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No viable bacterial communities reside in the urinary bladder of cats with feline idiopathic cystitis

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ABSTRACT

Urinary microbial diversities have been reported in humans according to sex, age and clinical status, including painful bladder syndrome/interstitial cystitis (PBS/IC). To date, the role of the urinary microbiome in the pathogenesis of PBS/IC is debated. Feline idiopathic cystitis (FIC) is a chronic lower urinary tract disorder affecting cats with similarities to PBS/IC in women and represents an important problem in veterinary medicine as its aetiology is currently unknown. In this study, the presence of a bacterial community residing in the urinary bladder of cats with a diagnosis of FIC was investigated. Nineteen cats with clinical signs and history of FIC and without growing bacteria in standard urine culture were included and urine collected with ultrasound-guided cystocentesis. Bacterial community was investigated using a culture-dependent approach consisted of expanded quantitative urine culture techniques and a culture-independent approach consisted of 16S rRNA NGS. Several methodological practices were adopted to both avoid and detect any contamination or bias introduced by means of urine collection and processing which could be relevant due to the low microbial biomass environment of the bladder and urinary tract, including negative controls analysis. All the cats included showed no growing bacteria in the urine analysed. Although few reads were originated using 16S rRNA NGS, a comparable pattern was observed between urine samples and negative controls, and no taxa were confidently classified as non-contaminant. The results obtained suggest the absence of viable bacteria and of bacterial DNA of urinary origin in the urinary bladder of cats with FIC.

1. Introduction

The combined use of next generation sequencing (NGS) and enhanced urine culture techniques allowed to detect microbes in urine samples of asymptomatic patients, overcoming the dogma that clinical urine specimens of asymptomatic humans are sterile (Hilt et al., 2014). Furthermore, urinary microbial diversities have been reported in human patients according to sex, age and clinical status, including painful bladder syndrome/interstitial cystitis (PBS/IC). For PBS/IC, some studies suggested a link between the urinary bacterial composition and the disease (Nickel et al., 2019; Siddiqui et al., 2012), while others found no evidence (Bresler et al., 2019; Jacobs et al., 2021). To date, to the best of the authors' knowledge, only six studies investigated the bacterial communities residing in the urine of healthy or sick dogs and cats (Balboni et al., 2020; Burton et al., 2017; Coffey et al., 2022; Kim et al., 2021; Lund et al., 2015; Melgarejo et al., 2021). Regarding cats, Lund et al. (2015) and Balboni et al. (2020) reported the absence of viable bacteria and bacterial DNA in urine samples of healthy cats and cats with a diagnosis consistent with feline lower urinary tract disease (FLUTD). Differently, Kim et al. (2021) reported that healthy cats and cats with urinary tract disease, such as chronic kidney disease (CKD) and feline idiopathic cystitis (FIC), harbour unique microbial communities in their urine, but found no evidence to suggest that the urinary bladder microbiome is implicated in the pathogenesis of FIC. In all these three studies, urine samples collected by ultrasound-guided cystocentesis and tested negative by standard urine culture (SUC) were analysed by 16S

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ribosomal RNA NGS (16S rRNA NGS). Whereas, only one study (Balboni et al., 2020) also adopted enhanced urine culture techniques detecting the absence of bacterial growth, therefore no data are currently available to support the presence of viable bacteria residing in the SUC-negative urine of cats.

FIC is a chronic lower urinary tract disorder affecting cats with similarities to PBS/IC in women (Westropp et al., 2019) and represents an important problem in veterinary medicine as its aetiology is currently unknown (idiopathic) and treatment is frustrating (He et al., 2022). The role of the urinary microbiome in the pathogenesis of PBS/IC in humans is debated. Similarly,the presence of viable bacteria in urine specimens of cats affected by FIC has not been demonstrated yet. Therefore, the presence of a bacterial community residing in the urinary bladder of cats with a diagnosis of FIC was investigated in this study using both culture-dependent and culture-independent methods.

2. Materials and methods

2.1. Study design

This was a prospective study carried out at the Veterinary University Hospital (VUH) of the Department of Veterinary Medical Sciences (DIMEVET), University of Bologna, in 2021–2022. Client-owned cats were included in the study after owner informed consent if they had signalment, history, clinical signs, and clinicopathological, imaging and microbiological findings suggestive of FIC (He et al., 2022). The bacterial community was investigated in urine samples using both culture-dependent and culture-independent methods to differentiate between live and dead bacteria or bacterial DNA fragments, as previously reported in a study on healthy cats by the same study group (2020), with some modifications. The culture-dependent approach consisted of expanded quantitative urine culture (EQUC) techniques, partially modified when compared to those reported in previous studies (Hilt et al., 2014; Price et al., 2016), and the culture-independent approach consisted of 16S rRNA NGS to explore microbial diversity with a high depth of analysis (Fukuda et al., 2016).

To both avoid and detect any contamination or bias introduced by means of urine collection and processing which could be relevant due to the low microbial biomass environment of the bladder and urinary tract (Glassing et al., 2016; Karstens et al., 2018), six methodological practices were adopted during the study: 1) ultrasound-guided cystocentesis was used for urine sampling, minimizing the introduction of extra-urinary bacterial contamination; 2) culture-dependent and quantitative real-time PCR (qPCR) tests were carried out on skin swabs, collected after skin aseptical scrubbing, to assess its effectiveness and to evaluate the potential urine contamination by cutaneous microorganisms; 3) negative controls were carried out for each culture-independent test, including two negative controls for the 16S rRNA gene sequencing (NCS) carried out at the beginning and at the end of the study, respectively, subjecting an aliquot of sterile phosphate-buffered saline (PBS) to each step of the DNA extraction procedure, bacterial 16S rRNA gene amplification and NGS sequencing; 4) all DNA extractions were performed in a pre-PCR laminar flow hood; 5) all samples subjected to NGS were sequenced at once on the same machine to minimise technical variations (Brubaker et al., 2021); 6) bioinformatic and statistical analysis were applied to identify bacterial DNA background contamination (Davis et al., 2018; Salter et al., 2014).

The animal study was reviewed and approved by Animal Welfare Committee (COBA) of the *Alma Mater Studiorum* - University of Bologna (ID 862, 16/05/2018) that confirm that the research does not fall within Directive 63/2010 of the European Parliament and of the Council on the protection of animals used for scientific purposes (transposed into Italian law by Legislative Decree 26/2014) and thus doesn't require any authorization from the national competent Authorities. The study was not carried out on experimental animals. All methods were carried out in accordance with relevant guidelines and regulations. Written informed consent was obtained from the owners for the participation of their animals in this study.

2.2. Animals

Cats with a confirmed diagnosis of FIC were included in the study. To evaluate the clinical status of each cat, signalment, history and clinical data were recorded, and complete blood count (CBC), serum chemistry and urinalysis were carried out. Imaging results were also recorded. Cats were enrolled if they showed at the time of inclusion, or in the previous three months, lower urinary tract signs (LUTS), including dysuria, stranguria, hematuria, pollakiuria, and periuria, not explained by other diseases (e.g. urolithiasis, urinary tract infection and anatomical abnormalities) (He et al., 2022). Urinary tract infection was ruled out by means of urine culture. Cats were excluded if bacteria grew in the SUC. Animals who had undergone antibacterial and/or anti-inflammatory treatments over the previous 30 days were also excluded from the study.

Cystocentesis was performed using a 10 mL syringe after clipping the hair and using surgical aseptical scrubbing of the skin in the abdomen. A surgical scrub was carried out using 4% chlorhexidine gluconate (Neoxidina, Nuova Farmec, Settimo Veronese, Italy) rinsed with isopropyl alcohol 70%. The procedure was repeated three times before cystocentesis. The effectiveness of skin disinfection performed before the ultrasound-guided cystocentesis had already been evaluated and confirmed in a previous study (Balboni et al., 2020), nevertheless, two skin swabs were sampled after the surgical scrub from each cat to evaluate potential urine contamination: one swab was independently suspended in 2 mL of brain heart infusion (BHI) broth (Biolife, Milan, Italy) and used for the standard culture and the other swab was suspended in 200 μL of PBS and used for DNA extraction and 16S rRNA gene qPCR amplification.

For each cat included in the study, the collected urine (10 mL) was divided into four aliquots: a) 5 mL for urinalysis, b) 500 μL in 2.5 mL of BHI broth for the SUC, c) 2 mL for the bacterial 16S rRNA gene sequencing and d) 2.5 mL for the EQUC.

Samples undergoing to bacterial culture were stored at 0–4 °C and processed within 8 h to minimise alterations in the bacterial concentration (Patterson et al., 2016). Samples undergoing to molecular analysis (16S rRNA gene qPCR amplification and NGS sequencing) were stored at 0–4 °C and processed immediately after collection (within 4 h) to avoid degradation of nucleic acids (Brubaker et al., 2021). Samples undergoing to clinicopathological analyses (CBC, chemical profile and standard urinalysis) were stored at room temperature and processed within 1 h. Samples collected from each cat included in the study and the analyses they underwent are summarised in Fig. 1.

2.3. Standard culture

For each cat included in the study, a SUC was performed by inoculating 500 μL of urine in 2.5 mL of BHI broth, 10 μL on a 5% sheep blood agar plate (BAP) (Biolife, Milan, Italy) and 10 μL in MacConkey agar (MCA) (Biolife, Milan, Italy). The entire surface of the BAP and the MCA was streaked to obtain quantitative colony counts, and the plates and broths were incubated at 35 °C in aerobic conditions and inspected after 24 and 48 h. In the absence of any growth after 24 h, 50 μL of the BHI broth were plated on a new BAP and new MCA and were incubated aerobically at 35 °C for 24 and 48 h.

For each cat included in the study, one skin swab suspended in 2 mL of BHI broth sampled after aseptical scrubbing was streaked on two plates of BAP, Eosin Methylene Blue (EMB) agar (Oxoid, Thermo Fisher Scientific, Waltham, MA, USA) and Baird Parker (BP) agar (Biokar Diagnostics, Allonne, France). One plate each of BAP, EMB, BP and the BHI broth were incubated aerobically at 37 °C. One plate of BAP was incubated at 37 °C under anaerobic conditions. In the absence of growth in the plates streaked directly, the BHI was plated in agarised media (BAP, MCA, BP), incubated aerobically and inspected after 24 and 48 h.

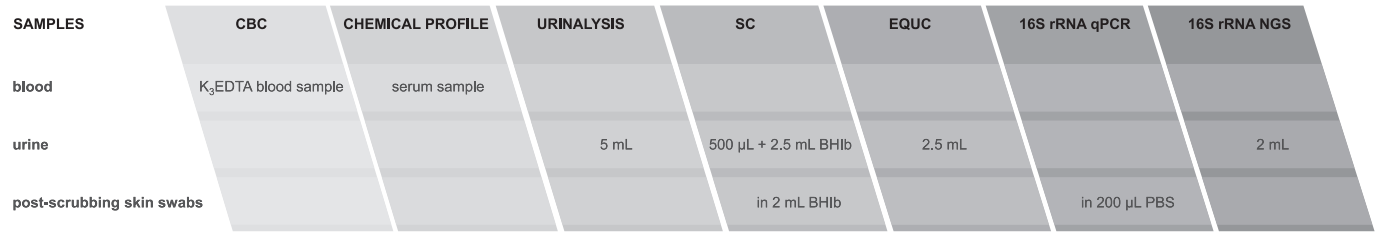

Fig. 1. Samples collected from each cat included in the study and analyses carried out. CBC, complete blood count; SC, standard culture; EQUC, expanded quantitative urine culture; 16S rRNA, 16S ribosomal RNA gene; qPCR, quantitative real-time polymerase chain reaction; NGS, next generation sequencing; K_3EDTA, K_3 ethylene diamine tetra-acetic acid; BHIb, brain heart infusion broth; PBS, phosphate-buffered saline.

The identification of the bacteria was carried out using the MALDI TOF MS (Biotyper Microflex LT, Bruker Daltonics, Billerica, MA, USA) with the MALDI Biotyper software package (version 3.0). Colonies with different morphology were sub-cultured to obtain an abundant and pure culture. Ten to 20 isolated colonies were picked up, suspended in 300 µl of sterile-filtered water (W3500, Sigma-Aldrich, St. Louis, MO, USA) and subjected to ethanol-formic acid protein extraction. Briefly, 900 µl of ethanol were added to the cell suspension, centrifuged at 20,000 rcf for 2 min, and the supernatant discharged. A second wash was performed, visible ethanol was discharged and the pellet was air-dried for at least one hour. The pellet was then suspended in 20–50 µl (depending on pellet size) of formic acid-water solution (70/30; *v*/v) and vortexed. An equal volume of acetonitrile (Sigma-Aldrich, St. Louis, MO, USA) was added and, after thorough vortexing, the solution was centrifuged at 20,000 rcf for 2 min. Then, 1 µl of the supernatant was transferred to the MALDI target plate, allowed to dry at room temperature and overlaid with 1 µl of matrix. The matrix was obtained by dissolving 2.5 mg of α-cyano-4-hydroxy-cinnamic acid in an organic solvent composed of 500 µl of acetonitrile, 25 µl of trifluoroacetic acid (Bruker Daltonics, Billerica, MA, USA) and 475 µl of deionized water. The following settings were applied: positive linear mode; laser frequency 30 Hz; ion source 1 voltage, 19.98 kV, ion source 2 voltage, 17.79 kV; lens voltage, 7.0 kV and mass range, 1960 to 20,137 Da. The instrument was calibrated using the bacterial test strains (Bruker Daltonics, Billerica, MA, USA) according to the manufacturer's instructions. One spectrum was generated for each isolate from 240 laser shots in an automatic acquisition mode. The spectrum of each strain was matched with those contained in the reference database V.3.1.2.0 (Bruker Daltonics, Billerica, MA, USA). Identification reliability was scored, and an arbitrary value from "0" to "3" was assigned. As specified by the manufacturer, a score value lower than 1.7 indicated that the identification was not reliable; scores between 1.7 and 1.999 that the identification was reliable at the genus level; scores between 2.0 and 2.299 that the identification was reliable at the genus level and probable at the species level and scores higher than 2.3 indicated highly probable species identification. In case of missed identification (score value lower than 1.7), the bacterial species was indicated as "unidentified bacterial species".

2.4. Quantitative PCR

The DNA from post-scrubbing skin swabs was extracted using the NucleoSpin Tissue Kit (Macherey-Nagel, Düren, Germany) according to the manufacturer's protocol. The presence of bacterial 16S rRNA gene DNA was evaluated in DNA extracts by SYBR Green qPCR carried out using the PowerUp SYBR Green Master Mix Kit (Life Technologies, Thermo Fisher Scientific, Waltham, MA, USA) and the StepOnePlus Real-Time PCR System (Life Technologies, Thermo Fisher Scientific, Waltham, MA, USA). Primers Bakt_341F (CCT ACG GGN GGC WGC AG) and Bakt_805R (GAC TAC HVG GGT ATC TAA TCC) were used (Herlemann et al., 2011; Klindworth et al., 2013). Extraction- and qPCR-negative controls were included to assess potential DNA contamination.

2.5. Investigation of the urine bacterial community using the culture-dependent approach

Urine samples (2.5 mL) underwent to EQUC procedure (Balboni et al., 2020; Hilt et al., 2014), using a variety of volumes of urine, culture media and incubation conditions to cultivate bacteria that do not grow under standard culturing conditions and allow the detection of microbes similar to that achieved with sequencing techniques (Brubaker et al., 2021; Karstens et al., 2018; Price et al., 2016). For EQUC, 100 µL of the urine samples were plated in (i) BAP incubated at 35 °C in a microaerophilic chamber (Bactron 300, Sheldon Manufacturing, Cornelius, OR, USA) with an atmosphere composed of 5% O_2, 10% CO_2 and 85% N_2; (ii) chocolate colistin and nalidixic acid (CNA) agar (Biolife Italiana, Mascia Brunelli, Milan, Italy) incubated in microaerophilic conditions; (iii) Brucella Agar (BA) (Becton Dickinson, Franklin Lakes, NJ, USA), supplemented with hemin (5 µg/mL), vitamin K1 (1 µg/mL), laked sheep blood (5% *v*/v) and L-cysteine (0.4 g/L) incubated for 48 h at 35 °C in an anaerobic cabinet (Bactron IV, Sheldon Manufacturing, Cornelius, OR, USA) with an atmosphere composed of 5% H_2, 10% CO_2 and 85% N_2 and (iv) two BAPs incubated under aerobic conditions, one at 35 °C and one at 30 °C. In addition, 0.5 mL of each sample was added to 4.5 mL of Veterinary Fastidious broth (CLSI, 2018) which was incubated under anaerobic conditions at 35 °C for 48 h and subsequently plated on a BA and a BAP, incubated at 35 °C under anaerobic and microaerophilic conditions, respectively, and both inspected at 24 and 48 h (CLSI, 2018; Hilt et al., 2014). The bacteria were identified using MALDI TOF MS with the instrument setting reported above.

2.6. Investigation of the urine bacterial community using the culture-independent approach

The DNA from urine samples (2 mL) and PBS (2 mL) for the two NCS samples was extracted using the QIAamp DNA Microbiome Kit (Qiagen, Hilden, Germany), a commercially available extraction kit. The extraction procedure included an initial centrifugation step at low speed (2600 rcf for 10 min) to concentrate the urine sample. The pellet was resuspended in 500 µL of PBS and subjected to multiple disruption steps and protease digestion allowing for a more comprehensive representation of the range of bacterial species detected (Ackerman et al., 2019). The DNA extracted was eluted in 50 µL of Buffer AVE and stored at −20 °C. Total DNA was quantified using a Qubit fluorometer (Life Technologies, Thermo Fisher Scientific, Waltham, MA, USA).

A DNA fragment containing the hypervariable regions 3 and 4 (V3-V4) of the bacterial 16S rRNA gene was amplified from each urine together with NCS samples and was sequenced using a custom protocol developed for the Illumina MiSeq sequencer platform (San Diego, CA, USA) by BioFab Research (Rome, Italy).

The bacterial 16S rRNA gene amplicon library was generated by the PCR amplification of the V3-V4 hypervariable regions carried out with PCRBIO HiFi Polymerase (PCR Biosystems, London, UK) and the universal primers Bakt_341F and Bakt_785 (GAC TAC HVG GGT ATC TAA TCC) complemented with Illumina adapter sequences (Herlemann et al., 2011; Klindworth et al., 2013). Each sample was amplified with 25 PCR

cycles under the following conditions: 95 °C for 30 s, 55 °C for 30 s and 72 °C for 30 s. A negative control in the form of a PCR-amplified ultrapure water sample was also included in the reaction.

The amplicons were analysed by gel electrophoresis and purified. The DNA concentration of the eluted product was determined using a Qubit fluorometer (Life Technologies, Thermo Fisher Scientific, Waltham, MA, USA). The DNA samples were sequenced with an Illumina MiSeq sequencer platform using a paired-end 2 × 300-bp reagent cartridge according to the manufacturer's instructions (MiSeq Reagent Kit v3, Illumina, San Diego, CA, USA). The PCR amplification and sequencing of all the urine and the NCS samples were carried out simultaneously in the same run to avoid introducing variability between different reactions.

The raw sequences were processed using the open-source program DADA2 version 1.26.0 pipeline (Callahan et al., 2016). In particular, the reads were trimmed to remove primer sequences; reads with ambiguous, poor quality bases (phred quality score lower than 20) and >2 expected errors were discarded. The reads obtained were dereplicated and denoised using the DADA2 algorithm after estimating the error rates from the data, by alternating between sample inference and error rate estimation until convergence. The paired reads were then merged, and the chimeras identified and removed. Finally, the taxonomy was assigned to the sequence variants using the naive Bayesian classifier method implemented in DADA2. To this purpose, the Silva taxonomic training data formatted for DADA2 (version 138; McLaren, 2020) was used as a reference. Different metrics of alpha diversity ("Chao1", "ACE" and "Shannon") were calculated using phyloseq R packages (McMurdie and Holmes, 2013). The presence of contaminant reads was assessed by comparing the taxa prevalence in the urine samples with the NCS samples, using the statistical approach implemented in the *is Not Contaminant* function of the *decontam* package in R, which is recommended for low biomass samples, such as urine (Davis et al., 2018). A conservative $P < 0.1$ threshold was selected for removing the contaminant reads. Sample processing and graphics were created using the *phyloseq and microbiome* libraries implemented in R (Lahti and Shetty, 2017; McMurdie and Holmes, 2013).

The FASTQ reads generated in this study can be found in the National Center for Biotechnology Information and registered under SRA (https://trace.ncbi.nlm.nih.gov/Traces/sra) in the BioProject (https://www.ncbi.nlm.nih.gov/bioproject) accession no. PRJNA955387. From Cat01 to Cat19 under SRA accession nos.: SAMN34176564-SAMN34176601, and NCS01 and NCS02 under SRA accession nos.: SAMN34176602-SAMN34176605.

3. Results

3.1. Study population

Nineteen cats with FIC were included in the study. Fifteen out of the 19 cats included in the study were males, 11/15 were castrated, and 4/19 were spayed females. The median age was 5 years (range 11 months – 11 years). Fifteen out of 19 were domestic shorthaired cats and the other four animals were Siamese, Siberian, Bengal and British Shorthaired cats, respectively. Thirteen out of 19 cats were exclusively indoor and the other 6/19 also had access to the outdoors. The clinicopathological findings of the cats included in the study are reported in the Appendix A: Supplementary material. No bacteria grew in the SUC carried out for each urine sample collected from these cats.

3.2. Evaluation of the absence of skin contamination before the ultrasound-guided cystocentesis

No bacteria grew in the standard culture and no bacterial 16S rRNA gene DNA was revealed by qPCR in post-scrubbing skin swab samples.

3.3. Investigation of the urine bacterial community using the culture-dependent and culture-independent approaches

No bacteria grew with the EQUC techniques carried out on the urine samples taken by cystocentesis from each cat included in the study.

The DNA concentration in the eluted extracts obtained from the urine and NCS samples was lower than the limit of detection of the Qubit fluorometer (0.5 ng/mL) while the DNA concentration of the libraries obtained by the amplification of the hypervariable regions V3-V4 of the bacterial 16S rRNA gene is shown in Table 1. The total number of the reads (mean length of 301 nucleotides) obtained by Illumina MiSeq sequencing from each DNA sample, and the number of reads after filtering, denoising, merging and chimera removal are reported in Table 1. Good quality reads were not obtained from the urine sample of Cat17; therefore, this subject, an 8-year-old Siamese castrated male, was excluded from subsequent analyses. In the remaining 18 cats, the final number of merged reads ranged from 441 to 1377 (median 808,5). The final number of reads in the two NCS samples (821 and 1081, respectively) was in the range (Table 1 and Fig. 2). Likewise, the evaluation of several alpha diversity indices revealed similar values among the samples, including the negative controls (Fig. 2).

Bacteria belonging to several orders and genera could be identified in the urine and in the NCS DNA samples (Appendix A: Supplementary material), but all the urine and the NCS samples demonstrated a similar composition. The contaminant read analysis carried out with *decontam* evidenced that no read could be confidently considered as non-contaminant. Beta diversity (bacterial community composition) was not calculated in this study since the reads obtained from the urine samples of the 18 cats and from the two NCS samples did not pass the non-contamination test, thus making the potentially obtained results not reliable.

4. Discussion

In this study, the bacterial community residing in the urinary bladder of 19 cats affected by FIC was investigated using a culture-dependent approach which consisted of the EQUC techniques capable of identifying live bacteria not growing in a SUC (Hilt et al., 2014; Price et al., 2016), and a culture-independent approach which consisted of 16S rRNA NGS capable of exploring microbial DNA diversity with high resolution (Fukuda et al., 2016). To avoid the confounding factors of possible bacterial contamination introduced through urine collection, several precautionary measures were taken during the study. In particular, urine samples were collected by ultrasound-guided cystocentesis and the introduction of viable bacteria or bacterial DNA in the urine samples during collection was tested and reasonably excluded.

The urine sampled from the cats included in the study had no bacterial growth in the EQUC procedure, confirming the SUC results and suggesting that viable bacteria were not present.

in the urine of our FIC cases, as previously reported for healthy cats (Balboni et al., 2020). However, it must be considered that even with EQUC procedure some bacteria may not be cultivable (Vartoukian et al., 2010). In the urine and the NCS samples no DNA was detected by Qubit but bacterial DNA was amplified and sequenced by NGS, as reported for urine in dogs (Mrofchak et al., 2021). From the 16S rRNA gene sequencing, some reads were successfully classified at least at the genus level from the majority of the samples analysed, but a comparable pattern could be observed between the urine and the two NCS samples, both from a qualitative than a quantitative perspective. Accordingly, when the presence of contaminant sequences was statistically assessed, no reads could be confidently classified as non-contaminant.

The presence of a bacterial community in the feline urinary tract has been hypothesised by some authors, both in healthy and FIC cats (Kim et al., 2021). This hypothesis was formulated without the use of enhanced urine culture techniques to test the urine samples and detecting the bacterial DNA in urine samples using NGS techniques only,

Table 1
Summary of bacterial 16S rRNA gene amplicon library features before and after processing. Total number and length of the reads obtained from each DNA sample, and the number of reads after filtering, denoising, merging and chimera removal are reported.

Sample	Library concentration (ng/μL)*	Reads obtained (mean length of 301 nucleotides)	Reads filtered	Forward reads denoised	Reverse reads denoised	Reads merged	Chimera removal
Cat01	0.90	14,449	4182	3842	3813	1284	1111
Cat02	0.30	6967	1202	1085	1075	671	543
Cat03	0.30	5531	1466	1373	1332	651	635
Cat04	0.30	7004	1385	1245	1279	618	607
Cat05	0.20	5298	1204	1079	1068	560	551
Cat06	0.40	9432	1124	985	969	510	441
Cat07	0.80	15,068	3396	3077	3129	816	746
Cat08	1.00	18,635	2989	2595	2565	1402	1133
Cat09	0.60	18,922	2083	1944	1877	981	887
Cat10	0.40	10,695	1678	1554	1573	830	747
Cat11	0.50	11,158	1837	1683	1739	833	769
Cat12	0.60	13,642	2658	2499	2368	1010	920
Cat13	0.70	13,993	1635	1478	1506	684	617
Cat14	0.90	31,999	3258	2930	2994	1561	1212
Cat15	0.50	12,023	2095	1977	1926	962	857
Cat16	0.40	13,980	2721	2512	2460	1636	1377
Cat17	0.50	23	9	1	1	0	0
Cat18	0.60	14,958	1595	1405	1412	883	796
Cat19	0.80	23,460	2084	1777	1787	1154	961
NCS01	0.60	16,019	1922	1665	1649	855	821
NCS02	0.90	28,232	1853	1585	1531	1158	1081

The raw sequences were processed using the open-source program DADA2 version 1.26.0 pipeline (Callahan et al., 2016).
NCS01 and NCS02, negative controls to 16S rRNA gene sequencing carried out at the beginning and at the end of the study, respectively.
* Concentration of the bacterial 16S rRNA gene amplicon libraries determined using the Qubit fluorometer (Life Technologies, Thermo Fisher Scientific, Waltham, MA, USA).

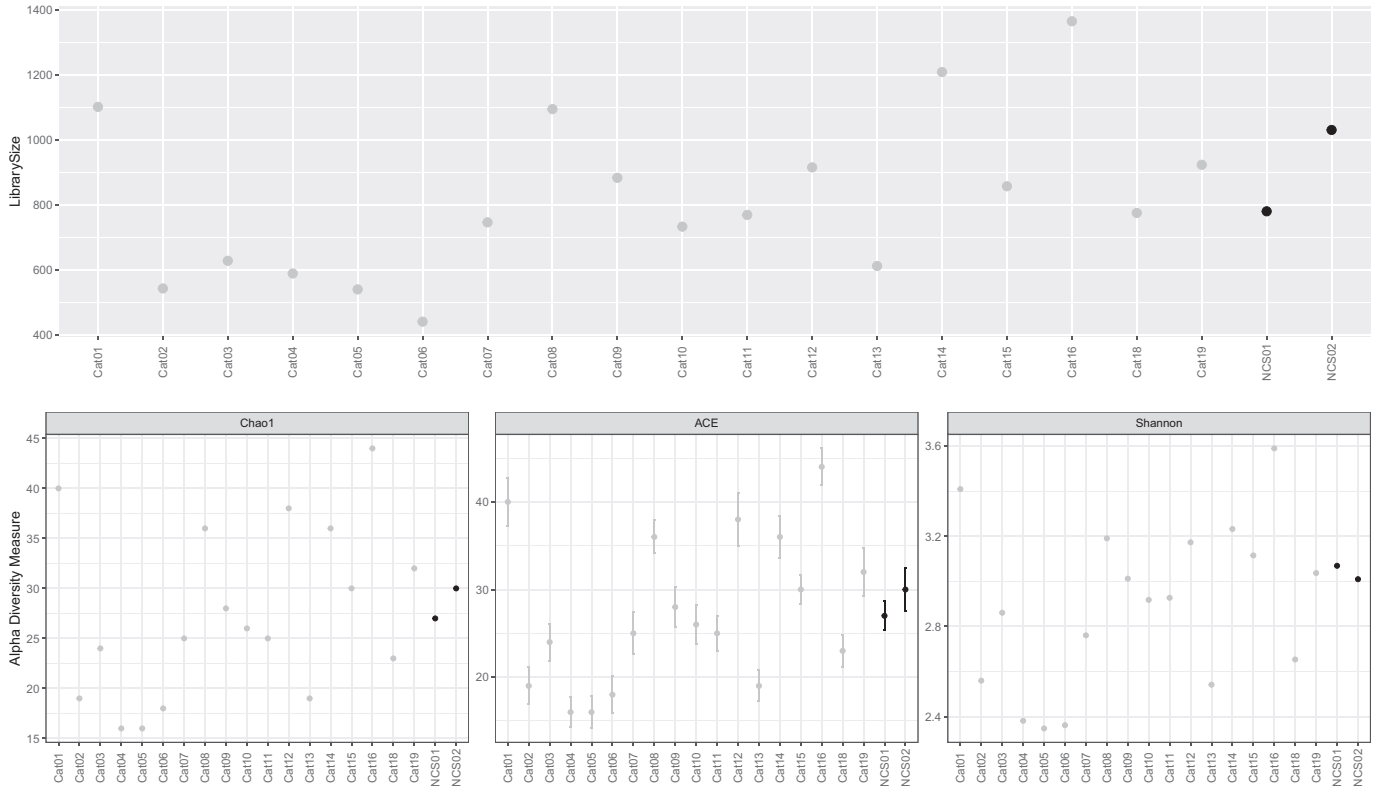

Fig. 2. Library size and alpha diversity evaluation. On the top: library size of the different samples included in the study. Below: different metrics of alpha diversity ("Chao1", "ACE" and "Shannon") calculated using the phyloseq R packages (McMurdie and Holmes, 2013).
NCS01 and NCS02, negative controls to 16S rRNA gene sequencing carried out at the beginning and at the end of the study, respectively.

analysing animal populations which did not show bacterial growth in SUC. Therefore, it remains to be clarified if urine were not sterile and whether the bacterial DNA detected in the urine of cats belonged to viable bacteria residing in the urinary tract. Data obtained in the present study suggest that the bacterial DNA detected in urine samples, in the absence of bacterial growth in the various culture-based tests performed, can be mainly attributable to ubiquitous and unavoidable DNA contamination of the reagents and materials used for the molecular

analyses (Salter et al., 2014), contaminants known as "the kitome" (Brubaker et al., 2021), and potentially to the presence of bacterial DNA circulating in the blood and ultrafiltered by the kidney in the form of residual fragmented DNA (Castillo et al., 2019; Païssé et al., 2016). This assumption is also supported by the fact that the bacterial DNA detected by NGS in cats in the present study, and previously by Kim and colleagues (Kim et al., 2021), mostly belonged to bacterial genera including commonly cultivable species or fastidious but cultivable species. Therefore, if the bacterial DNA detected was not of contaminant origin, these bacteria should have grown in the urine culture but they were not detected by SUC or EQUC. Differently, in humans, viable urinary bacterial community residing in the urine was confirmed not only by sequencing methods but also by culture-based diagnostics (Hilt et al., 2014; Jacobs et al., 2021; Storm et al., 2022). The presence of viable bacterial communities in humans can probably be linked to a different anatomical conformation of the urinary tract compared to that of cats, but also to other predisposing factors linked to the immune system, sexual habits, urinary pH and specific gravity, which differentiate the two species.

This study had some limitations. First of all, urine sample volumes analysed by culture and NGS assays were small and this may have affected the sensitivity of the tests, especially because urine has low microbial biomass (Ackerman et al., 2019). However, the amount of urine that can be collected routinely by cystocentesis from cats hardly exceeds 10 mL in total, and this volume had to be subdivided for the different tests carried out, so this limit is difficult to overcome for this animal species in a clinical setting. Second, a low amount of bacterial sequences of sufficient quality for analysis was obtained for most urine samples tested, including the urine of the Cat17 for which good quality reads were not obtained. However, this result is in line with data already reported for cats, dogs and humans (Ackerman et al., 2019; Kim et al., 2021; Mrofchak et al., 2021).While this could be related to the low volume of urine analysed and to the low microbial biomass of urobiome, on the other hand it may be due to the absence of viable bacteria in the urinary bladder of the enrolled cats and to the non-urinary origin of the little bacterial DNA detected. Finally, different DNA extraction methods were not initially evaluated to choose the best-performing one for cat urine samples. Although the choice of the extraction method is crucial for the analysis of low biomass substrates (Ackerman et al., 2019), it has been reported that the use of different commercially available DNA extraction methods could variably affect DNA concentration, but not bacterial diversity and composition of the urinary microbiota detected (Karstens et al., 2021; Mrofchak et al., 2021). For this reason, the commercially available extraction method specially developed to investigate the microbiome of the analysed samples herein used can be considered suitable for this study.

5. Conclusions

This study reported the absence of a viable microbial community and of bacterial DNA of urinary origin, suggesting that the urinary bladder of cats with FIC is sterile. The absence of a significant bacterial community in the 19 cats affected by FIC analysed in this study adds to the other ten healthy cats analysed with the same methodology in 2020, consolidating the data of the absence of viable bacteria in the urinary bladder of cats tested negative by SUC. Furthermore, this study evidenced that the adoption of both culture-dependent and culture-independent methods of analysis, associated with adequate sampling methods to ensure asepsis and with negative control samples to reveal bacterial 16S rRNA gene background contamination, are of fundamental importance for a correct evaluation of the bacterial communities, especially in low-biomass samples such as urine.

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CRediT authorship contribution statement

Andrea Balboni: Conceptualization, Supervision, Writing – original draft. **Giovanni Franzo:** Data curation, Formal analysis, Software, Writing – original draft. **Luca Bano:** Data curation, Formal analysis, Investigation, Methodology, Supervision, Writing – original draft. **Lorenza Urbani:** Data curation, Formal analysis, Investigation, Methodology. **Sofia Segatore:** Investigation, Methodology. **Alessia Rizzardi:** Formal analysis, Investigation, Methodology. **Benedetta Cordioli:** Formal analysis, Investigation, Methodology. **Matteo Cornaggia:** Formal analysis, Investigation, Methodology. **Alessia Terrusi:** Formal analysis, Investigation, Methodology. **Kateryna Vasylyeva:** Investigation, Methodology. **Francesco Dondi:** Conceptualization, Funding acquisition, Project administration, Writing – review & editing. **Mara Battilani:** Conceptualization, Funding acquisition, Project administration, Supervision, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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