

Uterine bacterial sterility in non-pregnant domestic cats – a pilot study

Esterilidade bacteriana uterina em gatas domésticas não gestantes – um estudo piloto

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ABSTRACT – In various physiological and pathological conditions, the uterine bacteriome in mammals generates debates regarding the presence of a permanent microbiome. In this sense, microbiome studies have been conducted in the upper reproductive tract in some species using modern molecular biology techniques. However, this knowledge is still lacking for cats. Thus, this study aimed to investigate the presence of a uterine bacteriome in healthy non-pregnant cats by investigating the 16S rRNA gene. Uterine fragments were collected from five healthy cats after total hysterectomy with salpingo-oophorectomy (HSO). After DNA extraction, the samples were subjected to conventional PCR amplification of the V3 and V4 regions of the 16S rRNA gene. Our analysis revealed no detectable amplification in the V3 and V4 regions of the 16S rRNA gene in any of the samples. These findings support the hypothesis that the feline endometrium may be sterile or harbor an ultra-low microbial biomass. Further studies with larger, more representative sample sizes are essential to validate these findings and to gain a deeper understanding of the uterine microbiome under different physiological conditions.

RESUMO – Em diversas condições fisiológicas e patológicas, o bacterioma uterino em mamíferos gera debates a respeito da presença de um microbioma permanente. Nesse sentido, os estudos vêm sendo conduzidos sobre o microbioma do trato reprodutivo em algumas espécies, utilizando técnicas modernas de biologia molecular. Entretanto, essas informações ainda não foram descritas em gatas. Desta forma, este estudo objetivou investigar a presença de um bacterioma uterino em gatas saudáveis e não gestantes, através da investigação do gene 16S rRNA. Fragmentos uterinos foram coletados de cinco gatas saudáveis após histerectomia total com salpingo-ooforectomia. Após a extração do DNA, as amostras foram submetidas à PCR convencional para amplificação das regiões V3 e V4 do gene 16S rRNA. Nossas análises revelaram ausência de amplificação detectável das regiões V3 e V4 do gene 16S rRNA em todas as amostras. Esses achados reforçam a hipótese de que o endométrio felino pode ser estéril ou abrigar uma biomassa microbiana em níveis ultrabaixos. Estudos futuros com tamanhos amostrais maiores e mais representativos são essenciais para confirmar esses resultados e compreender melhor o microbioma uterino em diferentes condições fisiológicas.

Keywords: 16S rRNA. Feline. Uterine environment. Bacteriome.

Palavras-chave: 16S rRNA. Felino. Ambiente uterino. Bacterioma.

Conflict of interest: The authors declare no conflict of interest related to the publication of this manuscript.



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INTRODUCTION

After decades, the "dogma" of the healthy uterus as a sterile environment was put into the discussion after research confronted data from the early twentieth century (BAKER; CHASE; HERBST-KRALOVETZ, 2018). Despite the lack of consensus in humans, studies are being developed on the uterine microbiome in physiological and pathological conditions, bringing new observations regarding the repercussions on fertility and reproductive losses (FRANASIAK et al., 2016; VERSTRAELEN et al., 2016). However, we know more about the uterine microbiome of women than about the microbial communities that reside in the uterus of other species (HOLYOAK; LYMAN, 2021). The healthy uterine microbiome has been researched in animals through culture techniques, microscopy (ASADI et al., 2023; CLEMETSON; WARD, 1990; SCHULTHEISS et al., 1999; STRÖM HOLST et al., 2003), and high-throughput next-generation sequencing (NGS) (AULT et al., 2019; HOLYOAK et al., 2022; LI et al., 2022; LYMAN et al., 2019; MOORE et al., 2017; ZOU et al., 2020).

Among the NGS methods commonly used in microbiome research, 16S rRNA gene sequencing is particularly prevalent because it is universal in bacteria and contains both conserved and variable regions (ALESSANDRI et al., 2020). These variabilities allow discrimination between different microorganisms by polymerase chain reaction (PCR) and 'universal' primers directed to conserved regions to amplify the greatest possible variety of microorganisms. The 16S rRNA gene sequence is about 1,550 bp long. However, for most bacterial isolates, the initial 500 bp sequence provides adequate differentiation for sufficient taxonomic identification at the genus level (WEINROTH et al., 2022).

This technique allows the extensive recovery of genetic material,

generating perspectives on the sterility and biological viability of the identified bacterial groups (WEINROTH et al., 2022). On the other hand, it still minimizes the greatest limitation of cultivation techniques, given that it is estimated that only 10% of bacteria are cultivable. Even so, it is necessary to emphasize that, regardless of the method used in the research, for example, there is still no consensus on whether the healthy uterus of women is sterile since most studies have involved pathological conditions (BAKER; CHASE; HERBST-KRALOVETZ, 2018; PEREZ-MUÑOZ et al., 2017).

Studies sought to identify microbial diversity in healthy wombs of cats at various stages of the estrus cycle using traditional cultivation techniques (CLEMETSON; WARD, 1990; SCHULTHEISS et al., 1999; STRÖM HOLST et al., 2003). However, studies have not yet described the bacteriome of the reproductive tract using next-generation sequencing techniques in cats. Therefore, this study aimed to investigate the presence of a microbial community in the uterus of healthy non-pregnant cats by analyzing the 16S rRNA gene by NGS.

MATERIALS AND METHODS

Biological samples

The research and methodology were approved by the Ethics Committee in Animal Use (CEUA-UFERSA) under license number 23091.006326/2014-88. Five healthy non-pregnant cats were randomly recruited from the veterinary service of the Federal Rural University of Semi-Arid Region in Mossoró, Rio Grande do Norte, Brazil. The animals were clinically evaluated for a total hysterectomy with salpingo-oophorectomy (HSO) to collect samples of uterine body fragments. Peripheral blood was collected for complete blood count (CBC) and preoperative clinical biochemistry. Notably, none of the animals had recently been exposed to antibiotics. The cats were induced and anesthetized with a combination of acepromazine, methadone, propofol, midazolam, and

isoflurane. The reproductive tract was removed using a sterile surgical technique. Subsequently, three fragments of the uterine body (~0.25 cm x 0.25 cm) were obtained with sterile Metzenbaum scissors and stored in individual sterile microtubes of 1.5 mL containing RNA Later® (Thermo Fisher Scientific) in the proportions recommended by the manufacturer. Finally, after proper identification, the samples were transported in Styrofoam boxes with dry ice to the Molecular Biology Laboratory of the Biotechnology Institute (IBTEC), São Paulo State University (UNESP), Botucatu, SP, Brazil.

Extraction of genetic material and quantification

Samples were weighed individually (25 mg) and macerated in the L-Beater 6® homogenizer (Loccus, Cotia, SP, Brazil) using 3.0 mm zirconium beads (Loccus, Cotia, SP, Brazil) and 160 µL PBS buffer pH 7.4.

Total DNA was extracted from 160 µL of macerated tissue using the ReliaPrep gDNA tissue Miniprep System® commercial kit (Promega), following the manufacturer's instructions. The genetic material was eluted in 50 µL of nuclease-free water and stored at -20 °C until processing.

DNA was quantified using the Qubit™ dsDNA BR Assay Kit® (Thermo Fisher Scientific), using 2 µL per sample and following the manufacturer's recommendations.

PCR of gene fragments

Conventional PCR reactions were performed for specific gene fragments of felines of different sizes to evaluate the integrity of the extracted DNA using the MYBPC3 and PKD1 genes (LONGERI et al., 2013; LYONS et al., 2004; WESS et al., 2010). The reactions were standardized to the final volume of 25 µL, containing 12.5 µL of 2x GoTaq Green G2 MasterMix (Promega) for each reaction, 0.75 µL of each primer (10µM), and 2.5 µL of DNA. The primers and PCR conditions are detailed in Table 1. All reactions had a positive (feline sample) and negative (nuclease-free water) control.

Table 1. Primers and respective PCR conditions used in the study to detect endogenous gene fragments from cats.

Genic fragment	Primers	Product size (bp)	PCR conditions	Reference
MYBPC3	5'AGT CTC AGC CTT CAG CAA GAA GCC 3' 5'GGT CAA ACT TGA CCT TGG AGG AGC C 3'	251	95°C, 5 min; 35 cycles (95°C 60 sec, 60°C 30 sec, 72°C 60 sec); 72 °C, 5 min	(WESS et al., 2010)
MYBPC3	5'GCT GGT GAC ACG ATC AGA A 3' 5'CTC ACT TCC TTC CTC TGT GAA A 3'	644	95°C, 5 min; 35 cycles (95°C 60 sec, 56°C 30 sec, 72°C 60 sec); 72 °C, 5 min	(LONGERI et al., 2013)
PKD1	5'CAG GTA GAC GGG ATA GAC GA 3' 5'TTC TTC CTG GTC AAC GAC TG 3'	558	95°C, 5 min; 40 cycles (95°C 60 sec, 59°C 30 sec, 72°C 60 sec); 72 °C, 5 min	(LYONS et al., 2004)

The amplified products were subjected to electrophoresis in a 1.5% agarose gel (weight/vol) containing SYBR™ Safe DNA gel stain (1 µL/10 mL) (Thermo Fisher Scientific) and visualized in a transilluminator UV light (GE, ImageQuant™ LAS 500). The bands were compared with a standard molecular weight 100bp DNA Ladder H3 RTU (Bio-Helix, Taiwan).

Amplification of the 16S rRNA gene

The DNA samples were subjected to amplification of the V3 and V4 regions of the bacterial 16S rRNA gene according to the Illumina 16S Metagenomic Sequencing Library Preparation protocol® (Illumina, San Diego, CA, USA). We used a positive sample for *Salmonella* spp. and

nuclease-free water as positive and negative controls, respectively. After the reaction, all products were subjected to 1.5% agarose gel electrophoresis (weight/vol), containing SYBR™ Safe DNA gel stain (1 µL/10 mL) (Thermo Fisher Scientific) and visualized in a transilluminator UV light (GE, ImageQuant™ LAS 500).

The KAPA HiFi HotStart ReadyMix KAPA kit (Biosystems, Wilmington, MA, USA) and Agencount AMPure XP® beads (Beckman Coulter Genomics, Brea, CA, USA) were used for PCR and purification after amplification, respectively. The initial PCR was performed using primers specific to the region of interest and that have compatibility with the index and Illumina sequencing adapters, forward primer: 5'-TCGTCGGCAGCGTCAGATGTGTATAAGAGA CAGTCGTCGGCAGCGTCAGATGTGTATAAGAGACAG CCTACGGGNGGCWGCAG-3'; reverse primer: 5'-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGT

CTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACT ACHVGGGTATCTAATCC-3'.

RESULTS AND DISCUSSION

The characteristics of included felines are detailed in Table 2. No alterations were observed in preoperative CBC and biochemistry analysis. In this study, the 16S rRNA gene did not amplify in the reactions by conventional PCR, making genomic sequencing impossible for further evaluation as performed in other research (FRANASIAK et al., 2016). Under the conditions of this study, these data suggest bacterial uterine sterility or ultra-low mass in five healthy non-pregnant cats, as recently described for the lower urinary tract of felines (BALBONI et al., 2020).

Table 2. General characteristics of the cats included in the study.

Animal	Age (years)	Breed	Weight (kg)	Reproductive history	History of reproductive failure
1	1	Mixed-breed	2.7	Nulliparous	No
2	1	Mixed-breed	2.5	Nulliparous	No
3	2	Mixed-breed	2.8	1 birth	No
4	1	Mixed-breed	2.0	Nulliparous	No
5	1	Mixed-breed	3.1	2 births	No

This study was initially proposed with the 16S rRNA gene sequencing technology, which is widely used to describe microbiomes in healthy pregnant and non-pregnant uteri in some species (AULT et al., 2019; HOLYOAK et al., 2022; LI et al., 2022; LYMAN et al., 2019; MOORE et al., 2017; ZOU et al., 2020). This research method is more accessible and employs universal primers to amplify and subsequently sequence one or more hypervariable regions of the 16S rRNA gene, a conserved phylogenetic marker composed of highly conserved sequences interspersed with nine hypervariable regions.

The lack of standardization regarding the most appropriate method for extracting genetic material for microbiome studies can be a challenge to the reproducibility and repeatability of research aiming to describe microbiomes. Thus, more standardization studies are needed to solve this issue (ALESSANDRI et al., 2020; MOOSSAVI et al., 2021).

Cell lysis is key in DNA extraction since it must guarantee access to the genetic material of all cells present in the evaluated samples (PEREZ-MUÑOZ et al., 2017). In this study, despite the lack of published cell lysis methods for uterine fragments of female cats, we used the combination of two methods to optimize DNA extraction: tissue maceration with 3.0 mm zirconium beads (mechanical) and Proteinase K (enzymatic) from the ReliaPrep gDNA Tissue Miniprep System® commercial kit.

It is worth mentioning that the mechanical technique used is considered excellent for lysing even the most complex bacterial cells, such as gram-positive and endospores. However, it has the possible disadvantage concerning the maintenance of DNA integrity if it induces the formation of

short fragments, leading to false-negative results in amplifying the 16S rRNA gene (WEINROTH et al., 2022). However, in addition to using positive and negative controls in amplification reactions, we performed conventional PCR of feline endogenous genes as an internal extraction control.

The DNA's integrity is crucial to ensure the PCR's success. This criterion is more challenging the larger the size of the sequence of interest (XIA et al., 2018). In this study, we amplified endogenous genes with sizes similar to 16S rRNA targets.

The recommendation in research with evaluations of endogenous genes is that at least two types be used (EISENBERG; LEVANON, 2013). We successfully amplified three gene fragments of different sizes (between 251 and 644bp) (LONGERI et al., 2013; LYONS et al., 2004; WESS et al., 2010). Therefore, the absence of amplification of the 16S rRNA gene is probably not linked to any bias regarding the quality of the extracted genetic material. In addition, all samples presented sufficient DNA yields (5 ng/µL) to perform the 16S rRNA gene amplification protocol (PSIFIDI et al., 2015) (Figure 1).

The possibility of contamination from sampling to laboratory processing can be a challenge for molecular techniques since there is low reliability with samples with low levels of DNA due to possible bacterial contamination of reagents and other inputs, which would generate the so-called "kitome" (WEISS et al., 2014; BAKER; CHASE; HERBST-KRALOVETZ, 2018; WEINROTH et al., 2022). Given the absence of detectable amplification of bacterial genetic material in our samples, it is possible to suggest that all our procedures were within sterility standards, with no

contamination during sample processing and handling. Thus, the surgical instruments and microtubes used in collecting and packaging the samples were autoclaved. Notably, maintaining intact bacterial genetic material through PCR after

autoclaving has already been published. However, our results do not support this possibility under the conditions of this study (YAP; GOLDSMITH; MOORE, 2013).

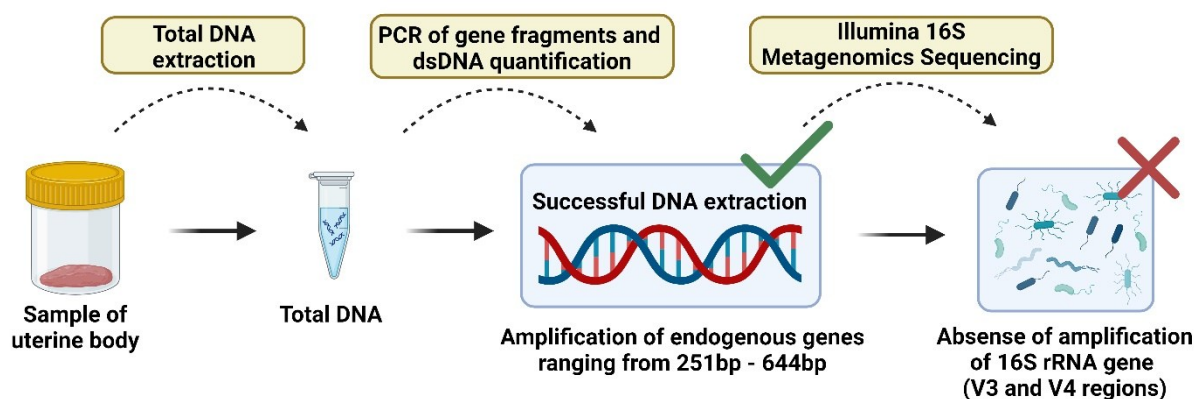


Figure 1. Exploring the uterine microbiome in healthy cats: Methodology reveals absence of microbiota or low bacterial mass.

Research on the potential microbiome of the feline uterus is scarce. Our findings are consistent with a previous study that reported no bacterial growth from uterine swabs (STRÖM HOLST et al., 2003). However, earlier studies reported the presence of a bacterial community in the uterus of healthy cats, with bacterial growth observed in approximately 10% (4 out of 37 cats) (SCHULTHEISS et al., 1999) and 7% (2 out of 29) (CLEMETSON; WARD, 1990). For the authors of these studies, low uterine biomass and sample contamination were considered causes of these results. With these important caveats, we can say that our result agrees with these studies because we did not detect bacterial genetic material, even using an independent high-yield culture technology, without contamination in our procedures.

Complementing this, research involving the uterine microbiome also addresses issues related to the uterus in the gestational period (PEREZ-MUÑOZ et al., 2017; ROTA et al., 2021). Thus, it is worth noting that the existence of the placental microbiome in mammals is also the subject of several studies, presenting a lack of scientific consensus. Researchers who argue that the healthy uterus is sterile regardless of its physiological state believe that the evidence for a placental microbiome is inconsistent, which reinforces our result (BAKER; CHASE; HERBST-KRALOVETZ, 2018; PEREZ-MUÑOZ et al., 2017).

However, despite our findings, if a microbiome does exist in the womb, the placental and uterine microbiomes are likely to be closely related (BARDOS et al., 2020). Our study suggests that the cat's placenta should also be sterile in healthy pregnancies.

The debate is advancing, and although our results do not support this hypothesis, studies do not rule out that the mammalian placenta may harbor a microbiome by translocating bacteria from the intestines or oral cavity, as demonstrated in mice, specifically during the gestational period (PEREZ et al., 2007). In this context, it is possible to

investigate the possibility of the non-pregnant uterus being sterile and harboring a detectable transient microbiome in the gestational period.

It is known that the uterus has a complex immune system with its own characteristics and response patterns that fluctuate as a consequence of hormonal changes. The predominant cells are T lymphocytes, macrophages/dendritic cells, NK cells, neutrophils, and mast cells (BARDOS et al., 2020). However, we still lack research on the composition and how these cells behave in the feline uterus according to its estrus cycle to maintain bacterial sterility or ultra-low bacterial biomass.

CONCLUSION

Under the conditions of this study, no bacterial DNA was detected in the endometrial samples from the uterine body of healthy, non-pregnant cats. These findings support the hypothesis that the feline endometrium may be sterile or harbor an ultra-low microbial biomass. However, given the limited number of samples analyzed ($n = 5$), definitive conclusions cannot be drawn. Further studies with larger and more representative sample sizes are essential to confirm these findings and better understand the uterine microbiome in different physiological conditions. Investigations involving healthy pregnant cats may also help clarify whether a sterile uterine environment is maintained during gestation.

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REFERENCES

- ALESSANDRI, G. et al. Catching a glimpse of the bacterial gut community of companion animals: a canine and feline perspective. **Microbial Biotechnology**, 13: 1708-1732, 2020.
- ASADI, B. et al. Isolated bacteria from the uteri of camels with different reproductive backgrounds: A study on sampling methodology, prevalence, and clinical significance. **Veterinary Sciences**, 10: 1-14, 2023.
- AULT, T. B. et al. Bacterial taxonomic composition of the postpartum cow uterus and vagina prior to artificial insemination. **Journal of Animal Science**, 97: 4305-4313, 2019.
- BAKER, J. M.; CHASE, D. M.; HERBST-KRALOVETZ, M. M. Uterine microbiota: Residents, tourists, or invaders? **Frontiers in Immunology**, 9: 1-16, 2018.
- BALBONI, A. et al. Culture-dependent and sequencing methods revealed the absence of a bacterial community residing in the urine of healthy cats. **Frontiers in Veterinary Science**, 7: 1-10, 2020.
- BARDOS, J. et al. Immunological role of the maternal uterine microbiome in pregnancy: pregnancies pathologies and altered microbiota. **Frontiers in Immunology**, 10: 1-14, 2020.
- CLEMETSON, L. L.; WARD, A. C. Bacterial flora of the vagina and uterus of healthy cats. **Journal of the American Veterinary Medical Association**, 196: 902-906, 1990.
- EISENBERG, E.; LEVANON, E. Y. Human housekeeping genes, revisited. **Trends in Genetics**, 29: 569-574, 2013.
- FRANASIAK, J. M. et al. Endometrial microbiome at the time of embryo transfer: next-generation sequencing of the 16S ribosomal subunit. **Journal of Assisted Reproduction and Genetics**, 33: 129-136, 2016.
- HOLYOAK, G. R.; LYMAN, C. C.. The equine endometrial microbiome: A brief review. **American Journal of Biomedical Science & Research**, 11: 532-534, 2021.
- HOLYOAK, G. R. et al. The healthy equine uterus harbors a distinct core microbiome plus a rich and diverse microbiome that varies with geographical location. **Scientific Reports**, 12: 1-14, 2022.
- LI, J. et al. Endometrial and vaginal microbiome in donkeys with and without clinical endometritis. **Frontiers in Microbiology**, 13: 1-14, 2022.
- LONGERI, M. et al. Myosin-binding protein C DNA variants in domestic cats (A31P, A74T, R820W) and their association with Hypertrophic Cardiomyopathy. **Journal of Veterinary Internal Medicine**, 27: 275-285, 2013.
- LYMAN, C. C. et al. Canine endometrial and vaginal microbiomes reveal distinct and complex ecosystems. **PLOS ONE**, 14: 1-17, 2019.
- LYONS, L. A. et al. Feline polycystic kidney disease mutation identified in PKD1. **Journal of the American Society of Nephrology**, 15: 2548-2555, 2004.
- MOORE, S. G. et al. Hot topic: 16S rRNA gene sequencing reveals the microbiome of the virgin and pregnant bovine uterus. **Journal of Dairy Science**, 100: 4953-4960, 2017.
- MOOSSAVI, S. et al. Repeatability and reproducibility assessment in a large-scale population-based microbiota study: Case study on human milk microbiota. **Microbiome**, 9: 1-10, 2021.
- PEREZ-MUÑOZ, M. E. et al. A critical assessment of the “sterile womb” and “in utero colonization” hypotheses: Implications for research on the pioneer infant microbiome. **Microbiome**, 5: 1-19, 2017.
- PEREZ, P. F. et al. Bacterial imprinting of the neonatal immune system: lessons from maternal cells? **Pediatrics**, 119: e724-e732, 2007.
- PSIFIDI, A. et al. Comparison of eleven methods for genomic DNA extraction suitable for large-scale whole-genome genotyping and long-term DNA banking using blood samples. **PLOS ONE**, 10: 1-18, 2015.
- ROTA, A. et al. Does bacteria colonization of canine newborns start in the uterus? **Animals**, 11: 1-12, 2021.
- SCHULTHEISS, P. C. et al. Normal bacterial flora in canine and feline uteri. **Journal of Veterinary Diagnostic Investigation**, 11: 560-562, 1999.
- STRÖM HOLST, B. et al. Characterization of the bacterial population of the genital tract of adult cats. **American Journal of Veterinary Research**, 64: 963-968, 2003.
- VERSTRAELEN, H. et al. Characterisation of the human uterine microbiome in non-pregnant women through deep sequencing of the V1-2 region of the 16S rRNA gene. **PeerJ**, 4: 1-23, 2016.
- WEINROTH, M. D. et al. Considerations and best practices in animal science 16S ribosomal RNA gene sequencing microbiome studies. **Journal of Animal Science**, 100: 1-18, 2022.
- WEISS, S. et al. Tracking down the sources of experimental contamination in microbiome studies. **Genome Biology**, 15: 1-3, 2014.
- WESS, G. et al. Association of A31P and A74T polymorphisms in the myosin binding protein c3 gene and hypertrophic cardiomyopathy in Maine Coon and other breed cats. **Journal of Veterinary Internal Medicine**, 24: 527-532, 2010.
- XIA, Z. et al. Conventional versus real-time quantitative PCR for rare species detection. **Ecology and Evolution**, 8: 11799-11807, 2018.
- YAP, J. M.; GOLDSMITH, C. E.; MOORE, J. E. Integrity of

bacterial genomic DNA after autoclaving: possible implications for horizontal gene transfer and clinical waste management. **The Journal of Hospital Infection**, 83: 247-249, 2013.

ZOU, X. et al. Exploring the rumen and cecum microbial community from fetus to adulthood in goat. **Animals**, 10: 1-17, 2020.