			0			0			0			12,5		
E. canis	0			0			0			0		X	0			0			0			0			0		
E. faecalis	0	X		11.1		X	0	X	X	11.1		X	87.5	X	X	25.0		X	0			33.3	X		25.0	X	X
E faecium	0			11.1			0			0			0			0			0			0			25.0		
E. hirae	0			0			0			0			0			0			0			0		X	0		
Macrococcus	0			0			0			22.2			37.5			25.0			0			50.0	X		25.0		
M. canis	0			0			0			11.1			25.0			25.0			0			50.0			25.0		
M. caseolyticus	0			0			0			11.1			0			0			0			0	X		0		
Lactobacillus	0			0			0			0			0			0			0		X	0			0		
L. murinus	0			0			0			0			0			0			0		X	0			0		
Micrococcus	0			0			0			11.1			0			0			0			0			0		
M. luteus	0			0			0			11.1			0			0			0			0			0		
Clostridium	0		X	22.2			0			0			0		X	0		X	0		X	0	X	X	0	X	
C. perfringens	0		X	22.2			0			0			0		X	0		X	0		X	0	X	X	0	X	
Bacillus	12.5	X		0			0			0			0			0			0	X		0			12.5		
B. cereus	0			0			0			0			0			0			0	X		0			0		
B. pumilus	12.5			0			0			0			0			0			0			0			12.5		
Aerococcus	12.5			0			0			33.3			0			75.0			0			0			0		
Kurthia	0			0			0			0	X		0			0			0			0			0		
K. zopfii	0			0			0			0	X		0			0			0			0			0		
Escherichia	12.5	X	X	66.7		X	100	X	X	0		X	25.0	X	X	0		X	0		X	0	X		0	X	X
E. coli	12.5	X	X	66.7			100	X	X	0		X	25.0	X	X	0		X	0		X	0			0	X	X
E. coli (hemolytic)	0			0		X	0			0			0			0			0		X	0	X		0		
Klebsiella	25.0	X		66.7		X	0			0			0			0			0			0			0		
K. oxytoca	0			11.1			0			0			0			0			0			0			0		
K. pneumoniae	12.5	X		44.4			0			0			0			0			0			0			0		
K. variicola	0			0		X	0			0			0			0			0			0			0		

Proteus	50	X	X	11.1		0		0	X	25.0	X	X	0		0		0		X	12.5	X	X
P. mirabilis	50	X	X	11.1		0		0	X	25.0	X	X	0		0		0		X	12.5	X	X
Leclercia	37.5			0		0		0		0			0		0		0			0		
L. adecarboxylata	37.5			0		0		0		0			0		0		0			0		
Acinetobacter	12.5			11.1		25.0		11.1		0			0		0		0			25.0		
A. lwofii	12.5			0		0		11.1		0			0		0		0			0		
A. radioresistens	0			0		25.0		0		0			0		0		0			25.0		
Enterobacter	12.5			22.2		25.0		0		0			0		0		0			0		
E. cloacae	0			22.2		25.0		0		0			0		0		0			0		
Citrobacter	0			0	X	0		0		0			0		0		0		X	0		
C. freundii	0			0	X	0		0		0			0		0		0		X	0		
Psychrobacter	0			11.1		50.0	X	66.7		37.5			75.0	X	25.0	X	83.3	X		87.5		X
P. pasteurii	0			11.1		0		0		0			0		0		0			0		
P. sanguinis	0			0		50.0		0		0			0		0		83.3	X		12.5		X
Pantoea	0			0		0		0		12.5			0		0		0			0		
Glutamicibacter	0			0		0		11.1		0			0		25.0		0			0		

M: meconium samples; V: vaginal samples from the dam; R: rectal samples from the dam.
Bacteria genera are written in bold in the first column.
Meconium samples: the percentages referred to bacterial genera are calculated based on the ratio of positive puppies to litter size. The same calculation is done for the bacterial species: when not all the species belonging to a genus were identified, the sum of the percentages of the identified species is lower than the percentage of the corresponding genus.
Vaginal and rectal samples: X = positive sample.
[1]Samples of these animals were also analyzed using 16S rRNA bacterial gene sequencing.

6. Declarations

Declaration of competing interest: there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Ethical approval and consent to participate: The authors confirm that all methods were performed in accordance with relevant guidelines and regulations. The study was carried out in compliance with the ARRIVE guidelines and it was approved by the Ethical Committee of the Department of Veterinary Sciences of the University of Turin (Approval number 2200, 24/09/2019) and was performed in accordance with the EU Directive 86/609/CEE and with the guidelines of the Italian Ministry of Health for the care and use of animals (D.L. 4 March 2014 n. 26 and D.L. 27 January 1992 n. 116). Previous informed consent was obtained from the dog breeders.

Author contribution statement: Conceptualization, AR and LB; methodology, PB, BC, MC, AB, ADC, AR and LB; software, LB.; investigation, PB, AB, ADC; resources, AR and AVS; data curation, AB and PB; writing—original draft preparation, AB, PB, AR; writing—review and editing, AB, PB, AR, AVS and LB; supervision, AR, AVS and LB; project administration, AR; funding acquisition, AR. All authors read and approved the final manuscript.

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Chapter 7
Discussion

The Developmental Origins of Health and Disease (DOHaD) theory refers to a critical window of vulnerability from conception through early life, during which environmental exposures can profoundly influence long-term health outcomes (Stiemsma and Michels, 2018; Stinson, 2020; Gaillard *et al.*, 2022). Similarly, this period has emerged as crucial for the establishment and development of the gut microbiome, which is implicated in long-term health (Stiemsma and Michels, 2018; Stinson, 2020).

The host-microbiome relationship is a mutualistic symbiosis in which microbial communities perform essential functions, including nutrient digestion, immune stimulation, and protection against pathogens proliferation. While the study of individual pathogens and their association with specific diseases dominated the early history of bacteriology, the concept that microbial communities form complex ecosystems within the animal body has only emerged during the past three decades (Forum, 2013). This research field received a boost with the development of culture-independent techniques for bacterial detection, including 16S rRNA sequencing. In this context, the Human Microbiome Project, launched in 2007, enabled sequencing of the microbiomes associated with various body sites. Studies in humans were then followed by research in domestic animals, including dogs and cats (Ganz *et al.*, 2022; Suchodolski, 2022).
Accepting that bacteria physiologically inhabit the animal body and have a critical role in multiple life functions, led to challenge the dogma that the fetus develops in a sterile environment, and more questions were prompted by these novel findings.

What if a resident fetal microbiome influences the future development of the individual?

This question has prompted research in humans and animals, targeting fetal tissues to either confirm or deny the existence of a fetal microbiome, which is preliminary to the assessment of its potential effects on future health. In this frame, the present thesis describes the research path that aimed at investigating this controversy in dogs and cats.
Small animals have distinctive gestational characteristics, including an endotheliochorial placentation and multiparity. The biological reasons why this research area must be investigated in a species-dependent manner are reported in **Chapter 1**. These should be integrated with understanding how the gut microbiome influences the future health of companion animals.

According to the European Pet Food Industry Federation (FEDIAF) ('FEDIAF | Statistics'), the European pet population is increasing yearly, with 25% of European households hosting at least one dog, and 27% hosting at least one cat. This population of 231 million canine and feline pets has a significant economic impact, with pet food companies facing a 9% annual growth, and 71% of European veterinarians specializing in companion animals (Jansen, 2023).

In this context, health management of dogs and cats becomes a primary interest. Some very common gastrointestinal (e.g. canine inflammatory bowel diseases), metabolic (e.g., obesity, diabetes), and allergic diseases in dogs and cats (Kieler *et al.*, 2019; Rhimi *et al.,* 2022; Kim *et al.,* 2023; Kwong *et al.*, 2023; Sinkko *et al.,* 2023) are associated with gut dysbiosis. Therefore, a balanced and well-developed gut microbiome is a major concern in clinical practice. Early life exposures (i.e., factors to which the individual is exposed during development) have been linked to the risk of obesity and chronic enteropathy in small animals. Such exposures include birth weight, neonatal and pediatric nutrition, and early-life infections (Gaillard *et al*., 2022).
Although research is still in its early stages in dogs and cats, the recognized association between dysbiosis and adverse health outcomes later in life in humans (Stinson *et al.,* 2020) highlights the importance of determining the timing and dynamics of bacterial colonization.

Research on fetal tissues is challenging in clinical settings, since intrauterine samples must be collected, although prioritizing the well-being of the dam and the fetuses. During pregnancy, the fetus is enclosed within the amniotic sac, connected to the placenta through the umbilical cord, and located in a sealed uterine environment. Once the uterus is opened (either during cesarean section or when the cervix opens at natural birth), the risk of contamination from external bacteria becomes exceedingly high, making studying the unaltered fetal environment a real challenge.
A preliminary study (**Chapter 4**) was necessary to understand the challenges associated with sample collection, processing, and interpreting results. This, combined with a critical review of previous literature on humans and other domestic animals (**Chapter 1**), helped establishing best practices for researching the feto-maternal microbiome and refining the study design outlined in **Chapter 5**. Finally, investigating the presence of bacteria in the gut of newborn puppies before any maternal care or contact with the environment was essential to confirm that initial colonization by viable bacteria begins at birth (**Chapter 6**).

1. Setting the stage: study design *versus* reality

When the present research project started, only two studies investigating bacterial presence at birth in newborn puppies were available (Zakošek Pipan *et al.*, 2020; Rota *et al.*, 2021), whereas no literature had previously been published on kittens. Both studies applied only culture for bacterial detection. Specifically, Rota *et al.* (2021) sampled feto-maternal tissues during emergency and elective cesarean section, whereas Zakošek Pipan *et al.* (2020) included also naturally born puppies. Once the cervix opens in the first stage of parturition, ascending colonization from vaginal bacteria is possible. Therefore, studies conducted on naturally delivered newborns or those born during emergency cesarean sections cannot reliably assess the intrauterine microbiological environment during pregnancy. When designing the study in **Chapter 4**, we considered the high risk of contamination in the two published studies and chose to include only bitches and queens undergoing elective cesarean sections. This approach was similar to that used in humans by Kennedy *et al.* (2021) and in cows by Husso *et al.* (2021).

From an ethical standpoint, all the studies included in the present thesis were performed in a clinical setting, prioritizing the well-being of the dams and their puppies and kittens over the sample collection. For this reason, elective surgeries were performed only in clinically eligible cases. The majority of dams included in the study in **Chapter 4** had previously experienced dystocia, cesarean sections and/or belonged to a brachycephalic breed, whereas elective ovariohysterectomies due to unwanted pregnancies were performed only upon the owners' request and within an ethical limit of 45 days of gestation in the study in **Chapter 5**. No elective procedure was otherwise advised. However, this dramatically reduced the number of eligible animals, as majority of caesarean sections are performed to solve cases of dystocia, when parturition has already started or is imminent.

As explored in **Chapter 1**, the placenta, amniotic fluid, and meconium are representatives of the fetal environment. These tissues have been sampled in several studies in humans and animals (*Table 1* and *Table 2*, **Chapter 1**) and we decided to include them in our research. However, in the study in Chapter 4, the placenta was only swabbed on the endometrial side (i.e., the side attached to the maternal endometrium), allowing the assessment of bacteria on the surface, but not within the organ. This limitation was mitigated in the study in **Chapter 5**, as the placenta was swabbed and then the whole organ was collected for histological evaluation and FISH. Conversely, meconium collection was not possible in the study in **Chapter 5**, as the fetuses were sometimes extremely small making intestinal sampling difficult. Therefore, meconium was collected only in term puppies (**Chapters 4** and **6**). As this is not a routine clinical procedure, it may be seen as the most questionable part of the studies

included in the present thesis. However, efforts to limit any stress or pain for the newborns included the use of specific smaller 'mini' swabs and very delicate movements by the sampling operator. Both studies received ethical approval, and owners/breeders were always informed. Moreover, a useful consequence is that swabbing the rectum of neonates allowed excluding cases of *atresia ani*, which is a congenital abnormality of the rectum and anus with a 0.4% incidence in dogs, making it the sixth most frequently reported congenital malformation in puppies (Nobre Pacifico Pereira *et al.*, 2019). The mouth and anus of any puppy and kitten should always be checked for abnormalities at birth.

Opposite to humans, small animals are pluriparous species, meaning that multiple newborns are produced during the same gestation. Ideally, all the fetuses of each feto-maternal unit should be sampled, as done for newborn puppies included in the study in **Chapter 6**. This is useful to assess possible in-between litter differences, as shown by the results in **Chapter 5**, reporting some differences in bacterial culture and FISH data of fetuses belonging to the same feto-maternal unit. However, overall 16S sequencing results did not show significant differences between fetuses sharing the same uterine environment, in agreement with results obtained in mice (Winters *et al.*, 2022). Furthermore, the sample collection must be fast at cesarean section, reducing the time between extraction from the uterus and resuscitation of the neonate, to minimize any negative effect on the newborn. The fetus was extracted within the intact amniotic sac and moved to a surgically prepared sampling table, where the placenta was swabbed two times, the amniotic fluid was collected using a syringe and partially poured onto a swab, while another aliquot was dropped into an Eppendorf tube. Finally, the amniotic sac is ruptured, two 'mini' rectal swabs are rapidly collected, and resuscitation is immediately started. The whole procedure takes about one minute if the sampling operator and assistant are well coordinated. To achieve sample collection, the minimum personnel that is required includes the surgeon, the anesthesiologist, the sampling operator (wearing a surgical vest and surgical gloves), a sampling assistant opening the packaging of the sampling material, retrieving the collected samples within sealed tubes, and writing the correct identification on each tube, and at least one more assistant resuscitating the other newborns. Although newborn puppies are remarkably resistant to oxygen deprivation (Rickard, 2010), sampling all the fetuses was not possible and we decided to only include the first extracted one in the study described in **Chapter 4**. This is also the fetus that is less affected by the risk of cross-contamination during extraction by the surgeon, who touches all the fetuses wearing the same surgical gloves.

These limitations related to sampling procedures were mitigated in **Chapter 5**. In this study, the uterus was removed *en bloc* and moved to the sampling table. The pace of sampling was slower, as no

resuscitation was provided and only one sampling operator and one assistant were needed, along with the surgeon and the anesthesiologist.

2. Which technique is the best for fetal bacteria detection?

Investigating bacterial presence within the fetal environment requires highly sensitive techniques, due to the expected zero-to-low bacterial load (Kennedy *et al.*, 2023).

Bacterial culture is a key diagnostic tool in clinical practice, especially when specific culturable pathogens are investigated. The ensemble of culture-dependent techniques includes laboratory methods exploiting culture media to provide a suitable environment for certain bacteria to grow. These techniques are particularly advantageous for studying bacterial behavior, assessing antibiotic sensitivity, and isolating specific strains. In clinical cases of abortion, submitting placental samples for culture may help establish the etiology of the event (Lamm and Njaa, 2012). However, culture is limited by the fact that most bacteria do not grow in laboratory media (Kaeberlein *et al.*, 2002), including the majority of those forming complex microbiomes.

Therefore, important bacteria are missed, while the role of culturable ones may be overestimated thanks to their ability to grow in laboratory conditions. Furthermore, taxonomical identification is traditionally based on phenotypic tests (e.g., culture characteristics, Gram staining) and biochemical assays (Biswas and Rolain, 2013). However, this can lead to ambiguous results due to overlapping phenotypic and metabolic characteristics among different species. This limitation can be overcome by coupling bacterial isolation and biochemical identification with Matrix-Assisted Laser Desorption/Ionization-Time of Flight Mass Spectrometry (MALDI-TOF MS). This technique consists of ionization of bacterial proteins, measurement of their mass-to-charge ratios, and comparison of their mass spectrum with a reference database (Biswas and Rolain, 2013). This was implemented in the studies presented in **Chapters 4-6** and described in **Chapter 3**.

To solve the issue of unculturable bacteria, molecular methods targeting bacterial nucleic acids have been developed. These techniques are based on detecting specific segments of the bacterial genome and range from Polymerase Chain Reaction (PCR) to shotgun metagenomics, being the choice based on the research question. In the studies included in the present thesis, we applied 16S rRNA bacterial gene sequencing (**Chapters 4-6**, partially described in **Chapter 3**) and FISH (**Chapter 5**, described in **Chapter 3**). 16S sequencing analyzes bacterial DNA by targeting the 16S rRNA gene, which is highly conserved among bacteria, including unculturable ones. This method has a higher sensitivity for bacterial detection compared to culture and allows the description of microbiomes. It provides a

comprehensive overview of bacterial diversity within a tissue, although, importantly, 16S sequencing does not provide information on the viability of the detected bacteria. Additionally, it may miss closely related species due to the conserved nature of the 16S gene, limiting its resolution compared to techniques such as shotgun metagenomics (Durazzi *et al.,* 2021). Nevertheless, in the present research, 16S rRNA gene sequencing provided an optimal balance, offering sufficient sensitivity and specificity to detect bacterial DNA and assess microbial diversity. Techniques with higher resolution (e.g., shotgun metagenomics), require significantly more DNA, which is hardly obtained in zero-to-low biomass samples such as fetal ones (Sun *et al.*, 2022; Kennedy *et al.*, 2023).

In the study in **Chapter 5**, we implemented FISH, a culture-independent molecular technique exploiting fluorescent probes (i.e., complementary sequences of nucleotides) binding to specific bacterial DNA or RNA sequences. Samples are formalin-fixed, paraffin-embedded, and sliced for common histological preparation. Apposition onto a polarized glass slide is then followed by specific treatments, allowing the probes to link to bacterial nucleic acids, if present. A fluorescent bacterial signal is detected by observation under a fluorescence microscope. Sensitivity is higher compared to culture, extending to the detection of unculturable bacteria when generic probes are employed, but lower compared to 16S sequencing, especially for low-biomass samples. Nevertheless, the advantage offered by FISH in our research is the possibility to directly visualize the bacteria.

In summary (*Table 1*), culture is useful to identify viable, culturable bacteria, 16S sequencing has higher sensitivity for bacterial DNA detection and offers an overview of bacterial communities, whereas FISH offers direct visualization with a better understanding of contamination.

Overall, each technique has its strengths and limitations, and none is sufficient to investigate bacteria within fetal samples alone. Therefore, the best choice is to include different complementary methods.

Table 1. Main characteristics of the technique for bacterial detection used in the studies included in the present thesis.

Technique	Sensitivity for bacterial detection	Description of microbiomes	Bacterial visualization	Assessment of bacterial viability	Cost per sample
16S rRNA sequencing	High	✓	✗	✗	44.68 €
Fluorescence In Situ Hybridization	Medium	✗	✓	✗	170 €
Culture	Low	✗	✗	✓	25 €

3. Microbial DNA is present in the fetal environment, but pioneer microbiome is acquired at birth

Earlier studies by Zakošek Pipan *et al.*, (2020) and Rota *et al.*, (2021) provided foundational knowledge on the feto-maternal bacteria in dogs, reporting isolation rates of 57% and 53.3% in placental samples, and 86.5% and 80% in meconium samples of newborn puppies, respectively. Amniotic fluid was investigated only by Rota *et al.* (2021), who reported a 40% isolation frequency. However, these studies raised concerns about potential contamination from the vagina and the environment, particularly since samples were collected during natural births and emergency cesarean sections.

Recognizing the possibility of contamination, we collected samples during elective cesarean sections (**Chapter 4**). This methodological adjustment decreased the bacterial isolation frequency, being 40% for placental, 20% for amniotic fluid, and 60% for meconium samples, considering both dogs and cats. Interestingly, most isolated bacteria were common contaminants. This study was also the one in which we implemented 16S rRNA gene sequencing for the first time. This revealed the presence of bacterial DNA in both fetal samples and controls, with a similar or higher bacterial load detected in controls. This finding suggested that contamination might contribute to the observed bacterial sequences. Additionally, this analysis did not reveal any difference in bacterial composition based on the sample type (i.e., placenta, amniotic fluid, meconium, controls) but detected a statistically significant difference between canine and feline samples.

In light of these findings, the study described in **Chapter 5** was designed. This involved hysterectomy (i.e., removal of the uterus) followed by hysterotomy (i.e., opening of the uterine cavity) on a separate sampling table, rather than extracting the fetus in the surgical field (**Chapter 4**). We also included multiple sampling controls to monitor potential sources of contamination. Sampling controls were chosen based on the scenario of a common surgery in which samples were collected. Specifically, the dam was positioned on the surgical table and the incision area (abdominal region) was scrubbed with antibacterial agents (i.e., 70% ethanol, 2% povidone-iodine). The surgeon, wearing sterile gloves, retrieved the scalpel from the surgical tray, performed the first surgical incision of the skin, used clamps and scissors to detach the subcutaneous tissue from the abdominal wall, and performed a cut to access the peritoneal cavity. The uterine serosa was immediately visualized, and it was touched multiple times by the surgeon performing the ovariohysterectomy. The cervix was palpated, two clamps were applied, and the scalpel is used to cut between the two clamps isolating the uterus from the vagina. The uterus was passed to the sampling operator, who touched the uterine serosa, moved the organ on a table covered with sterile drapes, and used new surgical tools to perform a hysterotomy.

Subsequently, the first fetus was extracted within the amniotic sac, the placenta was detached from the endometrium. Sampling of the placenta and amniotic fluid followed, and the procedure was repeated for the second fetus. Therefore, we decided to collect sampling controls from the gloves of the surgeon (touching the scrubbed skin of the dam, the surgical tools, and the content of the abdominal cavity), the uterine serosa (touched by the surgeon and by the sampling operator), and the sampling table. Moreover, we collected the whole placenta to perform FISH.

With this new protocol (**Chapter 5**), culture-based methods indicated only a 12.5% bacterial isolation frequency in placental samples, while no bacteria were isolated from amniotic fluid samples. Once again, 16S sequencing results did not reveal any difference based on the sample type. As we anticipated this result, sequences detected in sampling controls were removed from the analysis. This still detected unique bacterial sequences in both placental and amniotic fluid samples and revealed differences in bacterial composition based on the feto-maternal unit. Interestingly, differences in bacterial composition were also observed in samples collected from the surgeon's gloves, potentially due to contact with the skin of the incision area. This possibly justifies the differences between canine and feline samples detected in the study in **Chapter 4**. Importantly, bacterial DNA detection in sampling controls does not imply that harmful bacteria were introduced during surgery; rather, it indicates that bacterial DNA fragments can persist on the skin and surgical tools even after standard sterilization procedures (Yap *et al.*, 2013; Calderón-Franco *et al.*, 2020). Importantly, culture results confirmed that no viable bacteria were present in any sampling control, supporting the idea that sterility, defined as the absence of living bacteria, was maintained during surgery and sample collection.

Only some placental samples were positive by culture (12.5%) and by FISH (50%), indicating the presence of few viable bacteria and low bacterial DNA. This finding is consistent with the notion that the placenta, as the primary feto-maternal interface, may act as a barrier, preventing the passage of living bacteria to the fetus while allowing some bacterial DNA fragments to reach the fetal environment. This aligns with human studies that have also identified bacterial presence in healthy placentae (Aagaard *et al.*, 2014; Seferovic *et al.*, 2019).

Therefore, our results suggest that while bacterial DNA can reach the fetus during healthy pregnancies, there is no evidence of a resident microbiome within the fetal environment during mid-gestation in dogs and cats. Instead, we are inclined to believe that the placenta acts as a checkpoint, filtering out potentially harmful elements while allowing some bacterial DNA to pass through.

The present thesis shows how we gradually improved the reliability of our results and emphasizes the importance of strict sampling procedures, as contamination may easily interfere with the interpretation of genuine results.

Findings from studies in **Chapters 4** and **5** suggested the absence of a living microbiota during healthy pregnancies. Therefore, we investigated bacterial presence in meconium samples of naturally delivered canine puppies in **Chapter 6**, confirming that a viable microbiota exists immediately after birth. Samples were collected immediately after the passage of the fetus through the vaginal canal of the dam, before any contact with the environment and any maternal care. In this study, bacteria were cultured from 93.3% of samples, which were dominated mainly by aerobic bacteria. Moreover, bacterial species cultured from meconium samples matched those isolated from the puppy's dam in 80% of cases (n = 48 out of 60 puppies), providing a higher match with vaginal samples than rectal ones (71.6% and 46.6%, respectively). The resemblance between the puppies and their dams was also confirmed by 16S sequencing, indicating similarity between a dam and her puppies, with meconium samples showing a higher resemblance in bacterial composition with vaginal samples compared to rectal ones. Accordingly, in-between litter diversity was lower compared to that between different litters.

These dynamics are valid for naturally delivered puppies and may be different in puppies born by cesarean section, as suggested in humans (Dominguez-Bello *et al.*, 2010; Liu *et al.*, 2019). We did not collect samples from the dam during cesarean sections, thus it is not possible to draw any conclusion on differences in microbial colonization based on the delivery mode in dogs.

These results show that newborn puppies have a microbiota at birth and that the dam shapes the pioneer microbiota of the puppy during birth. Similar conclusions may be hypothesized for newborn kittens, although specific research is needed.

4. General conclusions and future perspectives

The present thesis retraces the research path aiming at solving the debate on pioneer bacterial colonization in dogs and cats. This included a critical assessment of the literature on humans and domestic animals (**Chapter 1**), followed by a preliminary study revealing the challenges of sample collection and results interpretation (**Chapter 4**). Afterward, an optimized protocol unmasked the massive role of contamination in low-biomass fetal samples and proposed the presence of bacterial DNA fragments over a thriving community of bacteria during healthy canine and feline pregnancies

(**Chapter 5**). Finally, the existence of a microbiota at birth was confirmed in puppies, supporting bacterial colonization during birth mainly by the vaginal flora of the dam (**Chapter 6**) (*Figure 1*).

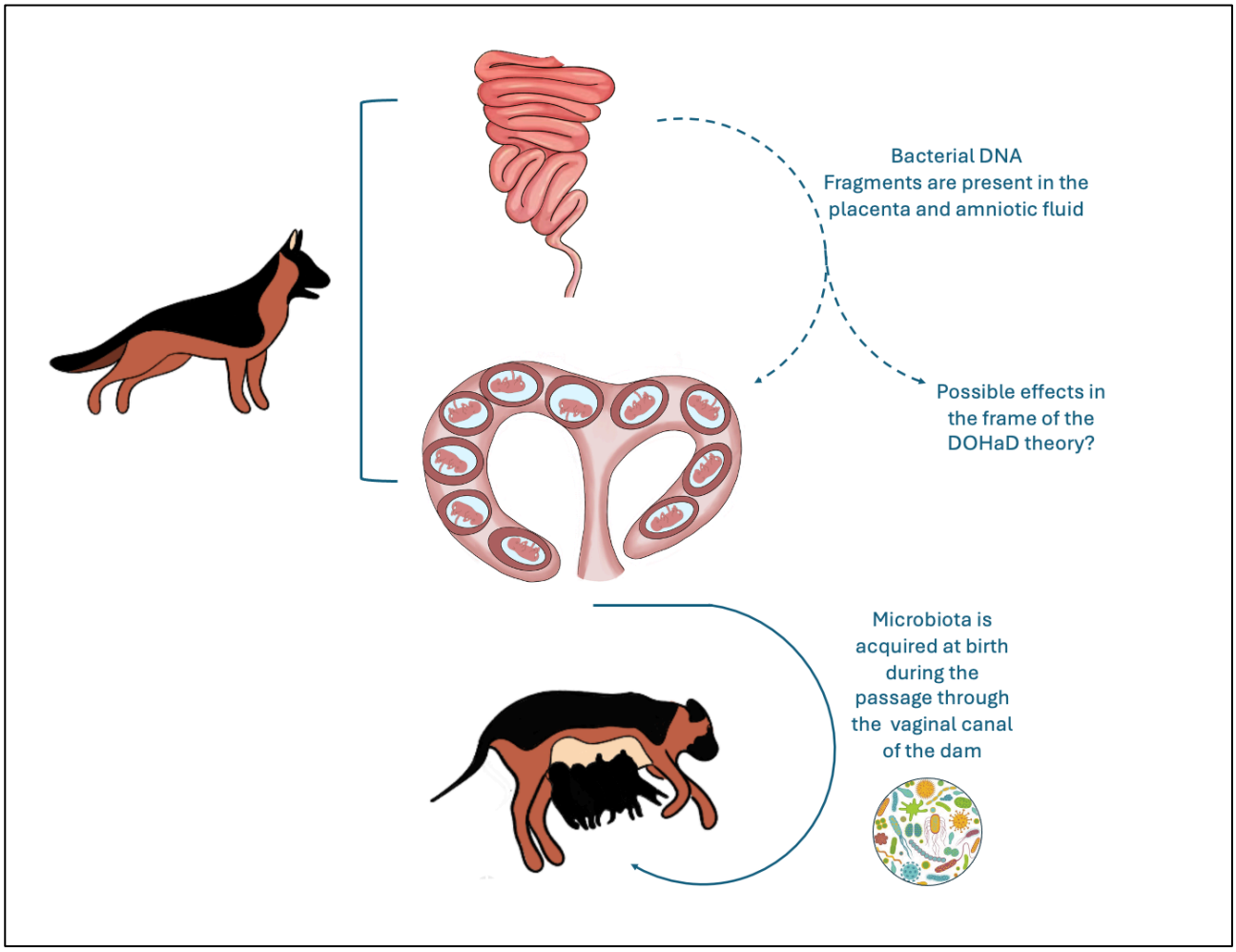

Figure 1. The figure summarizes findings on pioneer bacterial colonization of the fetus in dogs. Bacterial DNA is present in the placenta and amniotic fluid of canine fetuses, although its source and role in the frame of the DOHaD (Developmental Origins of Health and Disease) are uncertain. Pioneer bacterial microbiota is acquired during the passage in the vaginal canal of the dam in naturally born puppies.

The absence of fetal microbiota neither confirms nor refuses the role of bacteria in the DOHaD. It remains a possibility that DNA fragments and other bacterial components may affect fetal development *in utero*. The source of such components is likely the dam, as suggested by differences in bacterial composition between placental and amniotic fluid samples collected from different feto-maternal units (**Chapter 5**). Although our knowledge in dogs and cats is limited to these data, more advanced research has been performed in humans using mouse models. Specifically, a) the gut

microbiota of the dam was seen to affect gene expression profiles in her offspring (Gomez de Agüero *et al.*, 2016) and b) maternal microbiota was suggested to communicate with the fetus through microbiota-associated extracellular vesicles (Kaisanlahti *et al.*, 2023).
Briefly, these pioneering studies provide some interesting thoughts on possible mechanisms of transmission :

a) Gomez de Agüero *et al.* (2016) artificially colonized germ-free pregnant mice and compared different immune components and patterns of gene expression of their newborns with those from germ-free dams. Maternal microbial exposure during pregnancy caused extensive changes in the intestinal gene expression profile of the neonates, including genes linked to sugar metabolism, epithelial cell division and differentiation, mononuclear function, ion channels, and metabolism of dietary xenobiotics. These results were obtained through RNA sequencing of intestinal mucosal samples of 14-day-old mice.
These promising results cannot be obtained in companion animals, as germ-free animals are required and invasive sample collection (i.e., biopsies from the intestinal mucosa) would be ethically questionable in healthy neonates. However, it is hard to rely on research in species with different placental permeability. For instance, Gomez de Agüero *et al.* (2016) also checked differences in the passage of immunoglobulins, which is allowed by the thinner placental barrier in mice (i.e., hemochorial) but is very limited by the endotheliochorial placenta of dogs and cats.

b) Kaisanlathi *et al.* (2023) isolated and characterized extracellular vesicles (EVs) from amniotic fluid and fecal samples collected from pregnant women undergoing elective cesarean sections. EVs were also sequenced using 16S rRNA sequencing to investigate their bacterial origin. Finally, fluorescently labeled EVs were injected in pregnant mice and their translocation in the amniotic fluid was confirmed, indicating that microbiota-derived EVs can cross the placental barrier and reach the fetus. However, deeming whether EVs originate from bacteria or from the cells of the host is considered challenging. Often EVs derived from prokaryotic and eukaryotic cells overlap in size, morphology, protein, and lipid content (Cuesta *et al.*, 2021), and there might be cross-contamination due to the co-isolation of EVs originating from both types of cells.

We conclude that research on the source of bacterial DNA fragments detected in the fetal environment in dogs and cats is still at an embryonic stage, while knowledge of the role of such elements in the DOHaD is even less advanced. However, future perspectives are wide and promising.

This research project represented pioneer research in dogs and cats, although studies on larger populations would be interesting to confirm differences between feto-maternal units. Moreover, research conducted on naturally delivered puppies (Chapter 6), should be repeated in kittens to unveil species-specific dynamics.

Other future perspectives could include the application of probiotics. These are promising tools for gut microbiota manipulation (Hemarajata and Versalovic, 2013) and their effect could be investigated in case-control studies. For instance, changes in the dam's gut microbiota could be investigated, and related to results for microbiological and functional assessment of the placenta. Specifically, the multi-technique approach applied in **Chapter 5**, may be used on placental samples collected at cesarean sections, possibly integrating functional analyses of placental cells gene expression based on the treatment group.

Although practically difficult, longitudinal studies investigating the microbiota of the pregnant dam, that of her puppies from birth to adulthood, and the recurrence of specific diseases (e.g., obesity, diabetes, enteropathies) would help to assess whether the maternal and early gut microbiota should be considered as an exposure in the frame of the DOHaD concept.

Overall, our research added a piece to the puzzle defined as 'developmental programming' in dogs and cats by confirming the presence of bacterial DNA in the fetal environment during healthy pregnancies. Such a puzzle is far from being completed, and many questions still need to be addressed to understand possible interventions targeting the pregnant dam and improving the future health of her offspring.

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Appendix

Pioneer research on the reproductive microbiota of small animals

Chapter 1
The reproductive microbiome in dogs: Friend or Foe?

Adapted from:

The reproductive microbiome in dogs: Friend or Foe?

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1. Introduction

Bacteria inhabit the mammalian body, from the outer layers of the skin (Byrd *et al.*, 2018), the gastrointestinal tract (Cresci and Bawden, 2015), and all orifices (Wilson and Hamilos, 2014; Yamashita and Takeshita, 2017), creating real ecosystems within the organs (i.e., organ-specific microbiome). These bacterial populations contribute to the homeostasis in the body and must be safeguarded in the light of the One Health approach, aiming to balance and optimize the health of humans, animals, and ecosystems (Mettenleiter *et al.*, 2023). The inside of the body and the inner organs have been considered "sterile" for a long time (Yagi *et al.*, 2021). Although the presence of bacteria within the canine vagina has been confirmed already several decades ago by classical bacterial culture techniques (Leibold, 1954; Bjurström and Linde-Forsberg, 1992), the advent of culture-independent techniques (e.g., whole genome sequencing, 16s rRNA gene sequencing) boosted research on the microbiome of body niches, including the lumen of the reproductive tract. The description of a resident microbiome in the dog's genital tract by 16s rRNA bacterial gene sequencing (Lyman *et al.*, 2019), challenged the usual association that veterinarians make between bacterial presence and infection, and at the same time, it pointed to a possible beneficial role of microorganisms on organ homeostasis and general health (Lyman *et al.*, 2019). Nevertheless, the presence of abundant bacteria of a particular species, leading to over-proliferation and dysbiosis, and the detection of specific pathogens remains indicative for inflammation and infectious disease (Kustritz, 2006). When clinical signs of discomfort or organ dysfunction are present, proving an association between various reproductive pathologies (e.g., vaginitis, endometritis, orchitis, prostatitis) and bacterial dysbiosis is often pursued by means of microbial culture. Traditionally considered the gold standard diagnostic test in these cases and favored due to its practicality and ease of sample collection, it is also acknowledged that the results of a classical microbial culture cannot provide a comprehensive description of microbiomes. Often, when suspicion of impaired fertility exists, it can become challenging to reach a definitive diagnosis and implement appropriate treatment; this is particularly true when our knowledge of a defined standard (i.e., normal) microbiome is absent for a given species or individual.

Research on the microbiome of reproductive organs has become more important in human medicine, focusing on the bacterial populations in the genital tract of women (Kaluanga Bwanga *et al.*, 2023), in ejaculates of men (Altmäe *et al.*, 2019), and on the interactions between male and female microbiomes (Mändar *et al.*, 2015; Vodstrcil *et al.*, 2017). These studies have described the microbiome of healthy (Hou *et al.*, 2013; Mändar *et al.*, 2017) and diseased reproductive organs in

humans (Mändar *et al.*, 2017) and focused on the association between the reproductive microbiota and human fertility (Hou *et al.*, 2013; Ravel and Brotman, 2016). In the dog, which can be considered the closest companion of humans, research on the genital microbiome is still in its infancy and only a few studies have applied next-generation sequencing (NGS) techniques to describe and characterize it. This is surprising considering the impact reproductive health has on breeding performance in dogs and given the fact that any misuse of antimicrobial drugs can lead to the spread of antimicrobial resistance, both in dogs and, as for the One Health concept, in humans and the environment.

The aim of the present review is to summarize the current knowledge on the microbiome of reproductive organs in dogs, to expose the controversial role that some bacterial agents may play in canine subfertility, and to highlight future research perspectives in this field.

2. Reproductive microbiome in bitches

2.1 The vaginal microbiome: when are antibiotics necessary?

The vulva and vagina of the bitch are physiologically colonized by bacteria due to the direct and indirect contact with the skin, the rectum, and the environment (Wiebe and Howard, 2009; Groppetti *et al.*, 2012). Bacterial communities are then developed based on the chemical and physical characteristics of the skin of the external genitalia and those of the vaginal canal, with changes occurring based on the estrous cycle. Abnormal proliferation of bacteria is associated with the production of metabolites that can influence these characteristics. Similarly, abnormalities in metabolism and immunity, urinary infections, trauma, neoplasia, or malformations may lead to microbial imbalance and disease (Johnson, 1991; Hutchins *et al.*, 2013). This can manifest itself as clinically detectable inflammation (i.e., vaginitis) or subfertility/infertility.

The vagina is the site of sperm deposition during natural mating. In women, the vaginal microbiome is modulated by semen deposition. The human male and female genital microbiomes, although possibly extremely diverse, tend to equilibrate after a few sexual intercourses (Borovkova *et al.*, 2011; Mändar *et al.*, 2015). This has not yet been demonstrated in bitches, although a similar course of events would be expected. Nevertheless, the vaginal microbiome of domestic animals differs from that of women. In women, *Lactobacillus* spp. has a key role in protecting the vagina against proliferation of opportunistic bacteria (i.e., typically non-pathogenic bacteria that act as pathogen in certain circumstances) and pathogens and it is the most observed microorganism in the vagina, representing between 70 to 100% of the vaginal bacteria (Franasiak and Scott, 2015; Souza *et al.*,

2023). A decrease in the number of Lactobacilli and an increased bacterial diversity (i.e., in the vagina) has been associated with infertility and poor implantation outcomes following assisted reproductive technologies in humans (Carosso *et al.*, 2020; Fu *et al.*, 2020; Souza *et al.*, 2023). In bitches, *Lactobacillus* spp. only represent a very low proportion of the vaginal microbiota, ranging from 0.03 to 13.5% in healthy animals (Lyman *et al.*, 2019; Hu *et al.*, 2022). The vaginal microbiome of bitches is characterized by a high diversity and by different dominant genera. Studies reporting the vaginal microbiome composition of healthy bitches have limitations related to the enrolled animals, that may not fully represent the entire female canine population. Specifically, Lyman *et al.* (2019) included fifty stray bitches undergoing elective ovariectomy, whereas Hu *et al*. (2022) included only female beagles. Larger populations including different breeds and animals living in different environment are needed for a more accurate description of the reproductive microbiota. Nonetheless, the most reported bacteria were *Fusobacterium* spp. (Hu et al., 2022), *Ralstonia* spp., *Hydrotalea* spp., and *Mycoplasma* spp. (Lymann *et al*., 2019). *Ralstonia* spp. was also reported in women treated with intrauterine contraceptive levonorgestrel devices (Jacobson *et al.*, 2014). Since the structure of this molecule is similar to natural progesterone, we may hypothesize that the prolonged exposure to progesterone during canine metestrus (45-70 days) (Concannon, 2011) may play a role in selecting similar bacteria in bitches, possibly raising their relative abundance in the canine vaginal flora to 20.8% (Lymann *et al.,* 2019). Also in women, the dynamic nature of the reproductive microbiome is driven by hormonal fluctuations associated with the menstrual cycle, puberty, pregnancy, and menopause (Aagaard *et al.*, 2012; Smith and Ravel, 2017).

There is no data available on the specific composition of the microbiome in every phase of the estrous cycle in bitches, although Lymann *et al.* (2019) assessed intra-phases diversities in the vaginal and endometrial microbiome of female dogs undergoing ovariohysterectomy. Another recent study (Golińska *et al.*, 2021) used a cycle-phase approach to investigate the vaginal bacterial populations in healthy bitches by culture, whereas PCR (Polymerase Chain Reaction) was only applied for targeting microorganisms with difficult growth characteristics (i.e., slow growth, specific culture media needed). Specifically, *Mycoplasma* spp. was commonly found, showing a prevalence between 62% and 100%, without association with clinical signs of infection. Interestingly, bacteria belonging to the family *Chlamydiaceae* were never identified in these healthy bitches. Bacterial culture was also applied together with 16S rRNA sequencing to investigate differences in the vaginal microbiome of intact bitches in anestrus and spayed ones (Rota *et al.*, 2020). Although no significant difference was found between the two groups, *Mycoplasma* spp. was again commonly sequenced, whereas less

demanding bacteria were also isolated in culture, mirroring the results of (Golińska *et al.*, 2021). Specifically, these studies suggest that *Staphylococcus* spp. (especially *S. pseudointermedius*), *Streptococcus* spp. and some Gram-negative bacteria are the main culturable vaginal microorganisms in healthy bitches (Rota *et al.,* 2020; Golińska *et al.,* 2021). However, sequencing techniques show how culture overestimates the role of easily-culturable microorganisms at the expense of other bacterial species that may contribute to health, defense, or even to the etiopathogenesis of some reproductive diseases. Based on the applied technique, culture-independent methods can either target specific bacteria (e.g., PCR targeting *Mycoplasma* spp.) or provide a full picture of the microbiome in terms of composition and abundance (e.g., 16S rRNA bacterial gene sequencing).

2.2 The uterine microbiome: a cause of infertility in bitches?

While it is well established that the lower reproductive tract of the bitch is physiologically colonized by commensal bacteria (i.e., normal microbiome inhabiting the mucosal surfaces), there have been doubts about the presence of bacteria within the healthy uterus (Olson and Mather, 1978). Bacterial endometrial colonization has been confirmed later by culture (Watts *et al.*, 1996) and the uterine microbiome has been recently described by 16S rRNA sequencing (Lyman *et al.*, 2019). Furthermore, this organ was believed to be sterile during pregnancy as a prerequisite to successful birth of healthy neonates. Although research on the intrauterine colonization of the fetus challenged the 'sterile womb paradigm' (Perez-Muñoz *et al.*, 2017), the debate is still firing in humans (Aagaard *et al.*, 2014; Willyard, 2018; Zakis *et al.*, 2022; Banchi *et al.*, 2023; Kennedy *et al.*, 2023). There is at present no evidence of the passage of living microorganism to the canine fetus during gestation (Banchi *et al.*, 2023). In bitches, the cervix is patent in proestrus and estrus, favoring ascending colonization of the uterus by environmental bacteria via the vaginal canal, as well as permitting the introduction of bacteria during mating. The high blood progesterone during metestrus is then responsible for the closure of the cervix, for the proliferation and increased secretion of endometrial glands, and for a decrease in local immune defenses (Hagman, 2023) in the light of the steroidal nature of this hormone. In these instances, the presence of an established normal microbiota will play a key role in competing with pathogenic and opportunistic bacteria. Absence of a normal microbiota will increase the risk of developing a uterine disease in cows (Adnane and Chapwanya, 2022) and, in women, it will increase the likelihood of *Chlamydia trachomatis* or *Mycoplasma* spp. establishing uterine infections (Molenaar *et al.*, 2018). Nevertheless, the uterine microbiome is known to be less abundant (absolute abundance being defined as the number of bacteria in a specimen) compared to that of the vagina and external genitalia in women as in animals (Simon, 2018; Adnane and Chapwanya, 2022), and research

is limited by the low biomass of the endometrial microbiome (Mogheiseh *et al.*, 2020; Vanstokstraeten *et al.*, 2022). In bitches, collecting uterine samples can easily be performed during or immediately after ovariohysterectomy in non-breeding dogs (Maksimović *et al.*, 2012; Lyman *et al.*, 2019; Praderio *et al.*, 2019). However, spaying of healthy animals is more frequently performed in young bitches for reproduction control (Lyman *et al.,* 2019) than in older healthy bitches. This represents an inclusion bias limiting research on the normal uterine microbiome of older and breeding bitches. Furthermore, clinicians often prefer to perform ovariectomy over a more invasive ovariohysterectomy if the uterus does not present any macroscopic alteration (Van Goethem *et al.*, 2006). Transcervical uterine flushing with sterile saline solution has also been reported for bacteriological investigation in bitches (Mogheiseh *et al.*, 2020) (*Figure 1*), although a possible increased risk for pyometra has been reported when the procedure is performed in metestrus (Fontaine *et al.*, 2009; Christensen *et al.*, 2012). Other sampling techniques that are routinely used in women and large animals (e.g., endometrial swabs), are not feasible in breeding bitches, due to the high sensitivity of the endometrium to irritating stimuli (Watts and Wright, 1995). These sampling and technical limitations, together with reduced animal cohorts, make research on the endometrial microbiome challenging, especially in breeding bitches.

An altered endometrial microbiome was found in cows (Machado *et al.*, 2012; Miranda-CasoLuengo *et al.*, 2019) and in women (Liu *et al.*, 2019; Lozano *et al.*, 2021) diagnosed with endometritis. Endometritis has been proposed as a possible cause of reduced fertility in bitches (England *et al.*, 2021; Pascottini *et al.*, 2023). The association between endometritis and the uterine microbiome should be further investigated to understand their mutual relationship, and to determine if an impaired microbiome could lead to endometritis, or if the endometrium could be damaged by dysbiosis. Current knowledge on the healthy endometrial microbiome is that it differs from that of the vagina, presenting higher diversity (i.e., variety of species in a population) and evenness (i.e., relative differences in the abundance of various species in a population) (Young and Schmidt, 2008; Lyman *et al.*, 2019). Whereas culture-based studies reported isolation rates ranging from 3.8% (Olson and Mather, 1978) to 62.5% (Maksimović *et al.*, 2012), sequencing techniques revealed a characteristic microbiome, dominated by genera such as *Pseudomonas*, *Staphylococcus*, and *Campylobacter* (Lyman *et al.,* 2019).

Factors such as nutrition, living environment, sexual habits, phase of the cycle, medical treatment, pregnancy (Aagaard *et al.,* 2012), and disease can influence the female reproductive microbiome in women (Amabebe and Anumba, 2018) as in cows (Adnane and Chapwanya, 2022). The microbiome may interfere with fertility and could potentially serve as biomarker for pregnancy outcomes when

selecting bitches for breeding. For these reasons, the female reproductive microbiome may represent a prosperous field for future research in canine reproduction.

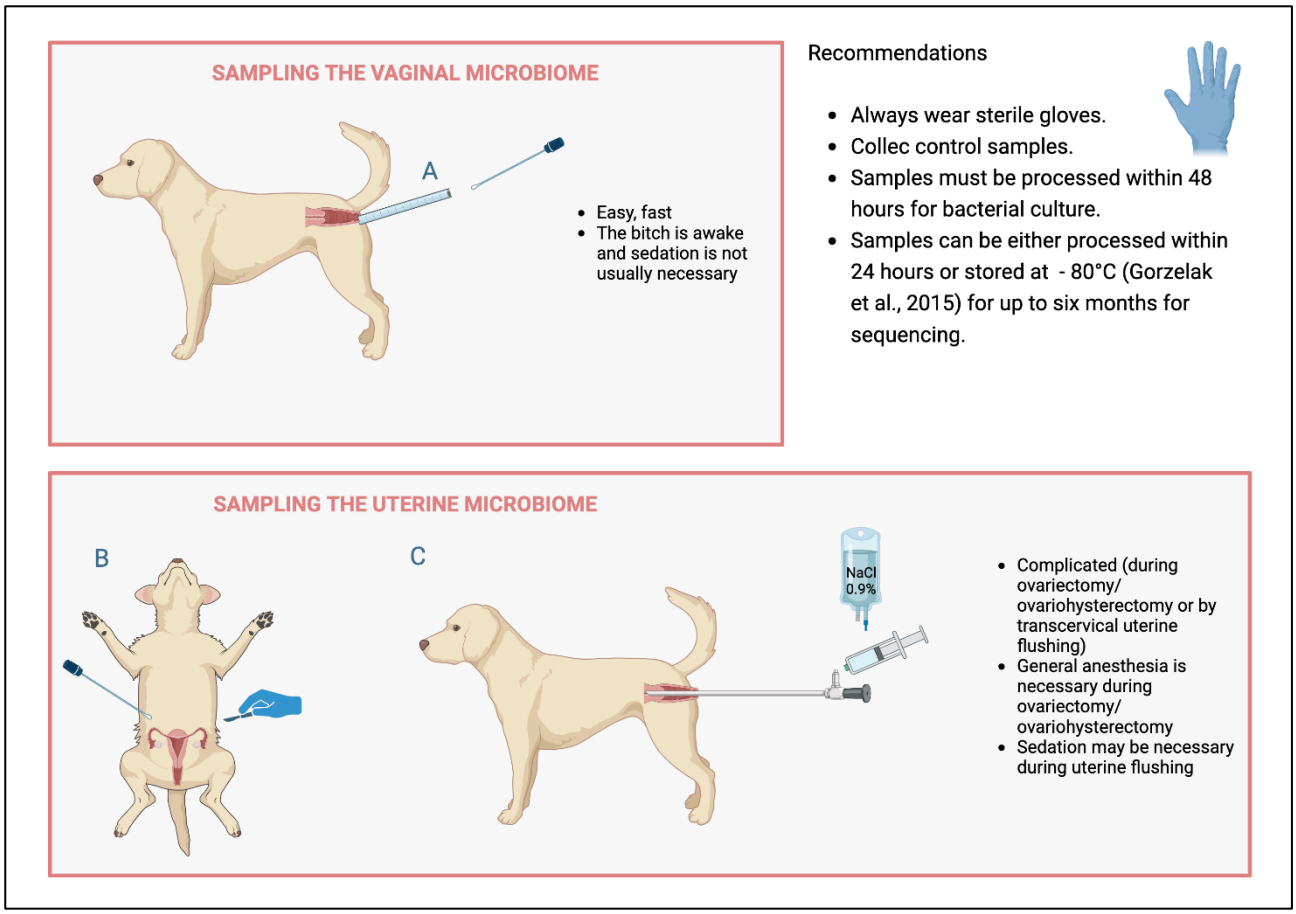

Figure 1. A schematic overview of the techniques currently available to sample the vaginal (A) and uterine (B) microbiomes including recommendations for correct sampling. To sample the vaginal microbiome the use of a sterile speculum or tube (A) is recommended to guide the swab through the vaginal canal and reach the cranial vagina. The uterine microbiome can be sampled during ovariectomy/ovariohysterectomy procedures (B) or by flushing the uterine lumen with sterile saline solution (C), although an increased risk for subsequent uterine infection has been reported. This representation was created with BioRender.com and it does not depict the reproductive organs of the bitch in an anatomically correct way.

3. Reproductive microbiome in male dogs

Research on the reproductive microbiome of male dogs is extremely scarce compared to that in humans and other domestic animals. Studies on the microbiome of the semen, prepuce, prostate, and testes have increased dramatically in humans and animals in recent years, following the introduction of NGS techniques (Weng *et al.*, 2014; Zhang *et al.*, 2020; Cojkic *et al.*, 2021; Quiñones-Pérez *et al.*, 2021; Koziol *et al.*, 2022; Mocé *et al.*, 2022; Magill and MacDonald, 2023; Malaluang *et al.*, 2023).

Nevertheless, only one study in dogs using culture-independent methods has been recently published by some of the authors of the present review (Banchi *et al.*, 2024, Appendix, Chapter 2). It is estimated that human infertility affects about 15% of couples, half of the time in association with male factors (Weng *et al.*, 2014; Brandão *et al.*, 2021). Such assessment is difficult in animals, as breeding pairs are changed frequently. Nevertheless, identifying the etiology of subfertility in domestic male animals is problematic (Rittenberg and El-Toukhy, 2010; Fontbonne *et al.,* 2011; Domosławska and Zdunczyk, 2020). Male subfertility can be caused by a variety of hormonal, metabolic, inflammatory, infectious, genetic, neoplastic, and toxic conditions, leading to a decrease in semen quality. While infectious diseases of various reproductive organs have been extensively documented for many years already (Doig *et al.*, 1981; Phongphaew *et al.*, 2021; Skorupski *et al.*, 2022), the role of the resident genital microbiome on reproductive function has been suggested only recently (Weng *et al.*, 2014; Monteiro *et al.*, 2018; Baud *et al.*, 2019).

Research on the male reproductive microbiome is primarily conducted on the ejaculate, as collection is routinely performed during fertility examinations in humans as in domestic animals. In dogs, semen is collected by digital manipulation, which is a simple, non-invasive procedure. Traveling along the male reproductive tract, semen can potentially convey bacteria colonizing different organs. For this reason, the origin of the semen microbiome is hard to define. Intuitively, bacteria from the external environment could colonize the prepuce, ascend via the urethra, eventually reaching the accessory sex glands (the prostate in dogs) and testes. Being ejaculated in three different fractions, dog semen contains more bacteria in the pre-spermatic one (i.e., the first fraction that is ejaculated), compared to the sperm-rich and prostatic ones (i.e., the second and third ejaculated fractions, respectively) when culture-dependent methods are applied (Goericke-Pesch *et al.*, 2011). This is not surprising, as the pre-spermatic fraction flushes the urethra, potentially removing bacteria and other contaminants. The sperm-rich and prostatic fraction may better mirror the prostatic and testicular environments, and they were reported to have similar bacterial composition in healthy stud dogs by 16S rRNA sequencing (Banchi *et al.*, 2024). Prostatic inflammatory processes may alter the microbiome of the ejaculate, although this has never been investigated in dogs. The prostate of men has been demonstrated to host a characteristic microbiome that is modelled based on the nature of the tissue (Cavarretta *et al.*, 2017). For instance, the tumoral and peritumoral prostatic tissue host a different microbiome compared to non-tumoral tissue in humans (Cavarretta *et al.*, 2017). Whereas prostatic cancer is relatively uncommon in dogs, and it mainly affects castrated animals (Schrank and Romagnoli, 2020; Iizuka *et al.*, 2022), benign prostatic hyperplasia (BPH) is one of the most common conditions in intact dogs

older than 4 years (Christensen, 2018; Ruetten *et al.*, 2021). Specifically, the prevalence of this para-physiological development of the prostate is higher than 80% in dogs older than 6 years (Schrank and Romagnoli, 2020). Although BPH can be subclinical, if left untreated, it may progressively lead to serious clinical signs as tenesmus, dysuria, urinary incontinence, anorexia, and pain, and it has been recognized as a predisposing factor to prostatitis (Niżański *et al.*, 2014). Hence, a comparison of the prostate microbiome in healthy dogs and animals with prostatic hyperplasia could reveal if the genitourinary microbiome is associated with BPH, as previously shown in men (Miyake *et al.*, 2022). Nevertheless, direct sampling of the prostatic gland can only be performed via blind or ultrasound-guided fine needle aspiration or biopsy or during laparotomic/laparoscopic procedures (Kustritz, 2006; Lévy *et al.*, 2014; Thiemeyer *et al.*, 2019) The first techniques are associated with a high risk of contamination from the gut microbiome, whereas the latter can only be performed when prostatic disease is already severe (i.e., large prostatic cysts, prostatic abscesses, prostatic tumors) (Powe *et al.*, 2004; Lévy *et al.*, 2014). Hence, sampling the prostatic fraction of the ejaculate may be an interesting alternative, although some bacteria exclusively colonizing the urethra may also be found. To limit this risk, sampling of the urethra before semen collection can be performed and the microbiome can be compared with that of the ejaculate (Ling *et al.*, 1990), although this has never been done in male dogs using culture-independent techniques.

The same idea can be applied to the testicular/epididymal microbiome, since spermatozoa are produced within the testes, mature along the epididymides, and sperm originating from both the right and left sides are ejected together within the second fraction of the ejaculate. Although testicular and epididymal microbial populations may contribute to the semen microbiome, research in men showed that the testicular microenvironment is characterized by a low bacterial biomass (Molina *et al.*, 2021). This has never been assessed in dogs, although tissue samples can be easily collected when orchiectomy is performed for reproduction control or surgical therapy following the diagnosis of testicular disease. Future research should clarify the role of the seminal and testicular microbiome in dogs using NGS techniques, paralleling research in men. Differences in the human testicular microbiome were found between individuals diagnosed with non-obstructive azoospermia (Alfano *et al.*, 2018), as the seminal microbiome of infertile men differs from that of fertile individuals (Gomes *et al.*, 2023). Similarly, the seminal microbiome can influence spermatozoa kinematics in stallions (Quiñones-Pérez *et al.*, 2021), may be related to seminal parameters in bulls (Cojkic *et al.*, 2021; Koziol *et al.*, 2022), and showed an association with the season in boars (i.e., winter versus summer) (Zhang *et al.*, 2020) and bucks (reproductive season versus anestrus) (Mocé *et al.*, 2022).

In twenty healthy stud dogs, the application of culture-independent techniques (i.e., 16S rRNA sequencing) revealed that the living environment influences the semen microbiome (Banchi *et al.,* 2024), as suggested by a recent study in horses (Malaluang *et al.*, 2024). The overall composition of the microbiome of the second and third fraction of the canine ejaculate included Proteobacteria, Firmicutes, and Actinobacteria as dominant phyla, similarly to that in men (Mändar *et al.*, 2017; Baud *et al.*, 2019). Nevertheless, further research is needed to assess differences in the microbiome of the ejaculate based on individual factors (e.g., breed, age, fertility outcomes) and reproductive conditions (e.g., BPH, azoospermia, testicular atrophy/degeneration/neoplasia, and inflammatory processes). Undeniably, next-generation sequencing increased the depth and coverage of research on microbial communities (Contreras *et al.*, 2023), although careful sampling using sterile techniques is needed to limit the risk of contamination and obtain reliable results. The sensitivity of NGS techniques is increased by the amplification of the bacterial genetic sequences. Nevertheless, both sequences belonging to the real samples and those belonging to contaminants are amplified, increasing the risk for unreliable results (Molina *et al.*, 2021). Specifically, when semen samples are analyzed, the amplification of sequences belonging to environmental and preputial contaminants is undesirable. This phenomenon could be limited by careful sampling done by operators wearing gloves, cleaning the prepuce before collection, using sterile funnels, and transferring the semen using sterile pipettes and tips into PCR-clean (i.e., DNase-, RNase-, and PCR inhibitor-free tubes).

3. Controversies on the reproductive microbiome

4.1 Mycoplasma and Ureaplasma spp.: enemies or scapegoats?

In recent years, bacteria belonging to the genera *Mycoplasma* spp. and *Ureaplasma* spp. have emerged in conjunction with many fertility issues in dogs. Although they are often opportunistic bacteria, these microorganisms are perceived as agents for sexually transmissible disease and are considered a threat to fertility in dogs by many breeders. Therefore, a common scenario in veterinary practice is that dog owners request bacteriological investigation in their breeding animals, sometimes specifically targeting mycoplasmas. Although it has been recognized that *Mycoplasma* spp. and *Ureaplasma* spp. may cause reproductive infections and play a role in fertility impairment (Cheng *et al.*, 2023; Domrazek *et al.*, 2023; Zheng *et al.*, 2023), these bacteria are common inhabitants of the reproductive tract in dogs (Doig *et al.*, 1981; Kustritz, 2006; Domrazek *et al.*, 2023) and their association with subfertility remains unclear (Fontbonne, 2020). Nonetheless, vertical transmission of Mycoplasmas via the birth canal has been reported in humans, possibly colonizing the newborn, and causing

neonatal disease, which may require immediate treatment (Waites *et al.*, 2005). These small bacteria lack a cell wall and preferably adhere to the mucous surfaces of the respiratory and genito-urinary tracts. Some species also developed the ability to colonize host cells, thereby escaping immune reactions and maintaining a parasitic relationship. Therefore, they are believed to cause indirect chronic inflammatory damage more than a direct toxic effect (Spergser, 2018).

An affinity between *Mycoplasma* species found in humans and dogs has been hypothesized, due to cohabitation within the same environment (Domrazek *et al.,* 2023). However, the occurrence of these microorganisms is more common in canine reproductive tissues than in humans (Domrazek *et al.,* 2023). This is not surprising since the environmental pH influences the growth of mycoplasmas (i.e., optimal pH 7.8-8) (Ren *et al.*, 2020), and this factor differs considerably between human and canine reproductive organs. With values between 7.4 and 8.0 (Antonov, 2014), the vagina of bitches represents an ideal growth environment for mycoplasmas, as opposed to women whose vaginal environment is characterized with a more acid pH (3.8 - 4.2) (Domrazek *et al.*, 2023). The semen of male dogs is slightly more acid compared to the vaginal environment in bitches, with pH values between 6.3 and 7.2, varying among the different fractions of the ejaculate (Berezovsky, 2015). The high prevalence of *Mycoplasma* spp. and *Ureaplasma* spp. in males and females is accompanied by low sensitivity in species identification. In humans, only some species were found to be associated with fertility problems, due to the variable inflammatory potential (Cicinelli *et al.*, 2014; Cheng *et al.*, 2023). In dogs, *M. canis* has been isolated from the reproductive organs of infertile animals (Spergser, 2018), whereas *M. spumans* and *M. maculosum* have been detected by 16S-23S rRNA sequencing in vaginal swabs collected from infertile Bernese Mountain dogs bitches (Tamiozzo, 2022) and *U. canigenitalium* has been associated with infertility in male dogs (Spergser, 2018). Furthermore, experimental infection with *M. canis* was shown to cause inflammation of the epididymis, prostate, and endometrium (Spergser, 2018). Nevertheless, these may not be the only species with pathogenic potential in dogs, although in-depth studies are lacking (Domrazek *et al.,* 2023).

Species-identification is extremely important to unravel the contribution of mycoplasmas to reproductive disease in dogs. All studies performed by sequencing in bitches (Lymann *et al.,* 2019; Rota *et al.,* 2020; Hu *et al.,* 2022) reported the presence of *Mycoplasma* spp. or Tenericutes (i.e., the taxon including the genus Mycoplasma) as normal components of the vaginal microbiome, depending on the taxonomic depth of the analysis, although never achieving species-level identification. Canine mycoplasmas present high similarities at 16S rRNA gene-level, making correct diagnosis by PCR and sequencing challenging (Chalker, 2005). Growth in culture is slow and has specific requirements

(Okella *et al.*, 2023), making isolation possible only when mycoplasmas are specifically targeted. Moreover, definitive species identification in culture requires further investigation by serological techniques, PCR-based methods (Spergser and Rosengarten, 2007), or MALDI-TOF MS (Matrix-Assisted Laser Desorption Ionization-Time Of Flight Mass Spectometry) (Pereyre *et al.*, 2013).

A cue for further research is the quantification of mycoplasma species within reproductive samples, as quantification of *Mycoplasma pneumoniae* by PCR was performed in human patients with respiratory infection, revealing a positive correlation with disease severity (Zhao *et al.*, 2023). Although this has never been tested in dogs with reproductive disease, it may represent a useful tool for monitoring the efficacy of antimicrobial treatments (Zhao *et al.*, 2023).

From a practical perspective, testing dogs for the presence of bacteria belonging to the *Mycoplasma* group is probably overestimating the role of these microorganisms in association with infertility, leading to exclude healthy animals from breeding programs or exposing them to unnecessary antimicrobial treatments. This practice may also cause an imbalance in the healthy microbiome of reproductive organs. This authors' recommendation would be that testing at species level should either be performed on all breeding dogs with the aim to conduct thorough epidemiological studies, or be limited to animals presenting poor conception rates, embryonic or fetal resorption, abortion, stillborn puppies, weak or dead neonates in their litters (Pretzer, 2008). Furthermore, as for any vaginal bacteriological investigation, samples should be collected from the cranial vagina, as the vestibulum may be contaminated with mycoplasmas causing urinary infections (Jang *et al.*, 1984). Communication with dog breeders and client education are key to avoid an overestimation of the role of these opportunistic bacteria, possibly missing the real cause for subfertility/infertility, and thus introducing an imbalance in the reproductive flora by inadequate antibiotic treatment, and as such inducing further health complications.

4.2 Antimicrobials and fertility: from hero to zero

Antibiotics have been considered fundamental to treat bacterial infections since penicillin was discovered almost 100 years ago (Fleming, 1929). Nevertheless, the World Health Organization has estimated that in approximately half of the cases, antibiotic use is inappropriate in humans (Zanichelli *et al.*, 2023). Awareness of antimicrobial drugs prescription and use is increasing in human as well as in veterinary medicine due to the emergence of antimicrobial resistance (Weese *et al.*, 2006). From the One Health perspective, the consequences of this phenomenon include severe risks for animals and humans, as they share the same living environment (Weese *et al.*, 2006; Boost *et al.*, 2007). Moreover, Regulation EU 2019/6 on Veterinary Medicinal Products discourages the preventive use

of antimicrobials, that can be administered only following veterinary assessment (Simjee and Ippolito, 2022). Nevertheless, in companion animals, it has been reported that broad-spectrum antimicrobials are often empirically prescribed (Guardabassi *et al.*, 2008), accelerating the spread of antibiotic-resistant bacterial strains.

The causes for infertility in dogs often remain undiagnosed (Fontbonne, 2011), leading to frustration and to unnecessary prescription of antimicrobials to comply with client requests (Frey *et al.*, 2023). In this regard, veterinarians should take responsibility and act in the best interest of the animal, acknowledging that insisting requests should not impact their clinical decisions. Whereas treatment with broad-spectrum antibiotics may be necessary in emergency situations (Hagman, 2023), this is not the appropriate practice for infertility management. Perhaps with the exception of *Brucella* spp., bacteria causing infertility in dogs rarely represent an impending risk for the health of humans and animals. All the other bacteria involved in reproductive conditions rarely cause disease in the absence of predisposing factors such as viral infections, malformations, mucosal alterations, immune-mediated conditions, stress, and endocrine diseases (Allen-Durrance *et al.*, 2022; Hagman, 2023). These predisposing factors should be identified and controlled to avoid recurrence (Weese *et al.*, 2015). Therefore, antimicrobial therapy should be implemented only in clinically evident cases of a bacterial infection or in cases when all the possible causes of subfertility/infertility have been excluded and culture is suggestive of dominance or dysbiosis of microorganisms that have been shown to impair fertility. Furthermore, the choice of the drug should always be based on the efficacy and the results of an antimicrobial-resistance assay (Guardabassi *et al.*, 2008). Communication with owners and breeders is once again key to avoiding antimicrobial misuse (Frey *et al.*, 2023) in canine reproduction. The veterinarian should convey information about the presence of a characteristic microbiome, each playing a role in maintaining the environmental characteristics of reproductive organs, preventing colonization from potential pathogens, and preventing opportunistic bacteria to over-replicate and prevail. Moreover, the detrimental effect of certain antimicrobial molecules on fertility has been recognized (Zdunczyk and Domoslawska, 2022). For instance, prolonged treatment with tetracyclines may lead to testicular atrophy and oligo-/azoospermia in dogs, whereas treatments with other molecules showed no significant effect on semen quality (Zdunczyk and Domoslawska, 2022). Nevertheless, further research to assess the effect of antibiotics on canine fertility is needed, and to research possible alternatives to antimicrobials.

4.3 Antibiotics and assisted reproductive technologies.

The use of antimicrobials is not limited to clinical settings for the treatment of reproductive pathologies, but it is also applied to increase the success of Assisted Reproductive Technologies (ARTs). In dogs, the use of antibiotics for this purpose is limited to their addition into extenders intended for chilled or frozen sperm preservation. Semen extenders are solutions in which the sperm are resuspended after the removal of the seminal plasma to improve preservation and protect from temperature- and osmotic pressure-related damage. Moreover, the presence of antimicrobials aims to prevent bacterial overgrowth during processing and preservation (Santos and Silva, 2020), as this phenomenon is associated with sperm agglutination, plasma membrane disruption, acrosome damage, and overall motility and viability in other species (Yániz *et al.*, 2010; Stankiewicz *et al.*, 2016). Bacteria may originate from the ejaculate itself or from collection procedures (Bussalleu and Torner, 2013). In canine extenders, the most prevalent molecules are gentamycin, tylosin, lincomycin, and streptomycin with concentrations ranging from 50 to 1200 μg/mL (Santos and Silva, 2020). A commonly used canine semen extender contains a combination of Na-benzylpenicillin and streptomycin sulphate (Linde-Forsberg, 1991; Rota *et al.*, 1995; Peña and Linde-Forsberg, 2000). β-Lactams may not be effective in controlling post-thaw bacterial growth alone (Barbosa *et al.,* 2010), for instance they would not allow preventing the possible proliferation of mycoplasmas in preserved semen, which display a natural resistance towards this class of antimicrobials.

Recently, alternatives to antimicrobials in semen preservation technologies have been investigated to avoid their possible toxic effect on sperm cells (Morrell and Wallgren, 2014) as well as the spread of antimicrobial resistance, as even antimicrobials in semen extenders have been reported to alter antimicrobial resistance patterns in inseminated mares (Malaluang *et al.*, 2023). These include antimicrobial peptides (Bussalleu *et al.*, 2017; Puig-Timonet *et al.*, 2018; Shaoyong *et al.*, 2019), physical methods such as single-layer centrifugation and microfiltration (Morrell and Wallgren, 2011; Morrell *et al.*, 2014, 2019; Barone *et al.*, 2016), and other natural or synthetic substances, such as aloe vera gel, iodine methionine, and Kojic acid (Brito et al., 2014; Fang *et al.*, 2017; Shaoyong *et al.*, 2019). Once again, studies are limited to farm animals and horses, but this topic also represents an interesting area for future research in dogs. Other than testing alternatives to antimicrobials in canine semen, researchers should also unravel the possible impact of extenders containing antibiotics on the reproductive microbiome and resistome (i.e., a collection of all the antibiotic resistance genes and their precursors in pathogenic and nonpathogenic bacteria) (Wright, 2007) of bitches.

4.4 Probiotics as promising but understudied tools in canine reproduction

Novel approaches to subfertility include the use of probiotics to improve reproductive performance (Feng and Liu, 2022; Darwish *et al.*, 2024). Probiotics are living microorganisms with beneficial effects on the microbiome and its homeostasis (Mahiddine *et al.*, 2023). They alter the microbiota composition in terms of abundance and diversity, influence the metabolism of hormones, lipids, and glucose, modulate the immune function, and modify the permeability of epithelial barriers (Feng and Liu, 2022).

In females, probiotics may be an alternative therapeutical tool to prevent opportunistic bacteria overgrowth, as reported in humans (Reid *et al.*, 2013; Helli *et al.*, 2022) and in in vitro studies in dogs (Edyta *et al.*, 2023). Furthermore, probiotics were shown to influence testosterone levels in male rabbits (Attia *et al.*, 2013), sperm oxidative stress in men (Aitken *et al.*, 2014), and spermatogenesis in mice (Poutahidis *et al.*, 2014).

In dogs, in vitro studies suggested a positive effect of lactic acid bacteria, including some belonging to the genus *Lactobacillus,* on the urogenital microbiome of bitches, as these bacteria effectively inhibited the growth of opportunistic pathogens such as *Escherichia coli*, *Proteus mirabilis*, and *Staphylococcus aureus* (Delucchi *et al.*, 2008; Edyta *et al.*, 2023). An in vivo trial on thirty-five healthy bitches showed that prolonged (i.e., two to four weeks) oral supplementation with lactic acid-producing bacteria belonging to the genera *Lactobacillus*, *Bifidobacterium*, and *Bacillus* did not increase their isolation from canine vaginal samples after culture (Hutchins *et al.*, 2013). However, the use of sequencing techniques revealed that the relative abundance of *Lactobacillus* spp. was increased following three days of vaginal administration of *L. rhamnosus GR-1* and *L. reuteri RC-14* in women (Bisanz *et al.*, 2014). The difference in vaginal pH between humans and dogs may be responsible for the better growth of Lactobacilli in women compared to bitches. Furthermore, in women and cows the local administration of lactic acid bacteria was suggested to be more effective than oral supplementation (Chandrashekhar *et al.*, 2021; Rosales and Ametaj, 2021). Further studies using culture-independent approaches would be useful to compare the effect of the oral and local supplementation of probiotics in bitches.

Probiotics may also act indirectly on reproductive functions by reshaping the gut microbiome (Mahiddine *et al.,* 2023). Intestinal resident bacteria produce a wide variety of metabolites affecting the microenvironment that they colonize, thus contributing to several physiological functions,

including gastroenteric regulation (Hasiri *et al.*, 2015) and spermatogenesis (Cai *et al.*, 2022). A study in dogs investigating this mechanism involved the dietary supplementation of *Lactobacillus rhamnosus* in combination with a prebiotic (i.e., a compound that exerts a positive effect on the growth and activity of microorganisms) in six Beagle dogs for six weeks (Mahiddine *et al.,* 2023). Although supplementation lasted less than a full spermatogenic cycle (i.e., about 60 days) (De Souza *et al.*, 2021), the authors reported enhanced gut microbiome diversity and improvements in viability, DNA integrity, and kinematic parameters of sperm cells. The hypothesis is that the gut microbiome may upregulate oxidative defense genes. In contrast, oral supplementation of probiotics does not affect the seme microbiome and sperm parameters in horses (Cooke *et al.*, 2024), although the supplementation of different strains may lead to different results.

The flourishing literature in humans and laboratory animals suggest that probiotics are a promising tool to influence fertility and sustain reproductive functions (Feng and Liu, 2022). This is still an underexplored but interesting area for further research in dogs.

5. Future perspectives

Although the reproductive microbiome is still considered a niche theme in canine reproduction, research possibilities are countless and promising, especially with the advent of sequencing techniques. The development of research on the reproductive microbiome of dogs is fraught with challenges, including sample availability and a lower economic interest compared to production animals. At present, knowledge is scarce on basic topics such as the semen microbiome of male dogs and its possible effect on the female microbiome following natural mating or even artificial insemination, as performed in humans and horses (Mändar *et al.*, 2015; Malaluang *et al.*, 2023). Furthermore, case-control studies are needed to unravel possible variations in the microbiome associated with more environmental (e.g., diet) and individual (e.g., age, breed, fertility performances, semen parameters) factors, as well as with reproductive conditions (e.g., poor semen quality, azoospermia, prostatic disease). Although a description of the vaginal microbiome in the bitch exists (Lyman *et al.*, 2019), it would be interesting to report a full description of bacterial populations based on the phase of the estrous cycle and to assess differences related to the living environment and diet. It has been demonstrated that the microbiome may play a role in endometritis and fertility in cows (Machado *et al.*, 2012; Miranda-CasoLuengo *et al.*, 2019) and women (Liu *et al.*, 2019; Lozano *et al.*, 2021), but no data are available in the bitch, since practical alternatives to sample the endometrium of infertile bitches are at present not at hand (Christensen *et al.*, 2012).

Epidemiological studies on the canine reproductive resistome are necessary to orient the use of antimicrobials, which is relevant for the individual animal as in the One Health perspective. A primary direction for research is the effect of local and systemic antimicrobials on microbiome composition and on the spread of resistance genes among bacteria within the reproductive tract. Further key research should aim to assess the effect of antibiotics in semen extenders on the vaginal flora of bitches, as already investigated in horses (Malaluang *et al.*, 2023).

Alternatives to antimicrobial treatments are needed in clinical practice and the use of novel tools as probiotics may represent an interesting research area, as already indicated in humans (Feng and Liu, 2022). This may lead to new harmless therapeutic protocols for fertility, meeting breeders' and owners' satisfaction and approaching a more responsible use of antimicrobials. In parallel, the use of antibiotics in semen extenders could be decreased if reasonable alternatives are tested, effective, and affordable.

6. Conclusions

Looking forward to further developments in research on male and female canine microbiome, the key to safeguard reproductive health of dogs is transparent communication with owners and breeders. Concepts as antimicrobial resistance and infectious disease should be conveyed using simple language and examples, highlighting the risks for animal health and for the increase of medical costs. In this context, the presence of mycoplasmas should be assessed only in cases of decreased reproductive performance or clinical signs. No antimicrobial treatment should be prescribed in undiagnosed cases of subfertility, as this may be detrimental for the normal reproductive microbiome. Finally, we should take responsibility for promoting a responsible use of antimicrobials, protecting the general and reproductive well-being of dogs, and indirectly safeguarding the health of humans sharing the same environment.

7. Declarations

Declaration of competing interest: None of the authors of this paper has financial or personal relationship with other people or organizations that could inappropriately influence or bias the content of the paper.

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Chapter 2
Canine semen microbiota

Adapted from:

Characterization of the semen microbiota of healthy stud dogs using 16S RNA sequencing

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Abstract

The reproductive microbiota of male dogs has never been investigated using culture-independent sequencing techniques. The purpose of the present study was to get seminal knowledge on the microbiota of the ejaculate. Specifically, factors as the fraction of the ejaculate, the sperm quality (normospermia, teratozoospermia), and the living environment were evaluated. The sperm-rich and the prostatic fractions of the ejaculate were collected from healthy stud dogs. Following the sperm analysis, samples from twenty animals (normospermic n=10 and teratozoospermic n=10) were stored at – 80°C until further processing including DNA extraction and 16S rRNA sequencing. Alpha- (Shannon index) and beta- (Bray-Curtis, Unweighted UniFrac) diversities were assessed and compared (PERMANOVA) based on the group of samples (biological samples from the ejaculate and controls), the fraction of the ejaculate (sperm-rich and prostatic fractions), the animal group (normospermia and teratozoospermia), and the living environment of the animal (kennel or pet living in-house). The most abundant bacterial phyla in canine semen samples were Proteobacteria, Firmicutes, and Actinobacteria. Overall, the dominant bacterial family was that of *Pasteurellaceae* The genus *Mycoplasma* was never detected. No differences in terms of bacterial composition were found based on the fraction of the ejaculate and based on the animal group ($P > 0.05$). On the other hand, differences in alpha and beta diversities were highlighted based on the living environment ($P = 0.001$).

Overall, the results of the present study provide preliminary insights on dog semen microbiota, opening a new chapter in the field of canine andrology. Our results suggest that the environment may play a role in influencing the reproductive microbiota of male dogs and that the prostatic fraction of the ejaculate can be used for further research as a representative of the semen microbiota.

1. Introduction

The spread and development of sequencing techniques, led to a shift in the paradigm of sterility of inner organs and fluids in humans and animals. Therefore, research on the presence of a resident microbiota in healthy organs has developed enormously in the last two decades (Yagi *et al.*, 2021). As a natural consequence, researchers started focusing on unveiling possible associations between the microbiota and various conditions, including fertility problems (Okwelogu *et al.*, 2021). Therefore, research on the reproductive microbiome has blossomed, targeting both male and female organs (Alfano *et al.*, 2018). In dogs as in humans, the underlying cause of up to 50% of of subfertility cases can be attributed to the male (Brandão *et al.*, 2021; Farahani *et al.*, 2021; Mason, 2023). Although bacterial infections represent only one element in the long list of underlying causes that can

affect fertility in dogs, empirical treatment with antimicrobials is often attempted by veterinarians to restore reproductive efficiency (Tompson *et al.*, 2021). However, antibiotics are not the solution to every problem and their effect can also be detrimental (Zdunczyk and Domoslawska, 2022), altering the resident flora in multiple organs and possibly causing unbalances and antimicrobial resistance, which is not desirable in a *One Health* perspective.

The lower reproductive tract of men and women presents with a characteristic microbiome (Altmäe *et al.*, 2019; Lyman *et al.*, 2019; Toson *et al.*, 2022) and recent studies have demonstrated an association between semen quality and bacterial communities in the ejaculate of healthy and subfertile men. Furthermore, acute and chronic infections of the genito-urinary tract account for up to 15% of infertility cases in men, possibly also affecting semen parameters (Weidner *et al.*, 2013; Weng *et al.*, 2014; Schuppe *et al.*, 2017). The ejaculate conveys bacteria possibly residing in any part of the canine genito-urinary tract, being the less invasive and most immediate substrate to analyze. Specifically, breeding male dogs are routinely subjected to reproductive examination to ensure optimal performances, which is of economic importance. Nevertheless, research on seminal bacteria is very limited and a description of the characteristic microbiota has never been provided. Canine semen contains up to 10^5 bacteria/mL and it has been suggested that a higher overall concentration of bacteria and the presence of potentially pathogenic microorganisms might be associated with lower semen quality (Goericke-Pesch *et al.*, 2011). However, previous research on canine semen microbial composition was only based on culture, thereby potentially missing some pathogens. Next generation sequencing (NGS) techniques allow for the description of novel microbiomes without prior knowledge of sequencing information or specific culture requirements. These techniques have been recently used to investigate the seminal microbiome of humans and domestic animals with compelling results (Baud *et al.*, 2019; Quiñones-Pérez *et al.*, 2021; Koziol *et al.*, 2022). To date, no data are available on the healthy dog semen microbiome although this is essential to investigate possible associations between seminal microbial populations and abnormalities in semen parameters, infertility, and reproductive conditions. Therefore, the aim of the present study is to explore the seminal microbiota of male dogs and to assess possible differences based on various factors (i.e., fraction of the ejaculate, semen characteristics, living environment).

2. Materials and methods

2.1 Animals and sample collection

This prospective study included purebred healthy client-owned stud dogs undergoing breeding soundness examination at the Faculty of Veterinary Medicine of Ghent University (Merelbeke,

Belgium) between December 2021 and March 2022. The study was conducted in accordance with the EU Directive 86/609/CEE for the care and use of animals and informed consent was obtained from the owners of the enrolled dogs. The dogs could be either in-house pets or animals from breeding facilities. A maximum of two dogs living in the same kennel or in the same household were included, to avoid cohabitation as a confounding factor. Dogs were at least 1 year of age and 10 kg of body weight and had not received any antimicrobial within the previous 6 months. All dogs underwent a general clinical exam and breeding soundness examination (visual assessment of the scrotum, palpation of the testes, examination of the penis and prepuce, ultrasound examination of the prostate) and were deemed healthy. Semen samples were collected by digital manipulation in the absence of a teaser bitch (i.e., a bitch in heat) and the sperm-rich fraction was used to assess semen quality. Dogs that presented both more than 80% morphologically abnormal spermatozoa and progressive motility lower than 40% were excluded from the study, as the same animals subsequently participated in a clinical trial with these criteria. The animals that were finally enrolled following the semen analysis, received a standard diet for adult dogs (provided by Royal Canin SAS) for at least 60 days (estimated duration of a full spermatogenic cycle in dogs) (Soares *et al.*, 2009). No dog received antimicrobial treatment during this period. Afterwards, the dogs underwent further clinical examination, and ejaculates were collected again using sterile plastic funnels. The operators wore gloves, and the prepuce was wiped with a clean gauze before collection. After the ejaculation of the pre-sperm fraction, the second (sperm-rich) and the third (prostatic) fractions were collected separately and immediately carried to the semen laboratory next to the collection room. Based on the estimated volume of the ejaculate, aliquots of 100-500 µL of each fraction were moved into two sterile 1.5 mL Eppendorf tubes (Safe-Lock tubes 1.5 mL PCR-clean, Eppendorf, Germany) using sterile tips and stored at – 80°C for further analyses. The rest of the sperm-rich fraction was used to assess semen quality.

Sampling controls (n=2) included sterile saline collected using a tip from the same batch of those used to collect the semen samples and a gauze stored with those used to clean the prepuce, and were stored at – 80°C.

Ten dogs that presented with normospermia (i.e., 60% or more morphologically normal spermatozoa) and ten dogs that presented with teratozoospermia (i.e., less than 60% of morphologically normal spermatozoa) were retrospectively selected based on the result of the semen analysis and the samples were thawed and processed by 16S sequencing, together with the sampling controls.

2.2 Fresh semen analysis

The sperm rich fraction of the ejaculate was used to assess semen parameters. The sperm concentration was measured using the Nucleocounter-SP100® (ChemoMetec, A/S, Allerød, Denmark) using a 10 µL aliquot of semen diluted into 1 mL lysis reagent S100 (ChemoMetec, A/S, Allerød, Denmark), according to the manufacturer's instruction (Domain *et al.*, 2022). The semen was then diluted to a concentration of 40×10^6 spermatozoa/mL into warm and sterile saline solution (NaCl 0.9%) and 4 µL were loaded in a pre-warmed ISAS®D4C20 disposable counting chamber to assess motility by the ISAS®v1 system (Proiser, Valencia, Spain) equipped with a heated stage set at 37 °C and a 10× negative phase-contrast objective (Rijsselaere *et al.*, 2012). The recorded kinematic parameters were total motility (TM, %), progressive motility (PM, %), average path velocity (VAP, µm/s), straight line velocity (VSL, µm/s), curvilinear velocity (VCL, µm/s), straightness (STR, %), and linearity (LIN, %).

Finally, eosin-nigrosin stain was used to evaluate the morphology and the viability of spermatozoa under bright-field microscopy at 1000× magnification (Olympus BX51TF, Tokyo, Japan). The percentage of living spermatozoa and the percentage of normal spermatozoa was determined by counting 200 spermatozoa. Specifically, spermatozoa were classified as dead when penetration of the staining into the sperm cell was observed (i.e., damaged plasma membrane). Morphological abnormalities were assessed and included abnormal heads, abnormal midpiece/tails, proximal protoplasmic droplets, and distal protoplasmic droplets.

2.3 16S rRNA bacterial gene sequencing

After internal testing of the extraction kit on test samples of different volumes and extraction negative controls, QIAamp® DNA Microbiome (Qiagen, Germany) was chosen to achieve DNA extraction on the 42 included samples (i.e., second and third fractions of 20 dogs and two controls) and on two laboratory blanks (i.e., lysis buffer from the extraction kit). Therefore, 44 samples were further processed. Specifically, 100 µL aliquots were used to achieve bacterial DNA extraction. Host DNA was depleted as for the instructions provided with the extraction kit. Afterwards, bacterial DNA was lysed following the QIAmp® DNA Microbiome (Qiagen, Germany) protocol. The concentration of the extracted DNA was assessed using a Nanodrop 2000 (Thermo Fisher Scientific, USA) spectrophotometer and samples were sent for sequencing of 16S rRNA to an external laboratory (BMR Genomics, Padua, Italy). After 30 cycles of amplification by RT-PCR targeting the hypervariable regions V3-V4 of the bacterial DNA, sequencing was performed using the Illumina MiSeq platform (Illumina, San Diego, CA).

2.4 Data analysis

Data analysis of the raw sequences was performed using Quantitative Insights into Microbial Ecology tool (QIIME2-2019.10) together with Greengenes database (DeSantis *et al.*, 2006; Caporaso *et al.*, 2010). Paired-end forward and reversed sequencing reads were assembled and assigned to their original sample based on the barcode. After removing primer sequences and barcodes, the denoising procedure using the Deblur method implemented in Qiime2 was applied. The sequences were classified in OTUs (Operational Taxonomic Unit) at 97% identity threshold against the Greengenes reference database (ver. 13-8-99-515-806) for taxonomy assignment. Alpha (i.e., intra-sample bacterial diversity) and beta (i.e., in-between sample diversity) diversities were assessed by Shannon-index and Bray-Curtis and Unweighted UniFrac, respectively. Differences in number of reads per sample based on different groups (i.e., type of sample, fraction of the ejaculate, normospermia and teratozoospermia groups, living environment) were assessed using Mann-Whitney U test. To understand the differences in the variation of samples, Wilcoxon test was used to compare the alpha diversity distribution values showed by groups of samples (biological samples from the ejaculate and controls), the fraction of the ejaculate (second and third fractions), the normospermia and teratozoospermia groups, and the living environment of the animal (kennel or pet living in-house).
A permutational multivariate analyses of variance (PERMANOVA) was run for each based on Bray-Curtis dissimilarity to assess differences based on sample type (biological sample from the ejaculate and controls), fraction of the ejaculate (sperm-rich and prostatic fractions), living environment (in-house pet and kennel dogs), and group (normospermic and teratozoospermic dogs).
Data were analyzed using R ver. 4.2.2 (Vienna, Austria). Significance was considered for $P < 0.05$. Differences among samples were calculated using alpha and beta diversity estimation with Qiime pipeline.

3. Results

All included animals were healthy at both clinical examinations, and semen collection was always successful. Dogs were aged 1 to 10 years (median 3.3, IQR 2-4.4 years), weighted between 10.7 and 70.1 kg (median 25, IQR 15.75-28.75), and belonged to 11 different breeds (*Table 1*). Eight animals came from breeding kennels, whereas the remaining twelve lived in households as pets. None of the dogs had mated in the previous week. Results from the semen analyses are summarized in *Table 2* as a mean and standard deviation. The mean percentage of morphologically normal spermatozoa was

76% (SD 22.4) in dogs classified as normospermic and 38.4% (SD 22.2) in dogs classified as teratozoospermic.

Table 1. Individual characteristics of twenty healthy dogs included in the present study

Animal	Breed	Age (months)	Body weight (kg)	Group[a]	Living environment[b]	Number of reads
A	Basset fauve de Bretagne	44	11.8	N	K	597
B	Basset fauve de Bretagne	50	13.7	N	K	749
C	Border collie	24	20.1	T	P	1,483
D	American Staffordshire terrier	38	28.6	T	K	788
E	Border collie	41	22.5	T	P	3,150
F	Basset fauve de Bretagne	33	14.4	N	K	1,486
G	Great Dane	49	62.6	N	P	4,589
H	Bassett hound	97	25.2	T	P	555
I	Viszla	15	28.5	N	K	757
L	Border collie	63	25.6	T	P	5,053
M	Pug	23	10.7	T	P	9,781
N	Border collie	36	24.4	N	P	7,677
O	Border collie	48	26.4	N	P	15,602
P	German shepherd	15	34.6	N	P	6,141
Q	English Springer Spaniel	28	24.8	T	P	6,471
R	Rottweiler	79	39.3	T	P	9,644
S	Bassett fauve de Bretagne	125	16.2	T	K	7,040
T	Bassett fauve de Bretagne	20	11.7	N	K	3,568
U	American Staffordshire terrier	23	29.2	N	K	1,909
Z	Bullmastiff	61	70.1	T	P	1,322

[a]Normospermia (N) or teratozoospermia (T); [b]Pet (P) or Kennel (K).

Table 2. Semen characteristics of twenty healthy dogs included in the present study.

Parameter	Mean	Standard deviation	Median	Interquartile range
Volume of the 2nd fraction of the ejaculate (mL)	2.07	1.09	1.80	1.55-2.20
Concentration (x 10^6 spermatozoa/mL)	305.23	190.61	274.35	139.47-436.95
Total Sperm Output (x 10^6 spermatozoa/ejaculate)	578.41	423.25	563.64	248.90-804.50
Total motility (%)	77	13.50	83	65.75-89.25
Progressive motility (%)	65.30	16.60	68.50	53.75-78.75
Viability (%)	92.50	5.98	95.50	90.75-96
Morphology (% of normal spermatozoa)	57.20	22.38	58.50	36.75-75.25
Abnormal heads (%)	10.90	7.90	9.50	5.50-14.25
Abnormal midpieces (%)	18.40	21.50	3	1-7.25
Abnormal tails (%)	13.35	13.60	8	7.75-15.25

DNA concentration per sample was between 3.7 and 42.1 ng/μL. The number of reads per sample is reported in *Table 1*. The most abundant bacterial phyla in canine semen samples were Proteobacteria,

Firmicutes, and Actinobacteria. Relative abundances for each sample are reported in *Figure 1*. Overall, the dominant bacterial family was *Pasteurellaceae* (phylum Proteobacteria), being identified in every sample, except in those collected from one normospermic dog and in controls and laboratory blanks. *Clostridiaceae* (7 dogs), *Lactobacillaceae* (5 dogs), and *Lachnospiraceae* (5 dogs) were the most prevalent families belonging to the phylum Firmicutes, whereas *Micrococcaceae* (7 dogs) and *Streptomycetaceae* (4 dogs) were the most represented ones belonging to the phylum Actinobacteria. Although most sequences were assigned only to higher taxonomic level (i.e., order, family), some relevant genera were identified. Specifically, the genus *Clostridium* and *Lactobacillus* were detected in the second and third fractions of the ejaculate of six and three dogs, respectively. Interestingly, these bacteria were found in pairs of dogs sharing the same living environment.

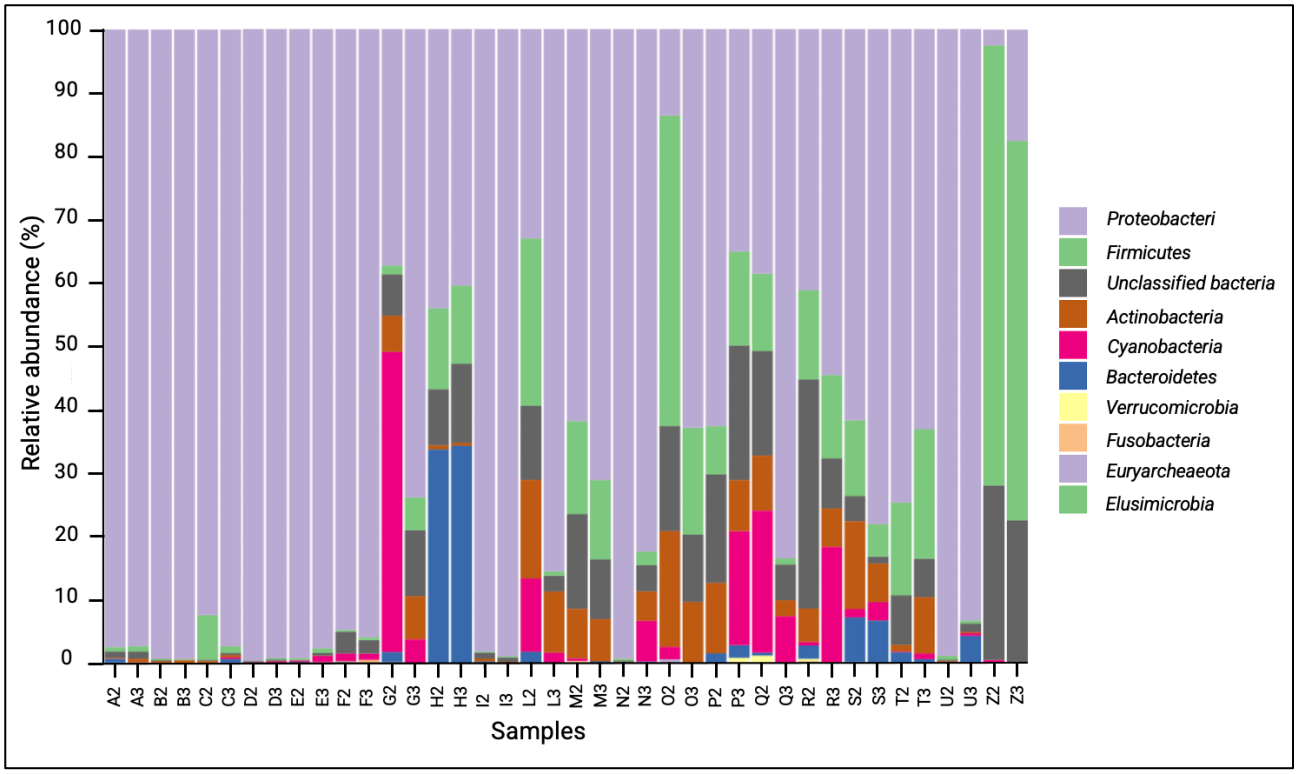

Figure 1. Phylum distribution as a percentage of the total number of identified sequences in individual samples. The same letter indicates samples collected from the same dog and ejaculate ("2" for the sperm-rich fraction and "3" for the prostatic fraction of the ejaculate, respectively).

The alpha-diversity, calculated by the Shannon index, did not differ between semen samples and controls, between the second and third fraction of the ejaculate (F2 and F3, respectively), and based on the group (i.e., teratozoospermia or normospermia) (Wilcoxon test $P > 0.05$). A significant

difference in alpha diversity was shown when living environments (i.e., pet or kennel) were compared (Wilcoxon test $P < 0.05$).

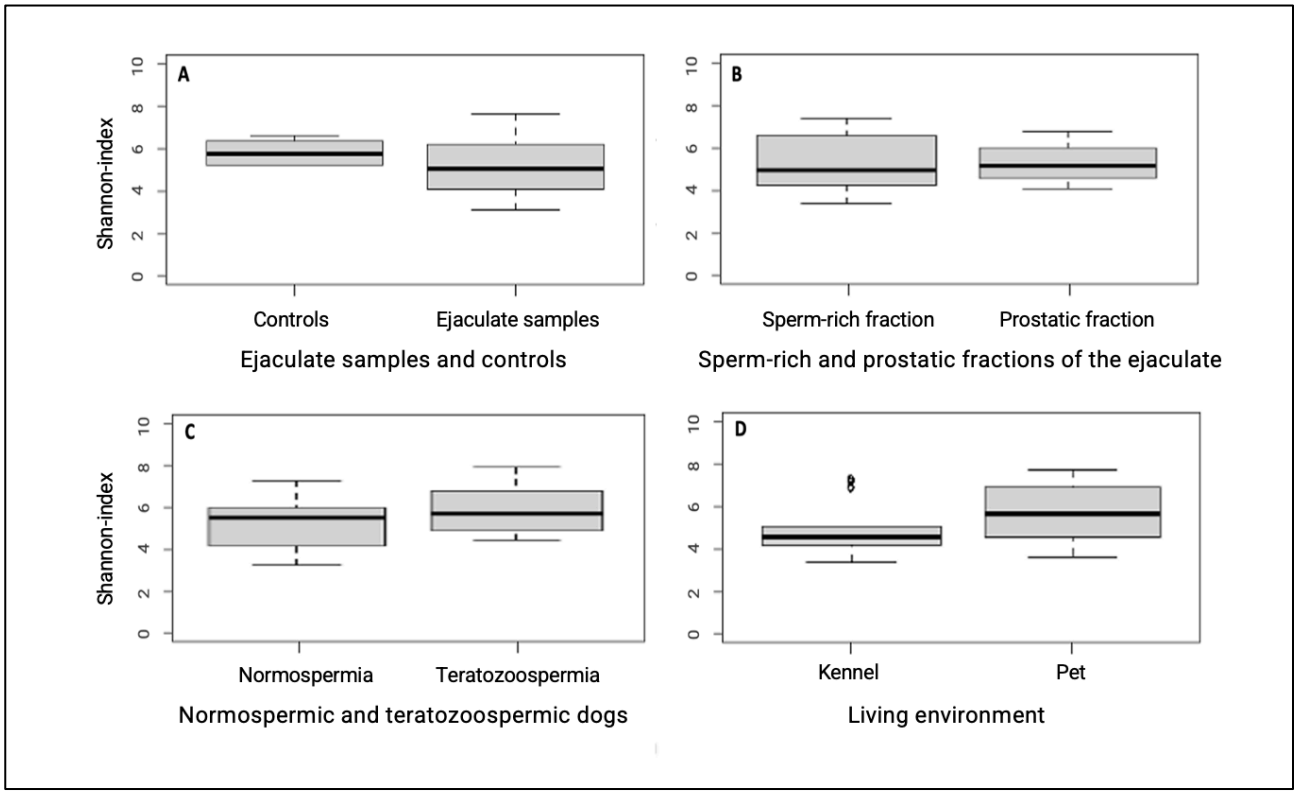

Figure 2. Alpha-diversities (Shannon index) comparisons based on A) type of sample (biological samples from the ejaculate and controls); B) fraction of the ejaculate (second and third fractions); C) group (normospermia and teratozoospermia); D) living environment of the animal (kennel or pet living in-house).

Bacterial population structure of dog semen was investigated evaluating beta-diversity assessed by Bray-Curtis dissimilarity and Unweighted UniFrac algorithms. Results are summarized in *Table 2*. The only comparisons showing significant differences using both algorithms were i) between biological samples (i.e., dog ejaculates) and controls and ii) between pet and kennel dogs (PERMANOVA $q < 0.05$ in both cases). No differences based on the fraction of the ejaculate or based on the group were found.

Table 3. Comparisons of beta diversities between/among sample features determined with Bray-Curtis and Unweighted UniFrac algorithms are reported.

Comparison	Group 1	Group 2	Bray-Curtis			Unweighted UniFrac		
			PseudoF	P value	PERMANOVA *q* value	PseudoF	P value	PERMANOVA *q* value
Biological samples (i.e., dog ejaculates)	Controls	Biological samples	2.90	0.007*	0.006*	1.7716	0.046*	0.058
Fraction of the ejaculate	Second (F2)	Third (F3)	1.045921	0.334	0.029*	1.1255	0.267	0.071
Group	Normospermia	Teratozoospermia	1.9469	0.070	0.011*	0.916570	0.499	0.115
Environment	Kennel	Pet	4.7336	0.001*	0.001*	2.399827	0.012*	0.004*

*Significance for $P < 0.05$

Two dimensions scaling plots were extrapolated for both algorithms and clustering by Bray-Curtis is represented in *Figure 2*.

The semen of pet dogs was enriched in *Pasterurellaceae*, *Bacillaceae*, and *Sphingomonadaceae* (*Sphingomonas*) and presented a lower bacterial load compared to kennel dogs, whose semen was mainly enriched in *Pasteurellaceae*. Interestingly, kennel dogs presented a higher bacterial load compared to pet dogs (median 9566 reads/sample, IQR 11769-8258.75 and 6296, IQR 7694-3985.25; $P = 0.003$).

The structure of bacterial communities did not differ significantly based on breed, fraction of the ejaculate, and group ($P > 0.05$).

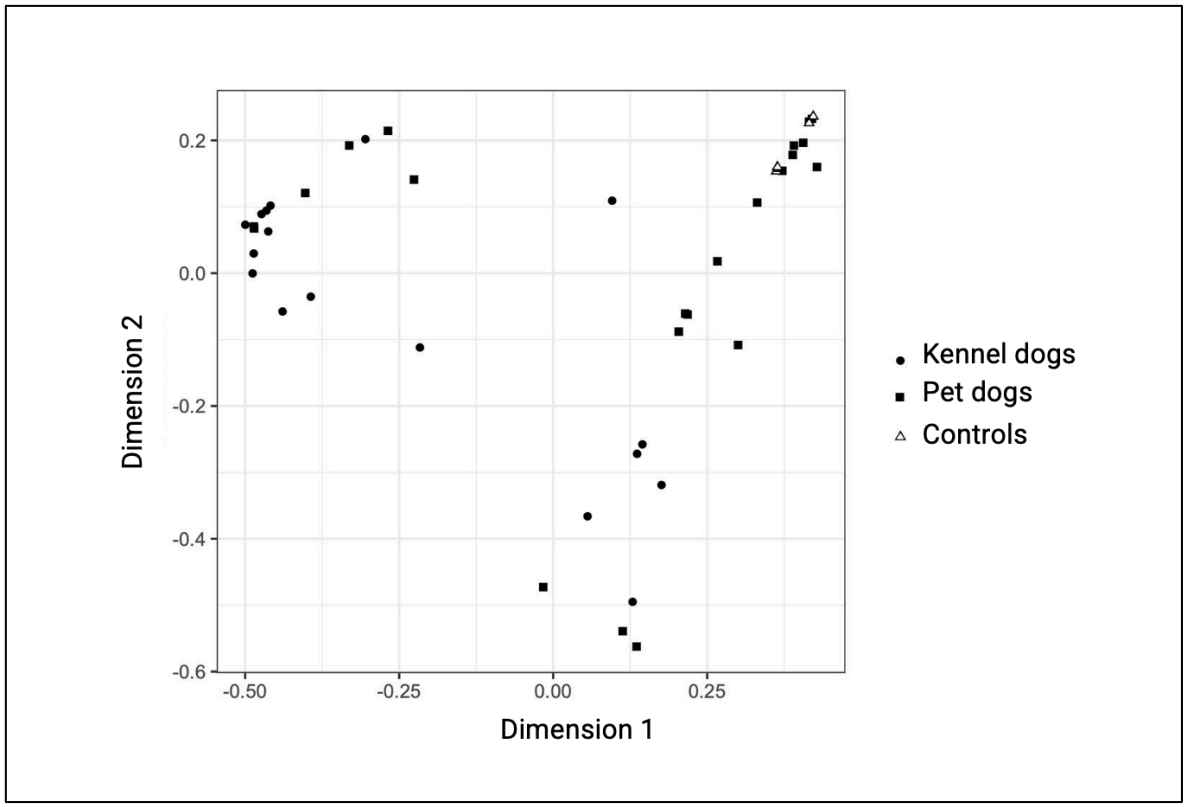

Figure 3. Two dimensions scaling plot by Bray-Curtis based on the living environment: kennel (black dots), pet (black squares), and controls (white triangles). Majority of kennel dogs clustered together, as majority of pet dogs clustered together, meaning that the bacterial diversity is higher between dogs coming from different type of living environment.

4. Discussion

In the present study, 16S rRNA sequencing was used to explore the microbiota of dog semen. Specifically, we described the most common bacterial populations in a group of overall healthy dogs housed either in breeding facilities or in a domestic environment.

Bacterial genetic material was sequenced in all the processed samples, although the bacterial load was generally low, and intra-sample diversity (i.e., alpha diversity) did not differ between semen samples and controls. However, bacterial composition of the semen was different when compared with controls in terms of beta-diversity (i.e., inter-sample diversity). Although these results must be carefully interpreted as the number of semen samples was higher compared to that of controls, our purpose was to confirm that bacterial presence was not caused by contamination during sampling and laboratory procedures. The prepuce was cleaned using a sterile gauze before collection, although few bacteria could remain on the prepuce. Future research should aim to compare the microbiome of the prepuce with that of the ejaculate, to detect which bacteria tend to swim up the urethra. Bacterial viability was not assessed, as growth in culture remains a gold standard to confirm viability, but

majority of bacteria does is considered unculturable (Kaeberlein *et al.*, 2002). This obstacle is overcome by sequencing techniques, that detect bacterial sequences belonging to unculturable microorganisms. Furthermore, the presence of bacteria in the ejaculate of healthy dogs has been previously described using culture-dependent methods (Goericke-Pesch *et al.*, 2011). Specifically, bacterial growth was previously found to be positive in all three fractions of the canine ejaculate (i.e., pre-sperm, sperm-rich, and prostatic). The pre-sperm fraction was indicated as the most contaminated one, presenting bacterial growth in 89.1% of cases, probably exerting the function of cleaning the urethra. We did not perform any analysis on the pre-sperm fraction, as it is always discarded when semen is collected for routine analyses or for ARTs. We focused on the second and third fractions, that mirror the fluid in which the spermatozoa are suspended and the status of the prostate, respectively. We found no differences in terms of bacterial populations between these two fractions. This means that analyses for microbiome studies in dogs can be performed on the third fraction of the ejaculate in dogs that present no clinically detectable prostatic disease. The advantage of using only the third fraction for the semen microbiome is twofold: it is making the whole second sperm rich fraction available for semen quality assessment and other procedures (e.g., artificial insemination, cryopreservation), and it is avoiding performing the extraction on a substrate highly contaminated with eukaryotic DNA (i.e., spermatozoa). Nevertheless, an intriguing target for future research could be the assessment of possible differences between fractions of the ejaculate in cases of prostatic disease. Parallelly, the hypothesis of the sperm-rich fraction mirroring the testicular microbiome and the prostatic one mirroring that of the prostate, should be also confirmed by studies specifically sampling these organs and investigating their microbiota, as done for men testicular tissue (Alfano *et al.*, 2018). The possibility that the bacterial sequences detected in the present study could derive from ascending migration from the urethra cannot be ruled out, although sperm is never collected bypassing this organ, except for epididymal sperm, which is an extremely different scenario out of the purpose of the present study.

The dominant phyla in the ejaculates of healthy dogs fed a standardized diet were Proteobacteria, Firmicutes, and Actinobacteria. These phyla, together with Bacteroidetes, match those that dominate the human semen microbiota (Mändar *et al.*, 2015, 2017; Chen *et al.*, 2018; Monteiro *et al.*, 2018; Baud *et al.*, 2019). Although freezing at -80°C and thawing all the sample at the same time for DNA extraction is a routine procedure (Monteiro *et al.*, 2018; Koziol *et al.*, 2022), previous research found that thawed samples have a slightly different microbiome composition in terms of relative abundance in stool samples (Chen *et al.*, 2022). This has never been investigated for dog semen. Nevertheless, the present study confirms that the microbiota of the canine semen and that of human semen have a

different microbial composition when analyzed at a deeper taxonomic level. For instance, the genus *Lactobacillus* was reported as an indicator of seminal health in men (Weng *et al.*, 2014; Monteiro *et al.*, 2018; Baud *et al.*, 2019) but it was never sequenced in the dogs included in the present study. However, many bacterial sequences remained unclassified at lower taxonomic levels, not allowing to exclude the presence of any specific bacteria. In dogs, sequences assigned to the bacterial family *Pasteurellaceae* were overall the most abundant and no other family was represented likewise. Interestingly, bacteria belonging to this family were rarely isolated in culture from canine ejaculates (Goericke-Pesch *et al.*, 2011) and molecular studies in humans highlighted their presence in samples from normospermic men (Hou *et al.*, 2013; Monteiro *et al.*, 2018) and individuals with hypomotile spermatozoa (Baud *et al.*, 2019). However, comparisons with previous literature are limited by the lack of studies applying molecular methods to describe the bacterial populations in dog semen. Furthermore, we found that the living environment influences the seminal microbiota, possibly complicating comparisons between different canine populations. Cohabitation is known to be associated with similarities in the gut microbiota (Song *et al.*, 2013; Mosites *et al.*, 2017; Finnicum *et al.*, 2019), not only within individuals of the same species, but also between dogs and humans (Song *et al.*, 2013; Zhao *et al.*, 2022). This strengthens the idea that the environment is a key factor in shaping the reproductive microbiota. In this regard, the diet is a possible confounding factor, for it is known to influence the bacterial populations in the gut. Hence, the present research was conducted on dogs that followed the same diet for the two months preceding the assessment of the semen microbiota. This suggests that living in-house or in a kennel influences the semen characteristics in terms of bacterial populations. On the other hand, the standardized diet, together with the fact that all the dogs were healthy, might have mitigated some differences in the seminal microbiota and this should be considered when conducting future research. Semen bacterial communities were not different between normospermic and teratozoospermic dogs, although this does not allow neither to exclude nor confirm that the semen microbiota influences semen parameters or fertility. Furthermore, some animals had a percentage of normal spermatozoa slightly higher or lower compared to the set cut off value of 60%, this could have reduced the possibility to detect real differences in the bacterial communities within the two groups. As mentioned, only healthy dogs were included in the present study and their fertility in terms of successful mating and litter production was not taken into account. Since the collection method influences the semen characteristics (Kustritz and Hess, 2007), we standardized our protocol by performing collection in the absence of a teaser bitch. Specifically, the presence of a female in estrus is associated with higher total sperm output, although it is not associated with sperm morphology (Kustritz and Hess, 2007). Morphological abnormalities of the spermatozoa

could lead to a decrease in motility parameters and fertility (Mickelsen *et al.*, 1993; Tesi *et al.*, 2018) and the inclusion of subfertile and infertile dogs will be a further step in this field of research. Moreover, targeted research is needed to investigate the presence and abundance of certain controversial bacteria, whose relationship with fertility is suspected but remains unclear. One specific example is that of bacteria belonging to the genera *Mycoplasma* and *Ureaplasma* (Domrazek *et al.*, 2023). When culture targeting *Mycoplasma* spp. is used, the prevalence of these bacteria in the genital tract of the canine population reaches almost 89% (Tesi *et al.*, 2018) and it seems higher in poor quality ejaculates compared to high quality ones (Laber and Holzmann, 1977; Johnston et al., 2001; Domrazek *et al.*, 2023). Hence, it is possible that only some species have a pathogenic potential on the canine reproductive tract, although it remains unknown which species this would be (Tesi *et al.*, 2018). Interestingly, sequences belonging to *Mycoplasma* spp. or to the phylum Tenericutes were not found in the present study. However, the presence of these bacteria cannot be completely ruled out because some sequences remained unclassified and because PCR protocols specifically targeting *Mycoplasma* spp. may be needed (Chalker and Brownlie, 2004; Hemmatzadeh *et al.*, 2019). The relationship between *Mycoplasma* and fertility in dogs should be a focus for future research, because the role of these bacteria may be overestimated in cases of infertility, leading to unnecessary antimicrobial treatments.

5. Conclusions

In conclusion, the present study aimed to provide seminal knowledge on the bacterial composition of ejaculates from healthy dogs, opening a novel chapter in canine andrology. The living environment affects the semen microbiome of dogs and further studies should include non-healthy animals and target specific bacteria. Further research can be conducted by performing molecular analyses using the third fraction of the ejaculate.

6. Declarations

Declaration of competing interest: VG and KH were employed by Royal Canin, which produces the dog food supplied to the dogs included in the present study and funded the research. They participated in the final revision of the paper, but their commercial affiliation does not interfere with the full and objective presentation of the results of this work. The other authors have no conflicts of interest to declare.

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Author contribution statement: PB designed the project in the frame of another project designed by AVS, KH, GD, HAH, and JL; PB, JL, GD, and RVL examined the included animals and collected the semen; PB and HAH collected the samples for molecular analyses; PB, HAH, JL, GD, and RVL performed the semen analyses; PB and LB performed the molecular analyses; LB and PB performed the data analysis; PB drafted the manuscript; VG, AVS, LS, AR, and LB edited the manuscript.

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Chapter 3
Feline vaginal microbiota

Adapted from:

The vaginal microbiota of healthy female cats

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Abstract

The vaginal microbiota of the queen (i.e., female cat) has never been described using culture independent methods. The objectives of the present research were to describe the vaginal microbiota of healthy domestic shorthair queens using both 16S rRNA sequencing and culture, and to assess the effects of age, living environment, and reproductive season on its composition. Thirty queens undergoing elective ovariectomy were included in the study. The vaginal samples were collected just before surgery, from animals under general anesthesia. Two consecutive mini-swabs were introduced in the queens' vaginal tract. A preliminary study with 10 healthy queens aimed to negate sampling order's effect. Two consecutive samples for sequencing (5 queens, 10 swabs) and culture (5 queens, 10 swabs) were collected, confirming a match (100% in culture, Bray-Curtis P=0.96 in sequencing). The experiment included 20 queens that were prospectively grouped based on age (prepubertal N = 10, adult N = 10), living environment (indoor N = 10, outdoor N = 10), and time of the year, whether during the reproductive season (N = 10) or during seasonal anestrous (N = 10). Bacteria were identified through metataxonomic analysis, amplifying the V1-V2 regions of 16S rRNA gene, and through standard culture followed by MALDI-TOF MS.

The feline vaginal microbiota is dominated by Proteobacteria, Firmicutes, Bacteroidota, and Actinobacteria. Escherichia-Shigella, Streptococcus, and Pasteurella were the most abundant genera. Although culture underestimated bacterial richness and diversity compared to sequencing, Escherichia and Streptococcus were the most isolated bacteria. No bacterial growth was observed in 15% of samples (N = 3/20), whereas growth of one or two bacterial species was observed in 64.7% (N=11/17) and 35.3% (N=6/17) of cases, respectively. No differences in terms of alpha (Kruskal-Wallis rank sum test P = 0.65) and beta diversity (Bray-Curtis, Unweighted and Weighted UniFrac analyses P>0.5) were observed. Although a difference in alpha diversity based on phylogenetic tree (P=0.02) was detected between indoor and outdoor queens. In conclusion, mixed and monoculture of *Escherichia coli*, *Streptococcus canis*, *Staphylococcus felis*, and *Enterococcus* spp. are normal findings within the cat vagina. Age and reproductive season do not influence the feline vaginal microbiota, whereas further research is needed to elucidate the role of the living environment.

1. Introduction

Bacterial communities inhabit the different parts of the animal body and play a key role in health and metabolism. Additionally, the vagina has a distinct microbiota, as evidenced by studies in both humans (Smith and Ravel, 2017; Simon, 2018) and animals (Maksimović *et al.*, 2012; Lyman *et al.*, 2019; Rota *et al.*, 2020; Adnane and Chapwanya, 2022; Hu *et al.*, 2022). The vaginal microbiota is

essential for maintaining reproductive health (Ravel *et al.*, 2011), influences fertility (Cocomazzi *et al.*, 2023), and affects pregnancy outcomes (Saadaoui *et al.*, 2023; Guo *et al.*, 2024). Research on the feline species is limited to some studies investigating vaginal bacterial flora using culture techniques (Ström Holst *et al.*, 2003; Mannion *et al.*, 2022). While culture remains the gold standard for diagnosing infectious agents, it is not the optimal method for investigating bacterial population, since it misses more than 90% of bacteria (Kaeberlein *et al.*, 2002; Almeida and De Martinis, 2019). Sequencing techniques offer higher sensitivity, allowing the description of the full microbiome of a specific environment (Woo *et al.*, 2008), and have therefore become widespread in human and animal research over the past two decades (Peixoto *et al.*, 2021; Gao *et al.*, 2023).

Domestic cats are popular companion animals, yet comprehensive studies exploring the composition and dynamics of their vaginal microbiota are missing, creating a gap with significant implications for feline reproductive management, reproductive diseases, and fertility issues, given that the vagina is the site of sperm deposition. Knowledge of the healthy vaginal microbiota could also orient the use of antimicrobials, which are often wrongly adopted as shortcut to improve fertility (Guardabassi *et al.*, 2008). Conversely, the balance of bacterial populations could be preserved or restored using probiotics, as demonstrated in humans and other mammals (Nader-Macías *et al.*, 2008; Ang *et al.*, 2023; Chung *et al.*, 2023; Di Pierro *et al.*, 2023; Mitra *et al.*, 2024).

The objective of the present research is to describe the feline vaginal flora using both culture and 16S rRNA bacterial gene sequencing, and to assess the effects of age, reproductive season, and the living environment on the vaginal microbiota of the queen.

2. Materials and Methods

2.1 Animals

Thirty clinically healthy queens undergoing elective ovariectomy either at the Veterinary Teaching Hospital of the University of Turin (Italy) or at a private practice in Turin (Italy) were enrolled in this research (*Figure 1*). The study was carried out between October 2022 and May 2023. The queens were either indoor pets (I) or stray cats living in outdoor colonies (O). No restrictions were placed on breed, age, or body weight of the animals. However, all the queens were domestic short-haired cats, either privately owned in-house cats or stray/colony cats.

A preliminary study aimed to assess the soundness of the sampling procedures. This included ten queens, while the experiment was conducted on twenty animals. The age of some stray queens was unknown and merely estimated. The animals were classified as prepubertal (P) or adult (A, i.e., after

puberty), based to history or, when missing, based on body weight, secondary sexual traits, the aspect of the ovaries following ovariectomy, and the season of the year.

This observational study was structured and reported in agreement with adapted ARRIVE guidelines (Animal Research: Reporting In Vivo Experiments) (Percie Du Sert *et al.*, 2020). Approval was obtained by the Ethical Committee of the Department of Veterinary Sciences of the University of Turin (Italy) (n. 0000284 - 01/02/2023). All the owners and cat colony managers provided informed written consent, and the procedures were carried out in accordance with the EU Directive 86/609/CEE and with the guidelines of the Italian Ministry of Health for the care and use of animals (D.L. 4 March 2014 n. 26 and D.L. 27 January 1992 n. 116).

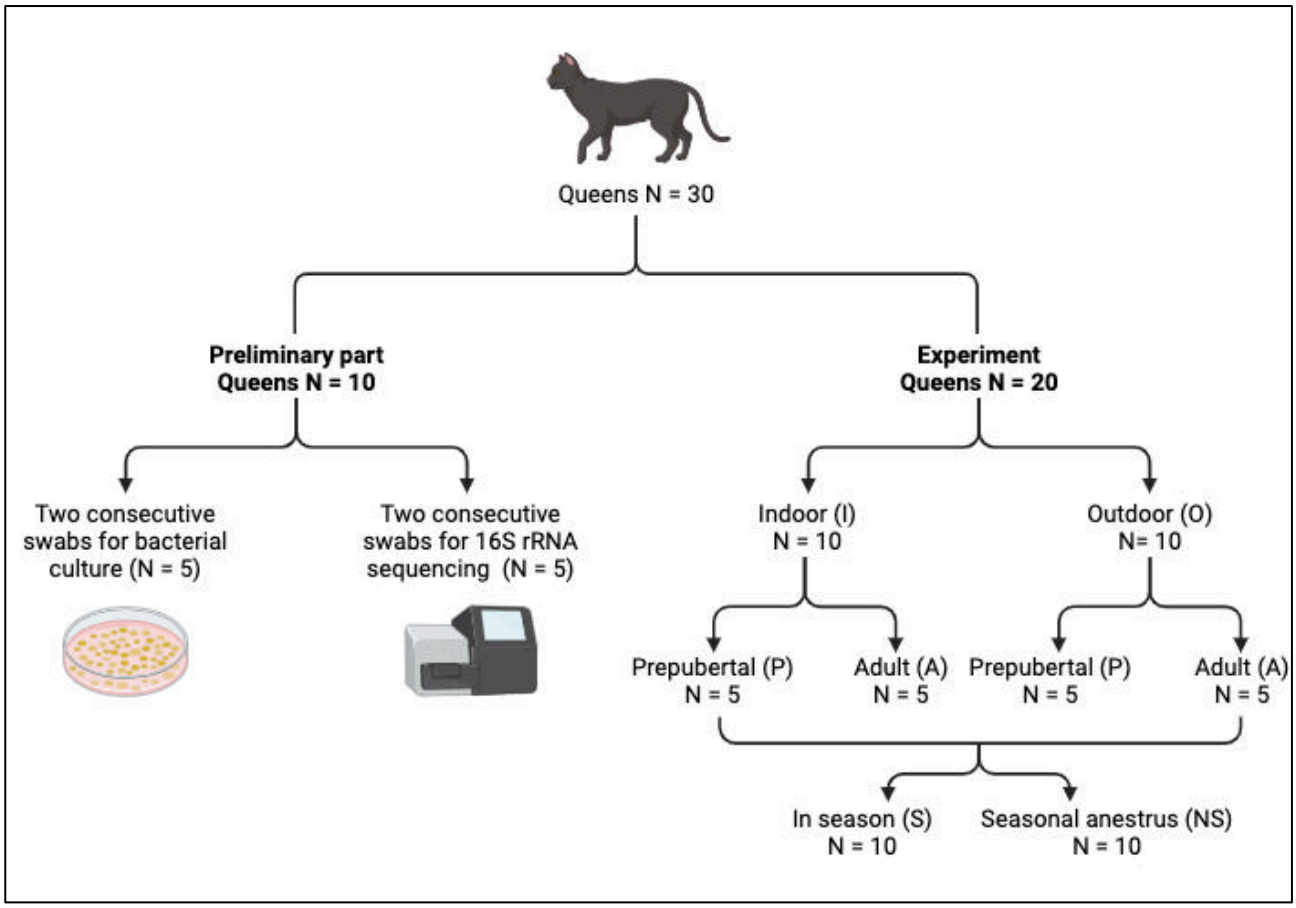

Figure 1. Diagram representing the animals included in the present study. A total number of 30 healthy queens was included either in the preliminary part (N = 10) or in the experiment (N = 20).

2.2 Sample collection

All the queens were clinically healthy; when they did not allow manipulation, a preliminary visual inspection was followed by proper clinical assessment at preparation for surgery. Ovariectomy

followed standard anesthesiologic and surgical procedures (Fossum, 2019). Vaginal swabs were collected immediately after the induction of general anesthesia: the procedure took between two and three minutes, without significantly extending the duration of anesthesia. Briefly, the perivulvar region was disinfected with 2% chlorhexidine, the vulvar labia were slightly separated, and a sterile 1 mL syringe without the plunger was gently introduced in the vulva to serve as a guide and protection for the sterile nylon 'mini' swab (ESwab® 484CE, Copan Italia Spa, Brescia, Italy) passed through for vaginal sampling. The 'mini' swab was introduced at about 1 cm depth, and the vaginal walls were delicately rubbed. Two consecutive samples were collected from each animal.

The preliminary technical study aimed to check for the possible effect of the order of swab collection. To this end, both swabs collected from five queens were placed into a 5 mL tube containing 1 mL of modified Liquid Amies Medium (ESwab® Copan Italia Spa, Brescia, Italy) for bacterial culture, and both swabs from five different queens were placed into a sterile 1.5 mL Eppendorf tube (Eppendorf Tubes® 3810X, Eppendorf s.r.l., Hamburg, Germany) for molecular analyses.

In the experiment, the first swab was placed into a sterile 1.5 mL Eppendorf tube (Eppendorf Tubes® 3810X, Eppendorf s.r.l., Hamburg, Germany) for molecular analyses, and the second swab was placed into a 5 mL tube containing 1 mL of modified Liquid Amies Medium (ESwab® Copan Italia Spa, Brescia, Italy) for bacterial culture. As positive and negative controls for molecular analyses, three more swabs were collected and immediately placed into a sterile 1.5 mL Eppendorf tube, one from the rectum and one from the mouth of a cat, the third just after extraction from its sterile envelope.

The samples intended for culture were immediately sent to the Istituto Zooprofilattico Sperimentale delle Venezie (Legnaro, Italy) and processed within 48 hours, whereas those intended for 16S rRNA sequencing were frozen at – 80°C and then processed all together once sampling was concluded.

2.3 16S rRNA bacterial gene sequencing

Sequencing of the bacterial 16S rRNA gene was performed on 30 vaginal swabs (two swabs from five queens in the preliminary part and 20 swabs from the cats in the experiment). A clean sterile 'mini' swab served as negative control to check for possible contamination from the swabs, whereas a rectal swab and an oral swab were processed to verify that the applied methods would lead to DNA extraction. DNA extraction from the frozen and thawed swabs 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. 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 (Walker *et al.*, 2020), such as in vaginal swabs from cats and horses (manuscript in preparation). 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 visualizing a ≈ 350 bp band. Except for negative controls, which yielded insufficient DNA, samples were then sent to an external laboratory (BMR Genomics, Padua, Italy) for barcoding and sequencing using the Illumina MiSeq platform (Illumina, San Diego, CA).

2.4 Bioinformatic 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 (Callahan *et al.*, 2016) 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 that were not assigned to the 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 normalized by performing subsampling based on the lowest number of sequences for a sample and by drawing a taxonomy rarefaction curve (*Figure 2*). Alpha diversity (i.e., intra-sample diversity) was assessed by observed ASV richness, Shannon, Simpson indexes, and by a phylogenetic tree using Kruskal–Wallis tests and pairwise Wilcoxon tests. Beta diversity (i.e., between-samples diversity) was estimated using Bray-Curtis, Unweighted and Weighted UniFrac. Permutational multivariate analyses of variance (PERMANOVA) were run to assess differences based on age (P and A), living environment (I and O), reproductive season (S and NS).

Significance was considered for $P < 0.05$.

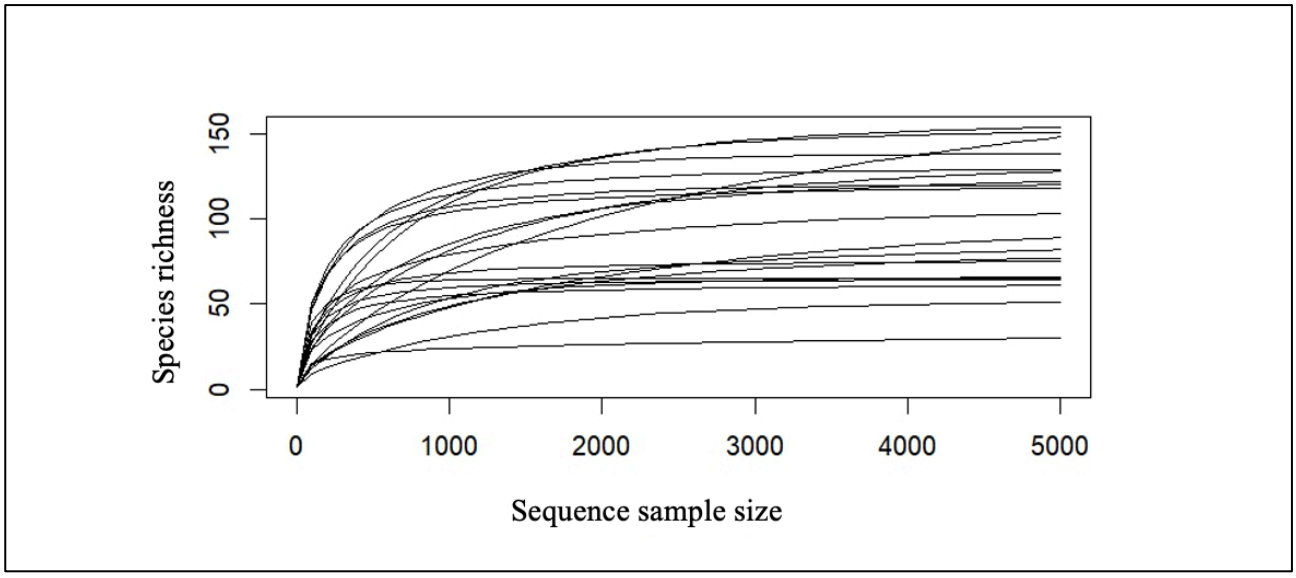

Figure 2. Taxonomy rarefaction curves for the feline vaginal samples included in the present study showing that the selected sequencing depth was sufficient to saturate species richness.

2.3 Bacterial culture

Culture was performed according to standard laboratory procedures on 30 vaginal swabs (two swabs from five queens in the preliminary part and 20 swabs from the cats in the experiment). Briefly, each swab was diluted in 1 ml of nutrient broth (Heart Infusion Broth). Then, 10 µL aliquots were inoculated into solid nutrient medium (three plates of 5% blood agar), and a selective medium for *Enterbacteriaceae* (MacConkey agar). All media were provided by Biolife, Milan, Italy. Blood agar plates were incubated at 37°C±1°C under aerobic, anaerobic, and microaerophilic conditions (5-10% CO_2), while the selective medium and the nutrient broth were incubated at 37°C±1°C under aerobic conditions. Culture media were checked after 24 and 48 h, in case of absence of bacterial growth on plates and turbidity in the nutrient broth, broth seeding was performed as described. Non-inoculated MacConkey agar plates were incubated in parallel in the same conditions as negative controls for culture.

All the bacterial colonies grown on the solid nutrient media were counted and identified. Species identification was performed by MALDI-TOF MS: Microflex LT instrument (MALDI Biotyper, Bruker Daltonics) equipped with FlexControl software (version 3.3, Bruker Daltonics).

3. Results

In the preliminary study, bacteria were isolated from all the vaginal swabs processes by culture (N=10), and the results of the first and second sampling matched in 100% of cases (N = 5), both for bacteria species and growth (*Table 1*).

Bacterial genetic material was detected in all the samples processed by 16S rRNA sequencing (N = 10). The DNA concentration was similar between sample A and B, regardless of the order of collection, and ranged between 5 and 50 ng/µL.

No differences were found in alpha-diversity calculated by Shannon and Simpson indexes (Wilcoxon rank sum exact test P = 0.55 for both indexes). Beta-diversity by Bray-Curtis analysis showed no statistically significant difference (PERMANOVA P = 0.96).

The characteristics and group assignment of the 20 queens included in the experiment (i.e., age, reproductive season, living environment) are summarized in *Table 2*.

Table 1. Bacteria species and number of Colony Forming Units (CFU) in two repeated vaginal swabs (A and B) from the same cat.

Cat	Swab (A or B)	Bacterium 1 species	Bacterium 1 CFU	Bacterium 2 species	Bacterium 2 CFU
1.1	A	*Escherichia coli* (haemolytic strain)	50	/	/
1.1	B	*Escherichia coli* (haemolytic strain)	50	/	/
2.1	A	*Escherichia coli* (haemolytic strain)	50	*Enterococcus fecalis*	50
2.1	B	*Escherichia coli* (haemolytic strain)	50	*Enterococcus fecalis*	50
3.1	A	*Escherichia coli* (haemolytic strain)	100	/	/
3.1	B	*Escherichia coli* (haemolytic strain)	100	/	/
4.1	A	*Pasteurella dagmatis*	>300	*Streptococcus suis*	>300
4.1	B	*Pasteurella dagmatis*	>300	*Streptococcus suis*	>300
5.1	A	*Escherichia coli* (haemolytic strain)	>300	*Klebsiella oxytoca*	>300
5.1	B	*Escherichia coli* (haemolytic strain)	>300	*Klebsiella oxytoca*	>300

Table 2. List of the included queens. The age (prepubertal or adult), the living environment (indoor or outdoor), and the reproductive season (in season or not in season) are reported, together with the four groups that were created (PO = prepubertal outdoor; PI = prepubertal indoor; AO = adult outdoor; AI = Adult indoor).

Animal	Age (P[a] or A[b])	Living environment (I[c] or O[d])	Reproductive season (S[e] or NS[f])	Group
1.2	P	O	NS	PO
2.2	A	I	NS	AI
3.2	P	O	NS	PO
4.2	P	O	NS	PO
5.2	A	I	NS	AI
6.2	P	O	NS	PO
7.2	A	I	NS	AI
8.2	P	I	NS	PI
9.2	P	I	NS	PI
10.2	A	I	NS	AI
11.2	P	I	S	PI
12.2	P	O	S	PO
13.2	A	O	S	AO
14.2	P	I	S	PI
15.2	P	I	S	PI
16.2	A	O	S	AO
17.2	A	O	S	AO
18.2	A	I	S	AI
19.2	A	O	S	AO
20.2	A	O	S	AO

[a]Prepubertal; [b]Adult; [c]Indoor; [d]Outdoor; [e]Reproductive season; [f]Out of the reproductive season.

3.1 16S rRNA bacterial gene sequencing

Bacteria DNA was extracted from all samples, except for negative controls, that were not sequenced. The mean number of ASVs per sample was 136.47 (standard deviation SD 59.38, minimum 39, maximum 257). The most abundant bacteria phyla in feline vaginal samples were Proteobacteria, Firmicutes, Bacteroidota, and Actinobacteria. Sequences belonging to 359 bacterial genera were detected and the most abundant genera were *Escherichia-Shigella* (mean relative abundance per sample 40.84% ± standard deviation SD 37.68; 80%, N = 16 out of 20 samples), *Streptococcus* (mean relative abundance per sample 7.64% ± SD 13.69; 90%, N = 18 out of 20 samples), *Pasteurella* (mean relative abundance per sample 5.68% ± SD 16.20; 30%, N = 6 out of 20 samples), *Bacteroides* (mean relative abundance per sample 4.49% ± SD 17.02; 35%, N = 7 out of 20 samples), and *Staphylococcus* (mean relative abundance per sample 3.47% ± SD 4.62; 70%, N = 14 out of 20 samples).

Relative abundances based on bacterial genera are reported in *Figure 3*.

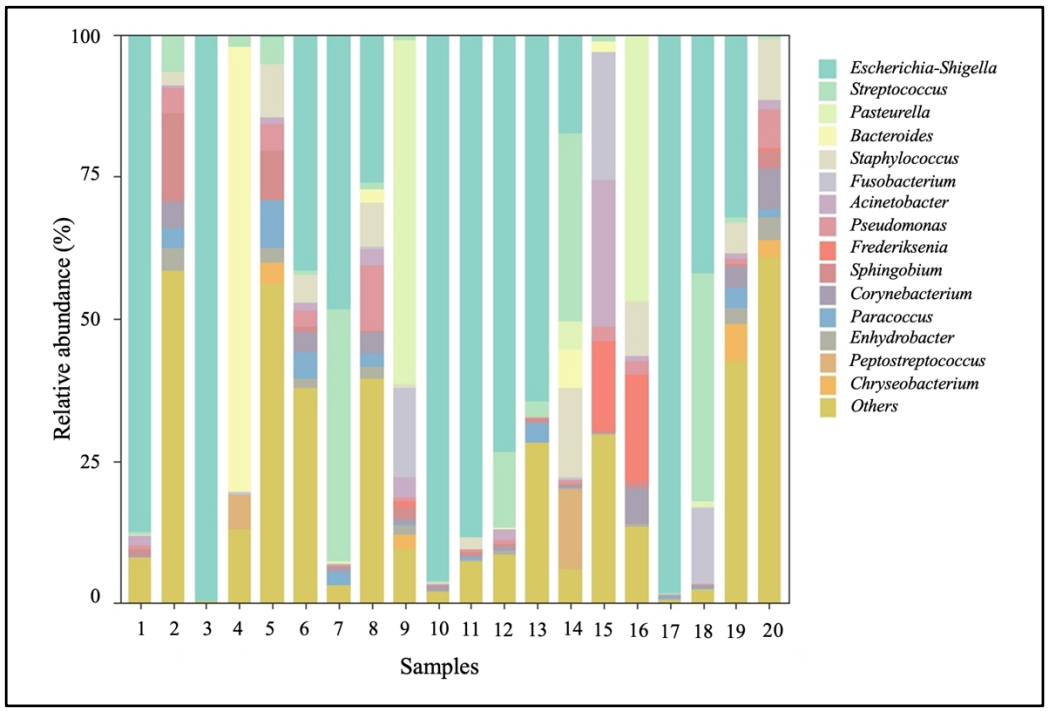

Figure 3. Relative abundance at genus level of the 15 most prevalent bacteria detected by 16S rRNA bacterial gene sequencing from twenty healthy queens are presented. Less relatively abundant bacteria are grouped as 'Others'.

No differences were found in observed ASV richness and in alpha-diversity calculated by Shannon and Simpson indexes based on the group (PO, AO, PI, AI; Kruskal-Wallis rank sum test: observed ASV richness P = 0.62, Shannon index P = 0.62, Simpson index P = 0.82).

Furthermore, when analyzed separately, no differences in alpha-diversity were found based on the living environment (Kruskal-Wallis rank sum test: observed ASV richness P = 0.28, Shannon index P = 0.87, Simpson index P = 0.65; *Figure 4*), age (Kruskal-Wallis rank sum test: observed ASV richness P = 0.82, Shannon index P = 0.76, Simpson index P = 0.70), and reproductive season (Kruskal-Wallis rank sum test: observed ASV richness P = 0.76, Shannon index P = 0.70, Simpson index P = 0.88). Analyses based on the phylogenetic tree showed a significant difference in the vaginal microbiota between indoor and outdoor queens (P = 0.02; *Figure 4*), although no differences were detected based on the age and reproductive season (P = 0.3 and P = 0.5, respectively). Beta diversity using Bray-Curtis statistics (*Figure 5*), Unweighted, and Weighted UniFrac did not reveal any significant difference either among groups (PO, AO, PI, AI; P > 0.5) or based on all the considered factors (*Table 3*).

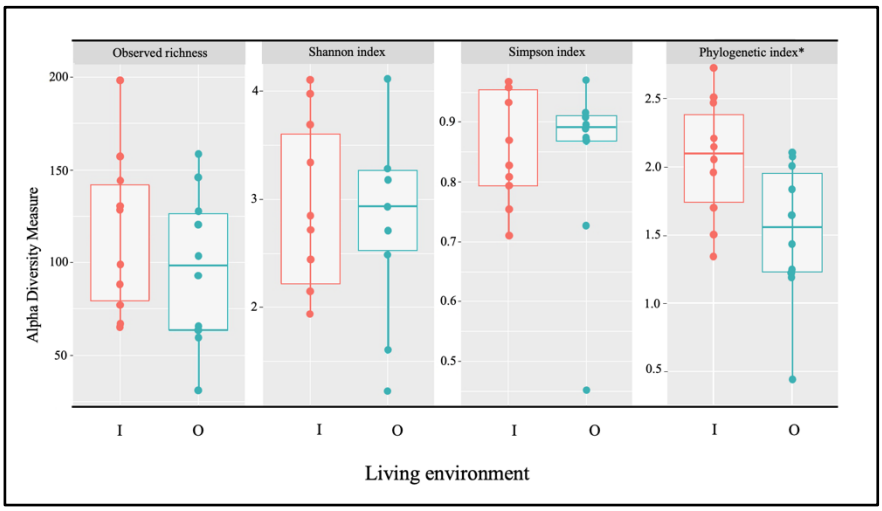

Figure 4. Variation in vaginal microbial diversity based on the living environment (indoor – I, outdoor – O) assessed by observed richness, Shannon, Simpson, and phylogenetic indexes in twenty healthy queens. The two groups of animals showed statistically significant (*) different phylogenetic indexes based on Kruskall-Wallis test (P = 0.02).

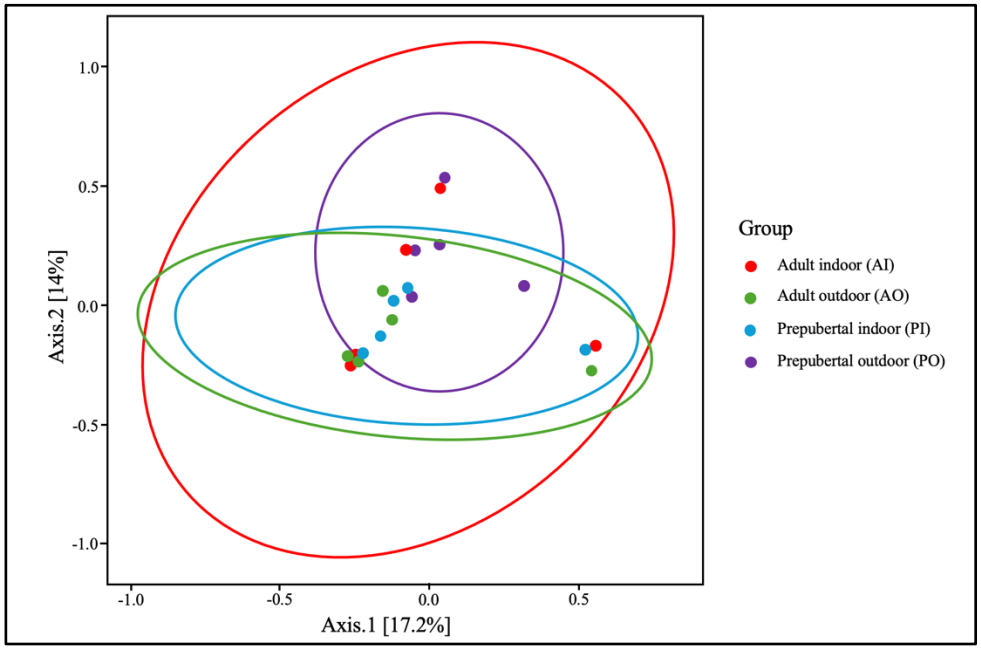

Figure 5. Two dimensions scaling plot by Bray-Curtis based on animal groups considering the age and the living environment of the included queens: adult indoor (AI), adult outdoor (AO), prepubertal indoor (PI), and prepubertal outdoor (AO). The majority of queens clustered together, showing no significant difference in beta-diversity (in-between sample diversity) based on the group.

Table 3. Results for permutational multivariate analysis of variance (PERMANOVA) based on Bray-Curtis, Unweighted, and Weighted UniFrac distances between living environment (indoor and outdoor), age (prepubertal and adult), and reproductive season (in season and seasonal anestrous) groups.

Groups	Bray-Curtis P-value	Unweighted UniFrac P-value	Weighted UniFrac P-value
Living environment (indoor – outdoor)	0.96	0.32	0.35
Age (prepubertal – adult)	0.46	0.55	0.60
Reproductive season (in season – seasonal anestrous)	0.58	0.72	0.75

*Significance for $P < 0.05$

3.2 Bacterial culture

Bacteria were isolated from 85% of the vaginal swabs (N = 17 out of 20). Culture was mixed in 64.7% (11 out of 17 positive plates) and pure in 35.3% of cases. Seven different bacterial species were isolated in culture. The most prevalent species was hemolytic *Escherichia coli* (75%; N = 15 out of 20 queens), which was isolated in pure culture from five out of twenty animals (25%). *Streptococcus canis* was the second most isolated bacterial species (30%, N = 6 out of 20 queens), followed by bacteria belonging to the genus *Enterococcus* (20%, N = 4 out of 20 queens). Finally, *Pasteurella* spp., *Staphylococcus felis*, and *Clostridium perfringens* were isolated one time each (5%, N = 1 out of 20 queens). Culture results are reported in *Table 4*.

Table 4. Bacteria isolation results from twenty queens included in the experiment. Swabs for culture were collected from the vagina of each animal.

Cat	Bacterium 1 Species	Bacterium 1 CFU[a]	Bacterium 2 species	Bacterium 2 CFU[a]
1.2	*Escherichia coli* (hemolytic strain)[c]	50	/	/
2.2	*Escherichia coli* (hemolytic strain)[d]	50	/	/
3.2	*Escherichia coli* (hemolytic strain)[c]	50	/	/
4.2	*Escherichia coli* (hemolytic strain)[c]	>300	*Enterococcus faecium*[d]	>300
5.2	Negative for bacterial growth	/	/	/
6.2	*Escherichia coli* (hemolytic strain)[c]	70	*Enterococcus fecalis*[d]	41
7.2	*Escherichia coli* (hemolytic strain)[c]	14	*Streptococcus canis*[c]	2
8.2	*Escherichia coli* (hemolytic strain)[c]	1	/	/
9.2	Negative for bacterial growth	/	/	/
10.2	*Escherichia coli* (hemolytic strain)[c]	100	/	/
11.2	*Escherichia coli* (hemolytic strain)[c]	4	*Streptococcus canis*[d]	3
12.2	*Escherichia coli* (hemolytic strain)[c]	100	*Clostridium perfringens*[d]	1
13.2	*Escherichia coli* (hemolytic strain)[c]	>300	*Enterococcus spp.*[d]	42
14.2	*Escherichia coli* (hemolytic strain)[c]	>300	*Streptococcus canis*[c]	1[b]
15.2	*Staphylococcus felis*[c]	1[b]	*Streptococcus canis*[c]	1[b]
16.2	Negative for bacterial growth	/	/	/
17.2	*Escherichia coli* (hemolytic strain)[c]	>300	*Streptococcus canis*[c]	4
18.2	*Escherichia coli* (hemolytic strain)[c]	23	*Streptococcus canis*[c]	7
19.2	*Escherichia coli* (hemolytic strain)[c]	>300	*Enterococcus fecalis*[d]	50
20.2	*Pasteurella spp.*[d]	1[b]	/	/

[a]Colony Forming Units; [b]isolation following enrichment in culture broth.
[c]Sequences belonging to the same bacterial genera were detected by 16S rRNA sequencing in samples collected from the same animal; [d]sequences belonging to these bacterial genera were not detected by 16S rRNA sequencing in samples collected from the same animal.

3.3 Comparison between 16S rRNA bacterial gene sequencing and culture

At genus level, culture underestimated bacterial richness. The mean number of bacterial genera detected by sequencing was 39.1 (standard deviation SD ± 16.75), ranging from a minimum of eight and a maximum of 75. The mean number of bacterial genera isolated in culture was 1.4 (standard deviation SD ± 0.75), ranging from none to a maximum of two.

Results for culture and 16S rRNA sequencing yielded matching results in terms of detected bacterial genera in 45% of cases (N = 9 out of 20), meaning that sequences belonging to the genera of the bacteria that were isolated in culture were also detected by sequencing. In 35% of samples sequencing failed to detect one of the two cultured bacterial genera. Finally, in 20% of cases the two techniques yielded completely unmatching results. These results are reported in *Table 4*, together with culture results. Overall, both techniques detected the bacterial genus *Escherichia* as the most common in the queens included in the present study. Nevertheless, the taxonomic depth of sequencing reached genus level, whereas culture led to the identification at species and strain level.

4. Discussion

The present study describes, for the first time, the vaginal microbiota of healthy queens by 16S rRNA sequencing and culture techniques. Since bacterial culture and sequencing were performed simultaneously, two consecutive swabs from the same site were collected. A previous study reported high, though not complete, similarity in repeated feline vaginal swabs for culture (Ström Holst *et al.*, 2003), but no information regarding sequencing was available. Therefore, a preliminary technical study was necessary: since the consecutive samplings led to similar results for both culture and sequencing, the validity of the design of the experiment was confirmed.

Culture led to the isolation of bacteria from most of the samples: the percentage of negative cultures was eight points lower than what was reported in a previous study on adult cats (Ström Holst *et al.*, 2003). It is possible that our mini-swabs are more efficient than those used in the previous experiment, which were moistened with sterile physiological saline solution (Venturi Transystem, Copan Italia, Brescia, Italy) (Ström Holst *et al.*, 2003). However, the same bacteria were isolated, with hemolytic *E. coli* being the prevailing species. In accordance with Ström Holst *et al*. (2003), we can affirm that pure culture, particularly of hemolytic or non-hemolytic *E. coli*, represents a common finding in healthy cats, even in case of high growth and monoculture. Furthermore, *Streptococcus canis* and staphylococci were among the most isolated vaginal bacteria, as in the study by Ström Holst *et al.* (2003) and (Clemetson and Ward, 1990). From a practical perspective, vaginal culture is commonly performed in cases of fertility problems, including conception failure and abortion. However, careful

interpretation of culture results is necessary to diagnose infection, since the presence of bacteria in the vaginal tract is normal, and antimicrobial treatment should be considered based on the history of the animal and clinical symptoms. Molecular analysis and assessment of bacterial population structure can be helpful because imbalances can be related to pathological conditions. Disruption of vaginal bacteria communities may indicate infection, but it can also be due to unwarranted antibiotics administration. Our results from16S rRNA sequencing showed that a distinct microbiota inhabits the vagina of healthy queens, suggesting that negative vaginal culture does not indicate that the vaginal environment is free from bacteria.

The vaginal microbiota of healthy queens resulted primarily composed by Proteobacteria, Firmicutes, Bacteroidota, and Actinobacteria, as analyzed by 16S sequencing at the phylum level. Analyses at the genus level revealed that the most abundant bacterial genera are *Escherichia-Shigella*, *Streptococcus*, *Pasteurella, Bacteroides, and Staphylococcus*. The feline vaginal microbiota obviously differs from the vaginal microbiota of humans, which is typically rich in Lactobacilli (Kumar and Ghosh, 2019), and from that of dogs, in which *Fusobacterium*, *Pasteurellaceae*, *Mycoplasma.* were the most reported bacteria (Lyman *et al.*, 2019; Rota *et al.*, 2020; Hu *et al.*, 2022). Species-specific anatomical and physiological characteristics are the plausible reason of the differences, as the bacterial populations colonizing the healthy vaginal mucosa are shaped by local pH and humidity, and contain members belonging to the typical skin, fecal, oral microbiota of the species, as confirmed by culture (Lansdell *et al.*, 1984) and sequencing (Sturgeon *et al.*, 2014; Swartz *et al.*, 2014; Older *et al.*, 2019; Moon *et al.*, 2023; Pappalardo *et al.*, 2024). In the present study, we included a rectal and an oral swab as positive controls for bacterial DNA extraction and detection by sequencing. However, further research, including paired vaginal, oral, and rectal swabs from the same animals, is needed to assess possible associations between the microbiota of these three niches.

As expected, sequencing results did not always match those obtained by bacterial culture, as the latter underestimated bacterial richness and diversity, with a maximum of two isolated bacterial species per sample. This is not surprising, as culture is based on the use of media that inevitably tend to select some bacterial species (Bonnet *et al.*, 2020). More than 90% of bacterial species are ‘unculturable’, as they do not grow in laboratory conditions (Kaeberlein *et al.*, 2002), and others are classified as ‘fastidious’, because they have very specific needs and slow growth in culture (Rishmawi *et al.*, 2007). Yet, this category includes species implicated in reproductive health and fertility conditions in animals and humans (e.g., *Mycoplasma* spp., *Brucella* spp., *Lactobacillus* spp.). Molecular techniques, including 16S rRNA sequencing, detect the presence of bacterial genetic material within an environment, although nothing is known about bacterial viability (Yagi *et al.*, 2021; Banchi *et al.*,

2024). In the present study, DNA sequences belonging to 359 different bacterial genera were detected by 16S rRNA sequencing, whereas only six were isolated in culture. Unexpectedly, some bacterial genera that were isolated in culture were not sequenced in the matching sample, including *Clostridium* spp., *Enterococcus* spp., *Streptococcus* spp., and *Pasteurella* spp. Although surprising, similar results have already been reported (Lagier *et al.*, 2012; Walker *et al.*, 2015; Sung *et al.*, 2018) and may be explained by the multitude of pre-analytical, analytical, and post-analytical factors influencing the outcome of 16S rRNA sequencing, including the extraction kit, the hypervariable region that is targeted by PCR, and the bioinformatic analyses (Sung *et al.*, 2018).

In the present study differences based of some characteristics on the queens' vaginal microbiota were assessed, including age (prepubertal and adult animals), reproductive season (in season and in seasonal anestrous), and living environment (indoor privately owned and outdoor colony cats).

Prepubertal and post-pubertal/adult queens had similar vaginal microbiota. This is in contrast with findings in bitches (Lyman *et al.*, 2019) and humans (Auriemma *et al.*, 2021), but similar results were obtained in minipigs (Lorenzen *et al.*, 2015). Domestic short hair queens can reach puberty as early as 4 months if the reproductive season begins (Kutzler, 2022). Therefore, post-pubertal 'adult' queens referred for spaying in our investigation could be very young. Our results may more precisely suggest that puberty does not affect the vaginal microbiota of queens.

The second factor that we investigated is season, without finding significant differences between queens during the reproductive season and those in seasonal anestrus, not only by culture but also when analyzing sequencing results, involving all bacterial population DNA. Queens are seasonally polyestrous induced ovulators, having repeated estrus periods during the reproductive season (Johnson, 2022): estrogen stimulation causes fast changes in the vaginal epithelium that could imply also changes in microbial populations. Ström Holst *et al.* (2003) reported a higher prevalence of *Pasteurellaceae* in estrous queens compared to anestrous ones by culture, although this was the only statistically significant difference, and the two groups of animals were unbalanced, with only 10 estrous queens versus 56 anestrous ones. The role of estrogens on vaginal bacterial growth and bacterial flora composition has been reported in women (Brotman *et al.*, 2018) but not in the vaginal microbiota of heifers (Messman *et al.*, 2020) or bitches (Lyman *et al.*, 2019). The canine species can represent a sound comparison for the effects of estrogens because, even if not a seasonal species, it has a long estrous period with prolonged estrogen stimulation of the vaginal mucosa, and a long anestrus (Concannon, 2011). The analogous results in the feline and canine species suggest that the vaginal microbiota is not sensitive to the modifications occurring in the vaginal epithelium because of estrogen stimulation in these species. Furthermore, hormonal deprivation following ovariectomy

did not significantly affect the vaginal microbiota in bitches, although the vaginal epithelium of spayed bitches can show signs of dystrophy compared to intact animals (Rota *et al.*, 2020), as occurs in women with postmenopausal vaginal atrophy (Bride *et al.*, 2010). Progesterone was reported to influence vaginal microbiota modelling in humans (Shen *et al.*, 2022), as does pregnancy (Shen *et al.*, 2022; Symul *et al.*, 2023), and its composition can represent a biomarker for gestational disease as chorioamnionitis, pre-term labor, and miscarriage (Symul *et al.*, 2023). Further research is needed to elucidate whether progesterone and pregnancy may influence the reproductive microbiota in queens, as it is well known that progesterone is involved in the development of some reproductive infections (Hagman, 2023).

Finally, the living environment of the queens may have an effect their vaginal microbiota, as we detected differences in terms of within-groups diversity (i.e., alpha diversity) based on the phylogenetic tree of vaginal bacterial populations of privately-owned in-house queens and stray cats living in feline colonies. The difference in phylogenetic diversity but not in other alpha-diversity indexes suggests a shift in ecological niches in indoor compared to outdoor animals, as differences are driven by the presence of phylogenetically distinct species rather than changes in bacterial richness or evenness. Furthermore, between-groups comparisons (i.e., beta diversity) did not show any difference based on the living environment based on both phylogenetic and non-phylogenetic metrics. Hence, further research is warranted to draw definitive conclusions on the effect of the living environment on the feline vaginal microbiota. In women the environment does not significantly influence the vaginal microbiota (Vargas-Robles *et al.*, 2020; Yao *et al.*, 2021), although mild differences in vaginal microbial composition were found based on ethnicity (Vargas-Robles *et al.*, 2020). In queens, breed would be a factor worth investigating, although this was not possible in the present study, as we enrolled only domestic shorthaired cats to avoid a confounding difference between privately-owned and stray cats, as the latter are obviously not pure-bred animals. The body condition score was not assessed in the present study, as we included only healthy queens, and none of the included animals was emaciated (i.e., BCS below 2/9) or obese (i.e., BCS above 8), although this would be worth investigating as obesity has been shown to influence the vaginal microbiome in women (Garg *et al.*, 2023). Moreover, in the present study, we investigated differences in the vaginal microbiota of queens based on age, season, and living environment as independent factors, although it is possible that they exert combined effects. Therefore, studies using multivariate models on larger populations are needed to draw definitive conclusions on the effect of multiple factors.

5. Conclusions

In conclusion, the vaginal microbiota of healthy queens includes *Escherichia-Shigella, Streptococcus, Pasteurella, Bacteroides*, and *Staphylococcus* as the most abundant bacterial genera. Mixed or monoculture of bacteria such as hemolytic *E. coli, S. canis, S. felis*, and *Enterococcus* spp. are normal findings in healthy animals. A distinct microbiota inhabits the vagina of healthy queens, and the absence of bacteria following vaginal culture does not indicate that the vaginal environment is free from bacteria. Antimicrobial treatments may unbalance these bacterial populations, possibly being more damaging than beneficial, although studies assessing the effect of antibiotics on the vaginal microbiome are needed. Age and reproductive season do not influence the vaginal microbial flora, whereas the living environment is a factor worth considering, according to these preliminary results. As research on larger populations is needed, future investigation should unveil the role of breed or pregnancy and, primarily, should describe possible changes within the vaginal microbiota in case of reproductive diseases.

6. Declarations

Declaration of competing interest: none.

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Ethical approval and consent to participate: Approval was obtained by the Ethical Committee of the Department of Veterinary Sciences of the University of Turin (Italy) (n. 0000284 - 01/02/2023). All the owners and cat colony managers provided informed written consent, and the procedures were carried out in accordance with the EU Directive 86/609/CEE and with the guidelines of the Italian Ministry of Health for the care and use of animals (D.L. 4 March 2014 n. 26 and D.L. 27 January 1992 n. 116).

Author contribution statement: PB designed the project; PB and AB examined the included animals and collected the samples; MC and ES performed the bacterial culture; AP and FG optimized the extraction protocol; AP adapted the primers; FG extracted DNA samples and performed the amplifications for sequencing; AP and GGD performed the data analysis; PB drafted the manuscript; AR and AB edited the manuscript; AR supervised the project.

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Summary

The microbiome of an individual may be a key player in the Developmental Origins of Health and Disease (DOHaD) theory, possibly influencing the future health of the animal in question. It has a crucial role in many life functions, including nutrition and immunity, and early life gut bacterial communities have been shown to be implicated in later disease in different species. Traditionally, sterility of the uterine environment is believed to be necessary for a successful healthy pregnancy. This dogma has been challenged in humans and other animal species, suggesting that *in utero* bacterial colonization may happen during fetal life. This thesis focuses on the time and dynamics of pioneering bacterial colonization in dogs and cats, investigating whether the placenta, amniotic fluid, and meconium of canine and feline fetuses and newborns host a resident microbiota.

Chapter 1 provides a comprehensive background on the microbiome's role in health, particularly in the context of the DOHaD theory. This chapter discusses the microbiome as a dynamic ecosystem that interacts with its host, contributing to his well-being. The microbiome is considered as a potential factor influencing the risk of future diseases in animals. Research on the feto-maternal microbiome in humans and domestic species is summarized in this chapter. Specifically, the limitations of current research and the anatomical and physiological differences of the feto-maternal connection between different species are discussed, emphasizing the need for species-specific research to investigate the feto-maternal microbiome in small animals. The chapter also introduces the technical challenges in detecting and analyzing bacterial presence in low-biomass samples like those found in the fetal environment.

Chapters 4, 5, and 6 of the thesis present the experimental work. Chapter 4 investigates the presence of bacteria in canine and feline feto-maternal units at term during elective cesarean sections using 16S rRNA gene sequencing and bacterial culture, in samples derived from the placenta, amniotic fluid, and meconium. The findings reveal the presence of bacterial DNA in these tissues, challenging the traditional view of a sterile fetal environment. However, the study also acknowledges the limitations of these findings, particularly the risk of contamination of low biomass samples during collection and processing. The chapter suggests that while bacterial DNA is present, the viability and significance of these bacteria remain uncertain, warranting further investigation.

Chapter 5 presents an improved study protocol, based on the results presented in Chapter 4. Specifically, the study aimed to detect bacteria in canine and feline fetuses during mid-gestation. This chapter addresses the issues associated with working with low-biomass samples, by collecting multiple control samples which are indicative for contamination control, and at the same time incorporating fluorescence in situ hybridization (FISH) as an additional technique to visualize bacteria within the placenta. This optimized protocol successfully detected bacterial DNA in mid-gestation fetuses but does not confirm the presence of a resident microbiome. Specifically, the placenta may be reached by a limited number of viable bacteria but since it works as a filter, it may avoid the establishment of a thriving microbiota within the fetal environment. The chapter also acknowledges the possibility for bacterial DNA fragments to have functional implications on fetal development, although this assumption still needs to be investigated.

Chapter 6 explores the influence of the birth process on the microbiome of newborn puppies, investigating whether the passage through the vaginal canal shapes the pioneer microbiome of naturally delivered puppies. Using 16S rRNA gene sequencing and bacterial culture, the study compares the meconium microbiota of naturally delivered puppies with the rectal and vaginal microbiome of their dams. The findings suggest that naturally delivered puppies are mainly colonized by bacteria from the vaginal canal. This chapter highlights the importance of the birth process in shaping the early microbiome and hypothesizes that the mode of delivery could also significantly influence the composition of the neonatal microbiome. This should be further investigated.

Conclusions based on the research conducted during this PhD project are presented in Chapter 7. The thesis concludes that while bacterial DNA is present within the feto-maternal unit in small animals, the significance of these fragments remains unclear. The fetus is not colonized by a thriving community of bacteria during gestation, although some bacterial components of uncertain origin can reach it.
The limitations and challenges of research on the feto-maternal microbiome are also discussed using a practical approach. The chapter emphasizes the need for further studies to determine whether bacterial fragments play a role in fetal development and future health of the offspring, and to fully understand the role of the dam and that of the mode of delivery.

The thesis also includes an appendix that reports pioneer research on the reproductive microbiome in small animals. Chapter 1 of the appendix is a review of the canine reproductive microbiome in both

male and female dogs. It discusses the current understanding of the vaginal and uterine microbiomes in female dogs and the seminal microbiome in male dogs, highlighting their potential roles in reproductive health and disease. The chapter also examines the controversies surrounding the reproductive microbiome, such as the roles of *Mycoplasma* and *Ureaplasma*, the impact of antibiotics on fertility, and some promising avenues for future research.

Chapters 2 and 3 of the appendix present original research on the semen microbiota in dogs and the vaginal microbiota in cats, respectively. Specifically, Chapter 2 examines the composition of the microbiota in canine semen, showing that the living environment affects the semen microbiota, and that the bacterial composition of the sperm-rich fraction is similar to that of the prostatic fraction in healthy stud dog. Chapter 3 explores the vaginal microbiota in cats, revealing that, once again, the living environment may influence the vaginal microbiota of queens, which remains consistent across different reproductive seasons. Both chapters contribute to the growing body of knowledge on the reproductive microbiome in small animals and represent a starting point for further research in this area.

Overall, this thesis makes a significant contribution to the field of reproductive microbiome research in small animals. By questioning the traditional belief in a sterile fetal environment and investigating the impact of the microbiome on reproductive health, this work opens new research pathways and provides important insights into the challenges within this field of study.

Samenvatting

Het microbioom van een individu kan een belangrijke rol spelen in de “Developmental Origins of Health and Disease (DOHaD)” theorie, en mogelijk de toekomstige gezondheid van het dier in kwestie beïnvloeden. Het speelt een cruciale rol bij veel levensfuncties, waaronder voeding en immuniteit, en van bepaalde bacteriële populaties in de darm is aangetoond dat ze betrokken zijn bij latere ziektes bij verschillende diersoorten. Traditioneel wordt aangenomen dat steriliteit van de baarmoederomgeving noodzakelijk is voor een succesvolle en gezonde zwangerschap. Dit dogma is ter discussie gekomen bij de mens en andere diersoorten, en er werd gesuggereerd dat bacteriële kolonisatie in utero al kan plaatsvinden tijdens het foetale leven. Dit proefschrift richt zich op de primaire bacteriële kolonisatie bij honden en katten, waarbij onderzocht wordt of de placenta, het vruchtwater en het meconium van foetussen en pasgeborenen van honden en katten al een microbioom herbergen.

Hoofdstuk 1 biedt een uitgebreid inzicht in de rol van het microbioom wat de gezondheid van het dier betreft, met name in de context van de DOHaD-theorie. Dit hoofdstuk bespreekt het microbioom als een dynamisch ecosysteem dat interageert met zijn gastheer en bijdraagt aan zijn welzijn. Het microbioom wordt beschouwd als een belangrijke factor die het risico op toekomstige ziekten bij dieren kan beïnvloeden. Onderzoek naar het foetomaternale microbioom bij mensen en gedomesticeerde diersoorten wordt in dit hoofdstuk samengevat. Specifiek worden de beperkingen van het huidig onderzoek en de anatomische en fysiologische verschillen van de foetomaternale interactie bij verschillende diersoorten besproken, waarbij de nadruk wordt gelegd op de noodzaak van soort specifiek onderzoek om het foetomaternale microbioom bij kleine huisdieren te onderzoeken. Het hoofdstuk bespreekt ook de technische uitdagingen die optreden bij het detecteren en analyseren van de aanwezigheid van bacteriën in stalen met een lage biomassa, zoals welke gevonden worden in de foetale omgeving.

Hoofdstukken 4, 5 en 6 van het proefschrift presenteren het experimentele werk. Hoofdstuk 4 onderzoekt de aanwezigheid van bacteriën in aparte compartimenten van foetussen, placenta’s en vruchtwater van honden en katten aan het einde van de dracht tijdens electieve keizersneden met behulp van 16S rRNA-gen-sequencing en bacteriële kweek, in monsters afkomstig van de placenta, het vruchtwater en het meconium. De bevindingen tonen de aanwezigheid van bacterieel DNA aan in deze weefsels, wat in tegenspraak is met de traditionele opvatting dat de foetale omgeving steriel is. De studie gaat ook dieper in op de beperkingen van deze bevindingen, met name het risico op

contaminatie van lage biomassa samples tijdens verzameling en verwerking. Het hoofdstuk suggereert dat, hoewel er bacterieel DNA gevonden werd, de vitaliteit en het belang van deze bacteriën niet is aangetoond, wat verder onderzoek noodzakelijk maakt.

Hoofdstuk 5 presenteert een verbeterd studieprotocol, gebaseerd op de resultaten uit hoofdstuk 4. Specifiek richtte de studie zich op het aantonen van bacteriën in foetussen van honden en katten tijdens het midden van de dracht. Dit hoofdstuk behandelt de problemen die optreden tijdens het werken met stalen met een lage biomassa, dit door meerdere controlestalen te verzamelen die indicatief zijn voor contaminatie, en tegelijkertijd fluorescentie in situ hybridisatie (FISH) als extra techniek toe te passen om bacteriën binnen de placenta te visualiseren. Dit geoptimaliseerde protocol detecteerde met succes bacterieel DNA in foetussen in het midden van de dracht, maar bevestigt niet dat er een lokaal microbioom aanwezig is. Meer bepaald kan de placenta waarschijnlijk door een beperkt aantal vitale bacteriën bereikt worden, maar omdat ze werkt als een filter, kan ze de vestiging van een vitaal microbioom binnen de foetale omgeving voorkomen. Het hoofdstuk erkent ook de mogelijkheid dat bacteriële DNA-fragmenten functionele implicaties kunnen hebben op de foetale ontwikkeling, maar dit vereist verder onderzoek.

Hoofdstuk 6 onderzoekt de invloed van het geboorteproces op het microbioom van pasgeboren puppy's, waarbij wordt onderzocht of tijdens de passage door het geboortekanaal het eerste microbioom van natuurlijk geboren puppy’s gevormd wordt. Met behulp van 16S rRNA-gen-sequencing en bacteriële kweek vergelijkt de studie de meconium microbiota van natuurlijk geboren puppy's met het rectale en vaginale microbioom van hun moeders. De bevindingen suggereren dat natuurlijk geboren puppy's voornamelijk worden gekoloniseerd door bacteriën uit het geboortekanaal. Dit hoofdstuk benadrukt het belang van het geboorteproces bij het vormen van het vroege microbioom en onderschrijft de mogelijkheid dat de wijze van werpen ook een significante invloed zou kunnen hebben op de samenstelling van het neonatale microbioom. Dit moet verder onderzocht worden.

Conclusies op basis van het onderzoek uitgevoerd tijdens dit PhD-project worden gepresenteerd in hoofdstuk 7. Het proefschrift concludeert dat hoewel bacterieel DNA aanwezig is binnen de foetomaternale eenheid bij kleine dieren, de betekenis van deze fragmenten onduidelijk blijft. De foetus wordt niet gekoloniseerd door een vitale populatie van bacteriën tijdens de dracht, hoewel sommige bacteriële componenten van onzekere oorsprong de foetus kunnen bereiken. De beperkingen en uitdagingen van onderzoek naar het foetomaternale microbioom worden ook besproken vanuit een meer praktische benadering. Het hoofdstuk benadrukt dat verdere studies nodig zijn om te bepalen of bacteriële fragmenten een rol spelen in de foetale ontwikkeling en toekomstige

gezondheid van het nageslacht, en om de rol van de moeder en die van de wijze van werpen volledig te begrijpen.

Het proefschrift bevat ook een appendix die baanbrekend onderzoek rapporteert over het reproductieve microbioom bij kleine huisdieren. Hoofdstuk 1 van de appendix is een review van het reproductieve microbioom bij zowel reuen als teven. Het bespreekt de huidige stand van zaken van het vaginale en uteriene microbioom bij teven en het seminale microbioom bij reuen, waarbij hun potentiële rol in reproductieve gezondheid en ziekte wordt belicht. Het hoofdstuk onderzoekt ook de controverses rond het reproductieve microbioom, zoals de rol van Mycoplasma en Ureaplasma, de impact van antibiotica op vruchtbaarheid en enkele veelbelovende richtingen voor toekomstig onderzoek.

Hoofdstukken 2 en 3 van de appendix presenteren origineel onderzoek naar het sperma-microbioom bij honden en het vaginale microbioom bij katten. Specifiek onderzoekt hoofdstuk 2 de samenstelling van het microbioom in sperma van honden, waarbij wordt aangetoond dat de leefomgeving het sperma-microbioom beïnvloedt, en dat de bacteriële samenstelling van de zaadrijke fractie vergelijkbaar is met die van de prostaatfractie bij gezonde dekreuen. Hoofdstuk 3 onderzoekt het vaginale microbioom bij katten, waarbij wordt aangetoond dat, wederom, de leefomgeving invloed lijkt te hebben op het vaginale microbioom van poezen, dat consistent blijft gedurende verschillende voortplantingsseizoenen. Beide hoofdstukken dragen bij aan de groeiende kennis over het reproductieve microbioom bij kleine dieren en vormen een startpunt voor verder onderzoek op dit gebied.

Al met al levert dit proefschrift een significante bijdrage aan het onderzoek naar het reproductieve microbioom bij kleine dieren. Door de traditionele overtuiging van een steriele foetale omgeving in twijfel te trekken en het effect van het microbioom op reproductieve gezondheid te onderzoeken, opent dit werk nieuwe perspectieven en biedt het belangrijke inzichten in de uitdagingen binnen dit onderzoeksveld.

Curriculum vitae

Penelope Banchi was born on September 27th, 1993, in Genova, Italy. She obtained her Doctor of Veterinary Medicine (DVM) degree from the University of Turin, Italy, in 2019. After her graduation she worked as a veterinarian at the Veterinary Teaching Hospital of the University of Turin, where she gained valuable experience in clinical and teaching activities related to exotic animals and wildlife. In 2020, she advanced her career as a research fellow at the same university first focusing on pain assessment in rabbits (for which she gained the Dechra Analgesia Prize in 2022) and then starting a research path in canine and feline reproduction. Alongside these roles, she started a residency of the European College of Animal Reproduction (ECAR) in 2021. In this context, she completed an externship at the École Nationale Vétérinaire d'Alfort in Paris (France), where she received clinical training in small animal reproduction. Later in 2021, she took on a new role as a research assistant at Ghent University in Belgium, where she started a joint Ph.D. under the supervision of Prof. Dr. Ann Van Soom and Prof. Dr. Ada Rota. Throughout these positions, she has engaged in clinical, research, and teaching activities, contributing significantly to the understanding of the reproductive microbiome and other aspects of small animal reproduction. During this time, she was awarded a grant from the American Kennel Club (AKC) and a grant from the European Society for Small Animal Reproduction (EVSSAR).

She is author and co-author of several publications in international peer-reviewed journals and abstracts for international conferences. Her goal is to continue her academic career, contributing to the advancement of veterinary reproductive medicine.

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