Theriogenology 224 (2024) 134–142

Contents lists available at ScienceDirect

Theriogenology

journal homepage: www.theriojournal.com

Original Research Article

The vaginal microbiota of healthy female cats

Penelope Banchi [a,b], Alessia Bertero [a,*], Fabrizia Gionechetti [c], Michela Corrò [d], Elena Spagnolo [d], Gian Guido Donato [a], Alberto Pallavicini [c,1], Ada Rota [a,1]

[a] Department of Veterinary Science, University of Torino, 10095, Grugliasco, Italy
[b] Department of Internal Medicine, Reproduction and Population Medicine, Faculty of Veterinary Medicine, Ghent University, 9820, Merelbeke, Belgium
[c] Department of Life Sciences, University of Trieste, Via Edoardo Weiss 2, 34128, Trieste, TS, Italy
[d] Diagnostics in Animal Health Department, Istituto Zooprofilattico Sperimentale delle Venezie, Viale dell'Università 10, 35020, Legnaro, Italy

ARTICLE INFO

Keywords:
Reproductive microbiota
Vagina
Cat
16S rRNA sequencing
Culture

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 anaesthesia. 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 anoestrous ($N = 10$). 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 [1,2] and animals [3–7]. The vaginal microbiota is essential for maintaining reproductive health [8], influences fertility [9], and affects pregnancy outcomes [10,11]. Research on the feline species is limited to some studies investigating vaginal bacterial flora using culture techniques [12,13]. 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 [14,15]. Sequencing techniques offer higher sensitivity, allowing the description of the full microbiome of a specific environment [16], and have

* Corresponding author.
E-mail address: alessia.bertero@unito.it (A. Bertero).
[1] These authors contributed equally to this research and share co-last authorship.

https://doi.org/10.1016/j.theriogenology.2024.05.021
Received 28 February 2024; Received in revised form 15 May 2024; Accepted 15 May 2024
Available online 16 May 2024

therefore become widespread in human and animal research over the past two decades [17,18].

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 [19]. Conversely, the balance of bacterial populations could be preserved or restored using probiotics, as demonstrated in humans and other mammals [20–24].

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 (Fig. 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 shorthaired 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) [25]. 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).

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 anaesthesiological and surgical procedures [26]. Vaginal swabs were collected immediately after the induction of general anaesthesia: the procedure took between two and 3 min, without significantly extending the duration of anaesthesia. 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.

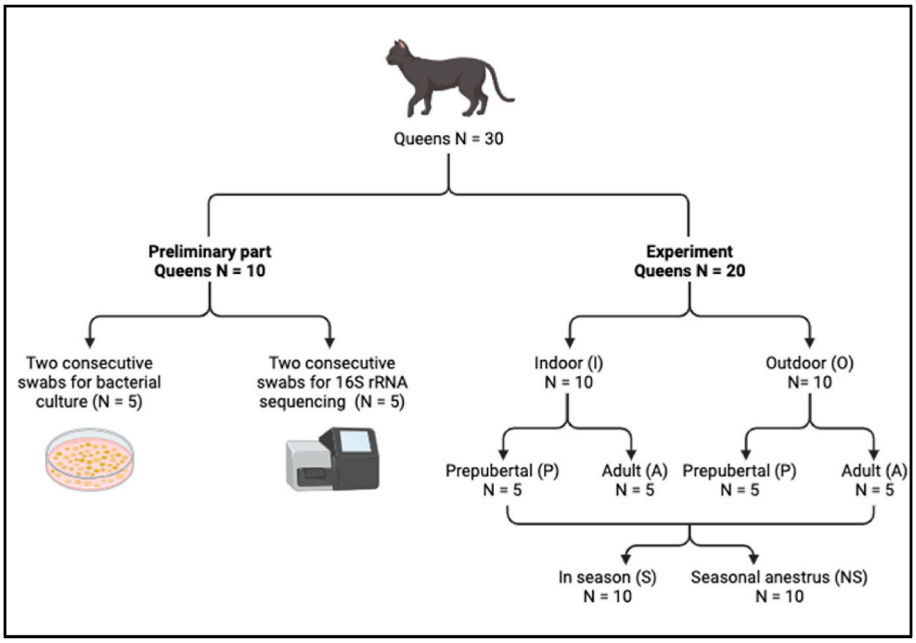

Fig. 1. Diagram representing the animals included in the present study. A total number of 30 healthy queens was included either in the preliminary part (N = 10) or in the experiment (N = 20).

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

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).

2.4. 16S rRNA 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 [27], 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).

2.5. Data analysis

Sequencing data were initially processed and analyzed using CLC Microbial Genomics Module version 23.0.3. Raw reads were quality filtered to remove adapters and low-quality sequences with a quality score threshold of 0.03. Paired-end reads were merged, and primers were trimmed. Paired-end reads were denoised into Amplicon Sequence Variants (ASVs) using the DADA2 algorithm [28] 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 (Fig. 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 $P < 0.05$.

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.

3.1. 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 haemolytic *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 3.

3.2. 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

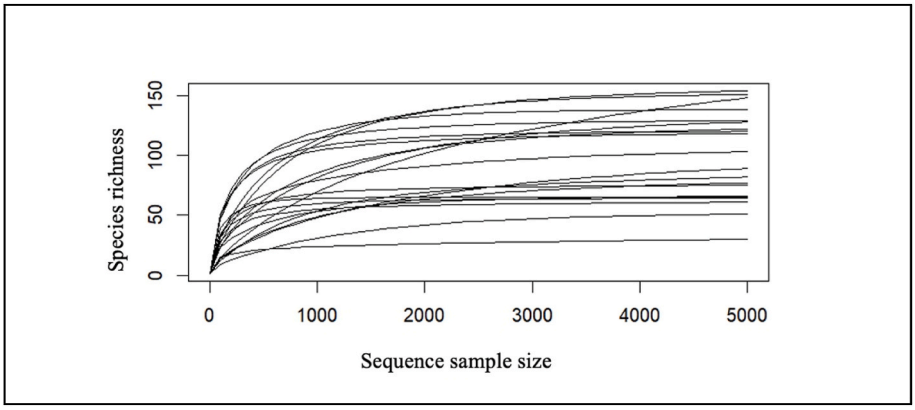

Fig. 2. Taxonomy rarefaction curves for the feline vaginal samples included in the present study showing that the selected sequencing depth was sufficient to saturate species richness.

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.

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 Fig. 3.

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; Fig. 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; Fig. 4), although no differences were detected based on the age and reproductive season (P = 0.3 and P = 0.5, respectively). Differential abundance analyses are reported as a supplementary material (S1). Beta diversity using Bray-Curtis statistics (Fig. 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 4).

Table 3
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* (haemolytic strain)[c]	50	/	/
2.2	*Escherichia coli* (haemolytic strain)[d]	50	/	/
3.2	*Escherichia coli* (haemolytic strain)[c]	50	/	/
4.2	*Escherichia coli* (haemolytic strain)[c]	>300	*Enterococcus faecium*[d]	>300
5.2	Negative for bacterial growth	/	/	/
6.2	*Escherichia coli* (haemolytic strain)[c]	70	*Enterococcus fecalis*[d]	41
7.2	*Escherichia coli* (haemolytic strain)[c]	14	*Streptococcus canis*[c]	2
8.2	*Escherichia coli* (haemolytic strain)[c]	1	/	/
9.2	Negative for bacterial growth	/	/	/
10.2	*Escherichia coli* (haemolytic strain)[c]	100	/	/
11.2	*Escherichia coli* (haemolytic strain)[c]	4	*Streptococcus canis*[d]	3
12.2	*Escherichia coli* (haemolytic strain)[c]	100	*Clostridium perfringens*[d]	1
13.2	*Escherichia coli* (haemolytic strain)[c]	>300	*Enterococcus* spp.[d]	42
14.2	*Escherichia coli* (haemolytic 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* (haemolytic strain)[c]	>300	*Streptococcus canis*[c]	4
18.2	*Escherichia coli* (haemolytic strain)[c]	23	*Streptococcus canis*[c]	7
19.2	*Escherichia coli* (haemolytic 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 3, 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 [13], 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 reported in a previous study on adult cats [13]. 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) [13]. However, the same bacteria were isolated, with haemolytic *E. coli* being the prevailing species. In accordance with Ström Holst et al. (2003) [13], we can affirm that pure culture, particularly of haemolytic or non-haemolytic *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) [13,29]. 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 from 16S 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 [30], and from that of dogs, in which *Fusobacterium*, *Pasteurellaceae*, *Mycoplasma.* were the most reported bacteria [4,6,7]. 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, faecal, oral microbiota of the species, as confirmed by culture [31] and sequencing [32–36]. 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 [37]. More than 90 % of bacterial species are ‘unculturable’, as they do not grow in laboratory conditions [15], and others are classified as ‘fastidious’, because they have very specific needs and slow growth in culture [38]. Yet, this category includes species implicated in reproductive health and fertility conditions in animals and humans (e.g., *Mycoplasma* spp., *Brucella* spp.,

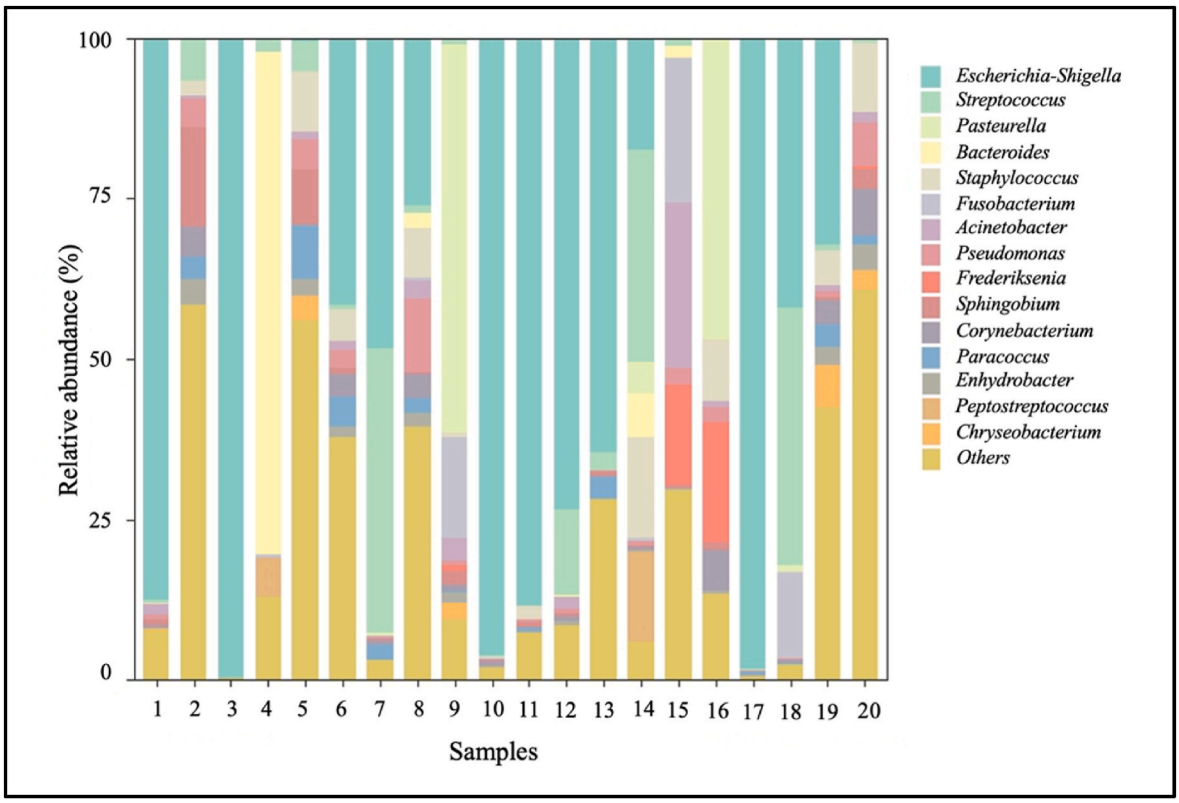

Fig. 3. Relative abundance at genus level of the 15 most prevalent bacteria detected by 16S rRNA bacterial gene sequencing from twenty healthy queens are presented. Less relatively abundant bacteria are grouped as 'Others'.

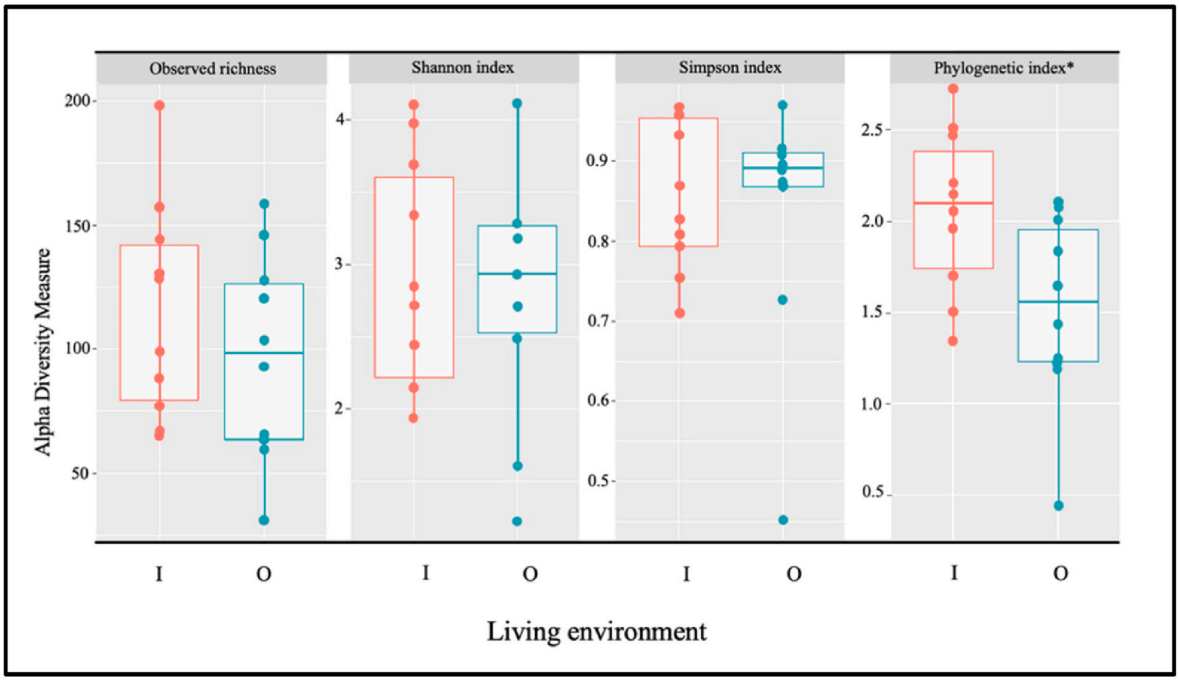

Fig. 4. Variation in vaginal microbial diversity based on the living environment (indoor – I, outdoor – O) assessed by observed richness, Shannon, Simpson, and phylogenetic indexes in twenty healthy queens. The two groups of animals showed statistically significant (*) different phylogenetic indexes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

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 [39, 40]. 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

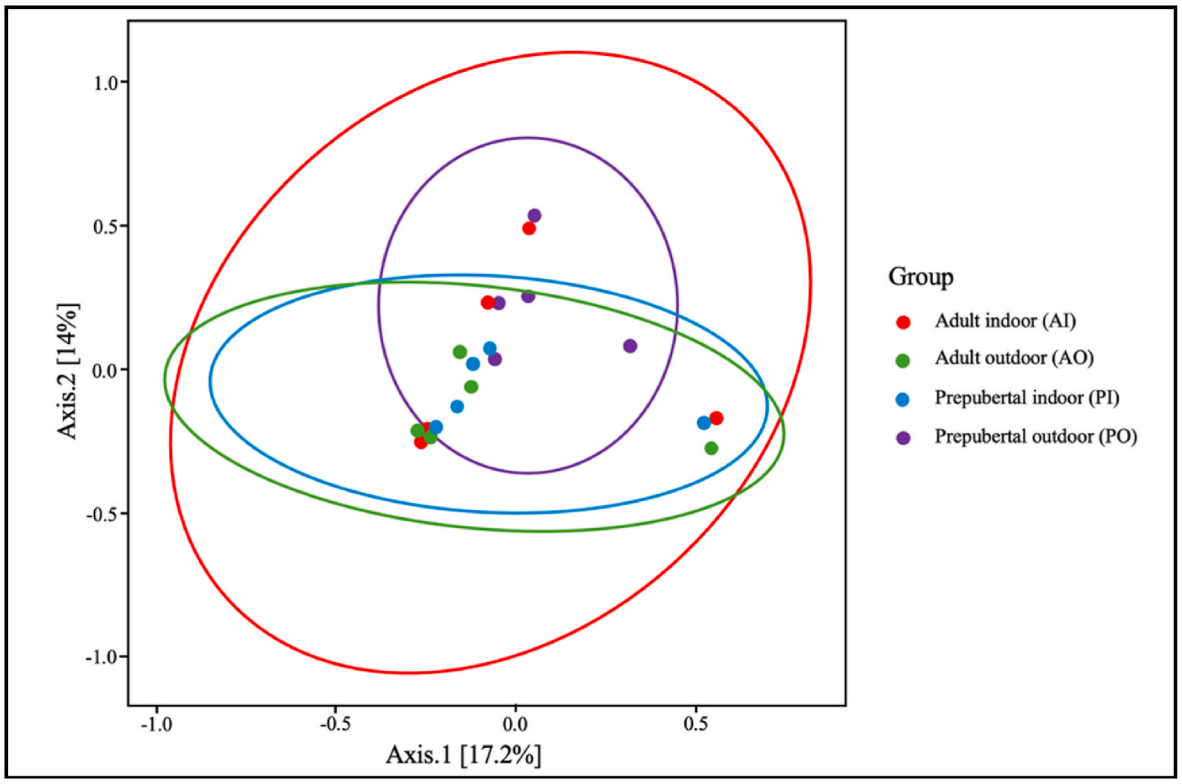

Fig. 5. Two dimensions scaling plot by Bray-Curtis based on animal groups considering the age and the living environment of the included queens: adult indoor (AI), adult outdoor (AO), prepubertal indoor (PI), and prepubertal outdoor (AO). The majority of queens clustered together, showing no significant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

Table 4
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

[a]Significance for $P < 0.05$.

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 [41–43] 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 [41].

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 anoestrous), 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 [7] and humans [44], but similar results were obtained in minipigs [45]. Domestic short hair queens can reach puberty as early as 4 months if the reproductive season begins [46]. 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 anoestrus, not only by culture but also when analyzing sequencing results, involving all bacterial population DNA. Queens are seasonally polyoestrous induced ovulators, having repeated oestrus periods during the reproductive season [47]: oestrogen 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 oestrous queens compared to anoestrous ones by culture, although this was the only statistically significant difference, and the two groups of animals were unbalanced, with only 10 oestrous queens versus 56 anoestrous ones. The role of oestrogens on vaginal bacterial growth and bacterial flora composition has been reported in women [48] but not in the vaginal microbiota of heifers [49] or bitches [7]. The canine species can represent a sound comparison for the effects of estrogens because, even if not a seasonal species, it has a long oestrous period with prolonged oestrogen stimulation of the vaginal mucosa, and a long anoestrus [50]. 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 oestrogen 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 [6], as occurs in women with postmenopausal vaginal atrophy [51]. Progesterone was reported to influence vaginal microbiota modelling in humans [52], as does pregnancy [52,53], and its composition can represent a biomarker for gestational disease as chorioamnionitis, pre-term labour, and miscarriage [54]. 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 [55].

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 [56,57], although mild differences in vaginal microbial composition were found based on ethnicity [57]. 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 [58]. 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.

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 haemolytic *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.

Funding

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

CRediT authorship contribution statement

Penelope Banchi: Writing – original draft, Investigation, Conceptualization. **Alessia Bertero:** Writing – review & editing, Investigation. **Fabrizia Gionechetti:** Validation, Methodology, Investigation. **Michela Corrò:** Investigation. **Elena Spagnolo:** Investigation. **Gian Guido Donato:** Formal analysis. **Alberto Pallavicini:** Validation, Methodology, Investigation, Formal analysis. **Ada Rota:** Writing – review & editing, Supervision.

Declaration of competing interest

None.

Acknowledgements

The authors express their gratitude to Dr. Cristina Levra Levron and Dr. Rhoda Arnò who helped in the enrolment of the animals included in the present research.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.theriogenology.2024.05.021.

References

[1] Smith SB, Ravel J. The vaginal microbiota, host defence and reproductive physiology. J Physiol 2017;595:451–63. https://doi.org/10.1113/JP271694.
[2] Simon C. Introduction: do microbes in the female reproductive function matter? Fertil Steril 2018;110:325–6. https://doi.org/10.1016/j.fertnstert.2018.06.041.
[3] Adnane M, Chapwanya A. A review of the diversity of the genital tract microbiome and implications for fertility of cattle. Animals (Basel) 2022;12:460. https://doi.org/10.3390/ani12040460.
[4] Hu J, Cui L, Wang X, Gao X, Qiu S, Qi H, Jiang S, Li F, Yin Y. Dynamics of vaginal microbiome in female beagles at different ages. Res Vet Sci 149;128-135. https://doi.org/10.1016/j.rvsc.2022.05.006..
[5] Maksimovic A, Maksimovic Z, Filipovic S, Besirovic H, Rifatbegovic M. Vaginal and uterine bacteria of healthy bitches during different stages of their reproductive cycle. Vet Rec 2012;171:375. https://doi.org/10.1136/vr.100886.
[6] Rota A, Corrò M, Patuzzi I, Milani C, Masia S, Mastrorilli E, Petrin S, Longo A, Del Carro A, Losasso C. Effect of sterilization on the canine vaginal microbiota: a pilot study. BMC Vet Res 2020;16:455. https://doi.org/10.1186/s12917-020-02670-3.
[7] Lyman CC, Holyoak GR, Meinkoth K, Wieneke X, Chillemi KA, DeSilva U. Canine endometrial and vaginal microbiomes reveal distinct and complex ecosystems. PLoS One 2019;14:e0210157. https://doi.org/10.1371/journal.pone.0210157.
[8] Ravel J, Gajer P, Abdo Z, Schneider GM, Koenig SS, McCulle SL, Karlebach S, Gorle R, Russell J, Tacket CO, Brotman RM, Davis CC, Ault K, Peralta L, Forney LJ. Vaginal microbiome of reproductive-age women. Proc Natl Acad Sci USA 2015;108:4680–7. https://doi.org/10.1073/pnas.1002611107.
[9] Cocomazzi G, De Stefani S, Del Pup L, Palini S, Buccheri M, Primiterra M, Sciannamè N, Faioli R, Maglione A, Baldini GM, Baldini D, Pazienza V. The impact of the female genital microbiota on the outcome of assisted reproduction treatments. Microorganisms 2023;11:1443. https://doi.org/10.3390/microorganisms11061443.
[10] Guo X, Hong X, Qian H, Qiao D, Wang B, Yu H. Relationship between vaginal microbiota and chorioamnionitis: a prospective cohort study. Microb Pathog 2024;186:106458. https://doi.org/10.1016/j.micpath.2023.106458.
[11] Saadaoui M, Singh P, Ortashi O, Al Khodor S. Role of the vaginal microbiome in miscarriage: exploring the relationship. Front Cell Infect Microbiol 2023;13:1232825. https://doi.org/10.3389/fcimb.2023.1232825.
[12] Mannion A, McGee W, Feng Y, Shen Z, Buckley-Jordan E, Dzink-Fox JL, Fox JG. Characterization of genotoxin-encoding Escherichia coli isolated from specific-pathogen free cats with impaired fertility. Vet Microbiol 2022;266:109337. https://doi.org/10.1016/j.vetmic.2022.109337.
[13] Ström Holst B, Bergström A, Lagerstedt AS, Karlstam E, Englund L, Båverud V. Characterization of the bacterial population of the genital tract of adult cats. Am J Vet Res 2003;64:963–8. https://doi.org/10.2460/ajvr.2003.64.963.
[14] Almeida OGG, de Martinis ECP. Bioinformatics tools to assess metagenomic data for applied microbiology. Appl Microbiol Biotechnol 2019;103:69–82. https://doi.org/10.1007/s00253-018-9464-9.
[15] Kaeberlein T, Lewis K, Epstein SS. Isolating "uncultivable" microorganisms in pure culture in a simulated natural environment. Science 2002;296:1127–9. https://doi.org/10.1126/science.1070633.
[16] Woo PC, Lau SK, Teng JL, Tse H, Yuen KY. Then and now: use of 16S rDNA gene sequencing for bacterial identification and discovery of novel bacteria in clinical microbiology laboratories. Clin Microbiol Infect 2008;14:908–34. https://doi.org/10.1111/j.1469-0691.2008.02070.x.
[17] Gao Y, Li D, Liu YX. Microbiome research outlook: past, present, and future. Protein Cell 2023;14:709–12. https://doi.org/10.1093/procel/pwad031.
[18] Peixoto RS, Harkins DM, Nelson KE. Advances in microbiome research for animal health. Annu Rev Anim Biosci 2021;16:289–311. https://doi.org/10.1146/annurev-animal-091020-075907.
[19] Guardabassi L, Houser GA, Frank LA, Papich MG. Guidelines for antimicrobial use in dogs and cats: chapter 11. In: Guardabassi L, Jensen LB, Kruse H, editors. Guide to antimicrobial use in animals. Oxford: Blackwell Publishing; 2008. p. 183–206.
[20] Ang XY, Roslan NS, Ahmad N, Yusof SM, Abdullah N, Nik Ab, Rahman NN, Woon JJ, Teh CS, Todorov SD, Liu G, Liong MT. Lactobacillus probiotics restore vaginal and gut microbiota of pregnant women with vaginal candidiasis. Benef Microbes 2023;14:421–31. https://doi.org/10.1163/18762891-20220103.

[21] Chung Y, Kang SB, Son D, Lee JY, Chung MJ, Lim S. Characterization of the probiotic properties of Lacticaseibacillus rhamnosus LR6 isolated from the vaginas of healthy Korean women against vaginal pathogens. Front Microbiol 2023;14:1308293. https://doi.org/10.3389/fmicb.2023.1308293.
[22] Di Pierro F, Sinatra F, Cester M, Da Ros L, Pistolato M, Da Parè V, Fabbro L, Maccari D, Dotto S, Sossai S, Fabozzi G, Bertuccioli A, Cazzaniga M, Recchia M, Zerbinati N, Guasti L, Baffoni A. Effect of L. crispatus M247 administration on pregnancy outcomes in women undergoing IVF: a controlled, retrospective, observational, and open-label study. Microorganisms 2023;11:2796. https://doi.org/10.3390/microorganisms11112796.
[23] Mitra A, Gultekin M, Burney Ellis L, Bizzarri N, Bowden S, Taumberger N, Bracic T, Vieira-Baptista P, Sehouli J, Kyrgiou M. Genital tract microbiota composition profiles and use of prebiotics and probiotics in gynaecological cancer prevention: review of the current evidence, the European Society of Gynaecological Oncology prevention committee statement. Lancet Microbe 2023. https://doi.org/10.1016/S2666-5247(23)00257-4. S2666-5247(23)00257-4.
[24] Nader-Macías ME, Otero MC, Espeche MC, Maldonado NC. Advances in the design of probiotic products for the prevention of major diseases in dairy cattle. J Ind Microbiol Biotechnol 2008;35:1387–95. https://doi.org/10.1007/s10295-008-0438-2.
[25] Percie du Sert N, Hurst V, Ahluwalia A, Alam S, Avey MT, Baker M, Browne WJ, Clark A, Cuthill IC, Dirnagl U, Emerson M, Garner P, Holgate ST, Howells DW, Karp NA, Lazic SE, Lidster K, MacCallum CJ, Macleod M, Pearl EJ, Petersen OH, Rawle F, Reynolds P, Rooney K, Sena ES, Silberberg SD, Steckler T, Würbel H. The ARRIVE guidelines 2.0: updated guidelines for reporting animal research. PLoS Biol 2020;18:e3000410. https://doi.org/10.1371/journal.pbio.3000410.
[26] Fossum TW. In: Small animal surgery. fifth ed. Philadelphia: Elsevier; 2019.
[27] Walker SP, Barrett M, Hogan G, Flores Bueso Y, Claesson MJ, Tangney M. Non-specific amplification of human DNA is a major challenge for 16S rRNA gene sequence analysis. Sci Rep 2020;10:16356. https://doi.org/10.1038/s41598-020-73403-7.
[28] Callahan BJ, McMurdie PJ, Rosen MJ, Han AW, Johnson AJ, Holmes SP. DADA2: high-resolution sample inference from Illumina amplicon data. Nat Methods 2016;13:581–3. https://doi.org/10.1038/nmeth.3869.
[29] Clemetson LL, Ward AC. Bacterial flora of the vagina and uterus of healthy cats. J Am Vet Med Assoc 1990;196:902–6.
[30] Kumar L, Dwivedi M, Jain N, Shete P, Solanki S, Gupta R, Jain A. The female reproductive tract microbiota: friends and foe. Life 2023;13:1313. https://doi.org/10.3390/life13061313.
[31] Lansdell LW, Taplin D, Aldrich TE. Recovery of Staphylococcus aureus from multiple body sites in menstruating women. J Clin Microbiol 1984;20:307–10. https://doi.org/10.1128/jcm.20.3.307-310.1984.
[32] Pappalardo VY, Azarang L, Zaura E, Brandt BW, de Menezes RX. A new approach to describe the taxonomic structure of microbiome and its application to assess the relationship between microbial niches. BMC Bioinf 2024;25:58. https://doi.org/10.1186/s12859-023-05575-8.
[33] Moon BY, Ali MS, Kwon DH, Heo YE, Hwang YJ, Kim JI, Lee YJ, Yoon SS, Moon DC, Lim SK. Antimicrobial resistance in Escherichia coli isolated from healthy dogs and cats in South Korea, 2020-2022. Antibiotics (Basel, Switzerland) 2023;13:27. https://doi.org/10.3390/antibiotics13010027.
[34] Older CE, Diesel AB, Lawhon SD, Queiroz CRR, Henker LC, Rodrigues Hoffmann A. The feline cutaneous and oral microbiota are influenced by breed and environment. PLoS One 2019;14:e0220463. https://doi.org/10.1371/journal.pone.0220463.
[35] Sturgeon A, Pinder SL, Costa MC, Weese JS. Characterization of the oral microbiota of healthy cats using next-generation sequencing. Vet J 2014;201:223–9. https://doi.org/10.1016/j.tvjl.2014.01.024.
[36] Swartz JD, Lachman M, Westveer K, O'Neill T, Geary T, Kott RW, Berardinelli JG, Hatfield PG, Thomson JM, Roberts A, Yeoman CJ. Characterization of the vaginal microbiota of ewes and cows reveals a unique microbiota with low levels of lactobacilli and near-neutral pH. Front Vet Sci 2014;1:19. https://doi.org/10.3389/fvets.2014.00019.
[37] Bonnet M, Lagier JC, Raoult D, Khelaifia S. Bacterial culture through selective and non-selective conditions: the evolution of culture media in clinical microbiology. New Microbes New Infect 2019;34:100622. https://doi.org/10.1016/j.nmni.2019.100622.
[38] Rishmawi N, Ghneim R, Kattan R, Ghneim R, Zoughbi M, Abu-Diab A, Turkuman S, Dauodi R, Shomali I, Issa Ael-R, Siriani I, Marzouka H, Schmid I, Hindiyeh MY. Survival of fastidious and nonfastidious aerobic bacteria in three bacterial transport swab systems. J Clin Microbiol 2007;45:1278–83. https://doi.org/10.1128/JCM.02110-06.
[39] Banchi P, Colitti B, Opsomer G, Rota A, Van Soom A. The dogma of the sterile uterus revisited: does microbial seeding occur during fetal life in humans and animals? Reproduction 2023;167:e230078. https://doi.org/10.1530/REP-23-0078.
[40] Yagi K, Huffnagle GB, Lukacs NW, Asai N. The lung microbiome during health and disease. Int J Mol Sci 2021;2219:10872. https://doi.org/10.3390/ijms221910872.
[41] Sung JY, Hwang Y, Shin MH, Park MS, Lee SH, Yong D, Lee K. Utility of conventional culture and MALDI-TOF MS for identification of microbial communities in bronchoalveolar lavage fluid in comparison with the GS junior next generation sequencing system. Ann Lab Med 2018;38:110–8. https://doi.org/10.3343/alm.2018.38.2.110.
[42] Walker AW, Martin JC, Scott P, Parkhill J, Flint HJ, Scott KP. 16S rRNA gene-based profiling of the human infant gut microbiota is strongly influenced by sample processing and PCR primer choice. Microbiome 2015;3:26. https://doi.org/10.1186/s40168-015-0087-4.
[43] Lagier JC, Armougom F, Million M, Hugon P, Pagnier I, Robert C, Bittar F, Fournous G, Gimenez G, Maraninchi M, Trape JF, Koonin EV, La Scola B, Raoult D. Microbial culturomics: paradigm shift in the human gut microbiome study. Clin Microbiol Infect 2012;18:1185–93. https://doi.org/10.1111/1469-0691.12023.
[44] Auriemma RS, Scairati R, Del Vecchio G, Liccardi A, Verde N, Pirchio R, Pivonello R, Ercolini D, Colao A. The vaginal microbiome: a long urogenital colonization throughout woman life. Front Cell Infect Microbiol 2021;11:686167. https://doi.org/10.3389/fcimb.2021.
[45] Lorenzen E, Kudirkiene E, Gutman N, Grossi AB, Agerholm JS, Erneholm K, Skytte C, Dalgaard MD, Bojesen AM. The vaginal microbiome is stable in prepubertal and sexually mature Ellegaard Göttingen Minipigs throughout an estrous cycle. Vet Res 2015;46:125. https://doi.org/10.1186/s13567-015-0274-0.
[46] Kutzler MA. Reproductive anatomy and puberty in the queen. In: Johnson AK, Kutzler MA, editors. Feline reproduction. first ed. CABI; 2022. p. 1–10. https://doi.org/10.1079/9781789247107.0001.
[47] Johnson AK. Normal feline reproduction: the queen. J Feline Med Surg 2022;24:204–11. https://doi.org/10.1177/1098612X221079706.
[48] Brotman RM, Shardell MD, Gajer P, Fadrosh D, Chang K, Silver MI, Viscidi RP, Burke AE, Ravel J, Gravitt PE. Association between the vaginal microbiota, menopause status, and signs of vulvovaginal atrophy. Menopause 2018;25:1321–30. https://doi.org/10.1097/GME.0000000000001236.
[49] Messman RD, Contreras-Correa ZE, Paz HA, Perry G, Lemley CO. Vaginal bacterial community composition and concentrations of estradiol at the time of artificial insemination in Brangus heifers. J Anim Sci 2020;98:skaa178. https://doi.org/10.1093/jas/skaa178.
[50] Concannon PW. Reproductive cycles of the domestic bitch. Anim Reprod Sci 2011;124:200–10. https://doi.org/10.1016/j.anireprosci.2010.08.028.
[51] Mac Bride MB, Rhodes DJ, Shuster LT. Vulvovaginal atrophy. Mayo Clin Proc 2010;85:87–94. https://doi.org/10.4065/mcp.2009.0413.
[52] Shen L, Zhang W, Yuan Y, Zhu W, Shang A. Vaginal microecological characteristics of women in different physiological and pathological period. Front Cell Infect Microbiol 2022;12:959793. https://doi.org/10.3389/fcimb.2022.959793.
[53] Symul L, Jeganathan P, Costello EK, France M, Bloom SM, Kwon DS, Ravel J, Relman DA, Holmes S. Sub-communities of the vaginal microbiota in pregnant and non-pregnant women. Proc Biol Sci 2023;290:20231461. https://doi.org/10.1098/rspb.2023.1461.
[54] Gryaznova M, Kozarenko O, Smirnova Y, Burakova I, Syromyatnikov M, Maslov A, Lebedeva O. Cervical and vaginal microbiomes in early miscarriages and ongoing pregnancy with and without dydrogesterone usage. Int J Mol Sci 2023;24:13836. https://doi.org/10.3390/ijms241813836.
[55] Hagman R. Pyometra in small animals 3.0. Vet Clin North Am Small Anim Pract 2023;53:1223–54. https://doi.org/10.1016/j.cvsm.2023.04.009.
[56] Yao T, Wang Z, Liang X, Liu C, Yu Z, Han X, Liu R, Liu Y, Liu C, Chen L. Signatures of vaginal microbiota by 16S rRNA gene: potential bio-geographical application in Chinese Han from three regions of China. Int J Legal Med 2021;135:1213–24. https://doi.org/10.1007/s00414-021-02525-y.
[57] Vargas-Robles D, Morales N, Rodríguez I, Nieves T, Godoy-Vitorino F, Alcaraz LD, Pérez ME, Ravel J, Forney LJ, Domínguez-Bello MG. Changes in the vaginal microbiota across a gradient of urbanization. Sci Rep 2020;10:12487. https://doi.org/10.1038/s41598-020-69111-x.
[58] Garg A, Ellis LB, Love RL, Grewal K, Bowden S, Bennett PR, Kyrgiou M. Vaginal microbiome in obesity and its impact on reproduction. Best Pract Res Clin Obstet Gynaecol 2023;90:102365. https://doi.org/10.1016/j.bpobgyn.2023.102365.