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Author(s)	Young, Young Gang; Guevarra, Robin B.; Jun, Hyung Lee; Wattanaphansak, Suphot; Bit, Na Kang; Hyeun, Bum Kim; Kun, Ho Song
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## Comparative analysis of the reproductive tract microbial communities in female dogs with and without pyometra through the 16S rRNA gene pyrosequencing

Young Gang Song<sup>1,#)</sup>, Robin B. Guevarra<sup>2,#)</sup>, Jun Hyung Lee<sup>2)</sup>, Suphot Wattanaphansak<sup>3)</sup>, Bit Na Kang<sup>4)</sup>, Hyeun Bum Kim<sup>2,\*)</sup> and Kun Ho Song<sup>1,\*)</sup>

<sup>1</sup> College of Veterinary Medicine, Chungnam National University, Daejeon, South Korea 34134

<sup>2</sup> Department of Animal Resource and Science, Dankook University, Cheonan, South Korea 31116

<sup>3</sup> Department of Veterinary Medicine, Faculty of Veterinary Science, Chulalongkorn University, Thailand 10330

<sup>4</sup> Abbvie Bioresearch Center, Abbvie, Worcester, MA USA 01605

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### Abstract

Canine pyometra is one of the most common illnesses in middle-aged to aged bitches. We used the 16S rRNA gene analysis to evaluate whether there are differences in bacterial compositions between dogs with and without pyometra. Control vaginal swabs were obtained from clinically healthy bitches (n = 5) while uterus of bitches (n = 5) with pyometra were obtained through ovariohysterectomy. Results from this study showed that bacteria belonging to families *Pasteurellaceae*, *Fusobacteriaceae* and *Porphyromonadaceae* were abundant in the pyometra group. It is likely that these families comprise bacterial species which may be involved in the pathogenesis of canine pyometra. This is the first report to investigate the microbial community in uterus of bitches with pyometra using high throughput next generation sequencing.

Key Words: microbiota, pyometra, 16S ribosomal RNA

Canine pyometra is one of the most common diseases in intact female dogs, and it is potentially life threatening in middle-aged to aged bitches<sup>8,17)</sup>. Pyometra is defined as the accumulation of pus in the uterine cavity and demands costly surgical or medical interventions to treat the disease<sup>3,6,13)</sup>. The disease is

accompanied by a number of complications including sepsis, septic shock, peritonitis, disseminated bacterial infection and multi-organ dysfunction<sup>8,11)</sup>. It has been reported that mortality in bitches with pyometra was approximately 10% when euthanasia is included, and as low as 1% when the disease is treated through surgery<sup>8)</sup>.

\*Corresponding author: Hyeun Bum Kim, Department of Animal Resource and Science, Dankook University, Cheonan, South Korea 31116, Kun Ho Song, College of Veterinary Medicine, Chungnam National University, Daejeon, South Korea 34134

E-mail: Hyeun Bum Kim, hbkim@dankook.ac.kr and Kun Ho Song, songkh@cnu.ac.kr

#Equal contributors

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**Table 1. Information of the animals enrolled in this study**

Sample name	Sample location	Health status	Breed	Age (years)
C1	vagina	healthy	Maltese	4
C2	vagina	healthy	Maltese	5
C3	vagina	healthy	Maltese	3
C4	vagina	healthy	Pomeranian	4
C5	vagina	healthy	Pomeranian	3
P1	uterus	with pyometra	Shih Tzu	5
P2	uterus	with pyometra	Maltese	4
P3	uterus	with pyometra	Shih Tzu	6
P4	uterus	with pyometra	Maltese	6
P5	uterus	with pyometra	Maltese	5

However, pathogenesis of pyometra is not completely understood despite numerous researches performed<sup>23</sup>. It has been suggested that hormonal and bacterial factors contribute to the development of pyometra and the disease occurs in the luteal phase of the estrous cycle<sup>5</sup>. A progesterone-induced uterus is susceptible to bacterial infections as this hormone changes the uterus for the development of embryo<sup>19,20</sup>. These changes make the uterus more vulnerable for the establishment of infection caused by pathogenic bacteria entering the uterus during estrus<sup>5</sup>.

A few studies have investigated the bacterial community associated with canine pyometra. Recently, *Escherichia coli* (*E. coli*) has been proposed to be the causative organism in 80% of the pyometra cases<sup>15,18</sup>. However, these studies are based on traditional culture methods and may have underestimated potential pathogenic bacteria that are currently uncultivable in the laboratory. In this study, we characterized the uterine microbiome of bitches with pyometra using high-throughput next generation sequencing. More specifically, we used the 16S rRNA gene sequencing to investigate the bacterial community structure in bitches with pyometra.

A total of 10 dogs were enrolled in this study including five uteri from dogs with pyometra and 5 vaginal swabs from healthy dogs that were used as control. The information of the animals is presented in Table 1. For the analysis of vaginal microbiome of healthy bitches, samples

from dogs without pyometra were collected at estrus period and vaginal cytology examination was then conducted to make sure that dogs do not have pyometra since pyometra often occurs in intact bitches after estrus<sup>19</sup>. The activity of cellular immunity is suppressed during the first half of diestrus due to increasing progesterone concentration and minimal estrogen release<sup>19</sup>. Furthermore, the increase in progesterone may lead to endometrial proliferation and uterine glandular secretions and decreased myometrial contractions. When the immune cells are inhibited in the uterus, it often supports bacterial growth<sup>17</sup>. For uterine microbiome analysis, the uterus of dogs with pyometra were obtained through ovariohysterectomy. The samples were collected from dogs with age ranges from 3 to 6 years. The dogs used in this study belonged to three breeds namely: Maltese, Shih Tzu, and Pomeranian. All samples were immediately transported to the laboratory and stored at  $-80^{\circ}\text{C}$  until analysis. All animal protocols were performed according to the Guidelines for the Care and Use of Laboratory Animals of Chungnam National University.

Total DNA was extracted using a commercial DNA extraction kit (Bioneer Inc., South Korea) according to the manufacturer's instructions. From each sample, 20 ng of DNA was used in a 50  $\mu\text{l}$  PCR reaction. The 16S universal primers 27F (5' GAGTTTGATCMTGGCTCAG 3') and 800R (5' TACCAGGGTATCTAATCC 3') were used to amplify 16s rRNA genes (V1-V4 regions).

**Table 2. Diversity indices and mean observed operational taxonomic units (OTUs) of control and pyometra groups with an OTU definition at 97% identity cutoff**

Parameter	Control (n = 5)	Pyometra (n = 5)
Shannon diversity index	0.34 ± 0.44	1.94 ± 0.34 <sup>a</sup>
Simpson diversity index	0.82 ± 0.25	0.25 ± 0.05 <sup>a</sup>
Chao1 estimator of species richness	2.40 ± 1.52	34.87 ± 5.56 <sup>b</sup>
ACE estimator of species richness	0.80 ± 1.79	33.27 ± 5.06 <sup>b</sup>
Mean observed OTUs	2.40 ± 1.52	30.6 ± 6.95 <sup>b</sup>
Total Observed OTUs	12	153 <sup>b</sup>
Number of reads	2971.20 ± 1486.87	3409.20 ± 1843.37 <sup>ns</sup>

Values are presented as mean ± SD (n = 5 per group).

OTU - operational taxonomic unit.

<sup>a</sup>  $P < 0.05$  between two groups.

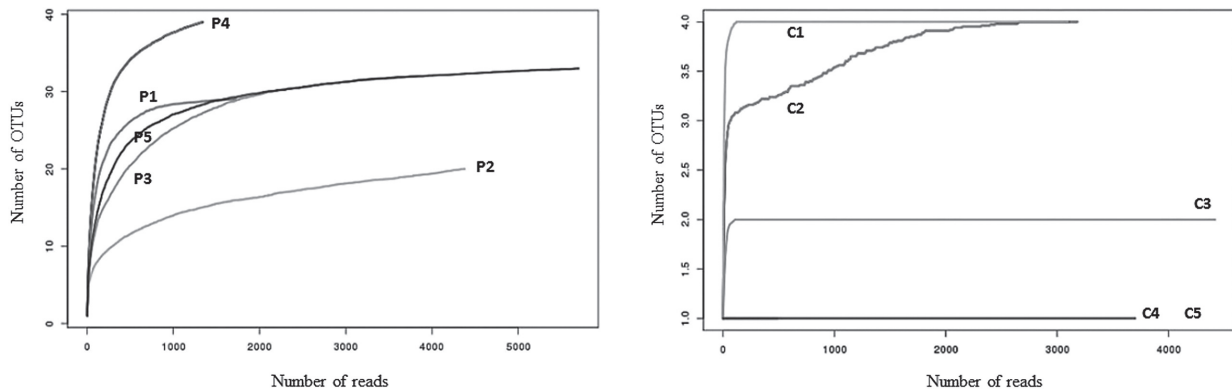
<sup>b</sup>  $P < 0.01$  between two groups.

<sup>ns</sup> not significant

The FastStart High Fidelity PCR System (Roche Applied Science, Mannheim, Germany) was used for PCR with the following reaction conditions: 94°C for 3 min followed by 35 cycles of 94°C for 15 sec; 55°C for 45 sec and 72°C for 1 min; and a final elongation step at 72°C for 8 min. The PCR amplicons were purified using AMPure beads (Beckman Coulter, Brea, CA, USA) and quantified using Picogreen assay (Victor 3, Santa Clara, CA, USA). Pyrosequencing was performed using Roche 454-GS-FLX Titanium chemistry (454 Life Sciences, Branford, CT, USA). Each sample was loaded in 1 region of a 70–75 mm PicoTiter plate (454 Life Sciences, Brandfort, CT, USA) fitted with an 8-lane gasket. The sequencing run was done by Macrogen, Inc. (Seoul, South Korea). The CD-HIT-OTU software was used to eliminate sequences containing ambiguous bases and chimeras, to remove sequence noise, and to cluster the sequences<sup>4</sup>. Operational taxonomic units (OTUs) were generated by the CD-HIT-OTU software using the following cutoff values for similarity: species, 97%; genus, 94%; family, 90%; order, 85%; class, 80%; phylum, 75%. The Mothur software (version 1.31.0) was used to evaluate microbial diversities<sup>16</sup>. The Shannon-Weaver and Simpson diversity indices were used to analyze species diversity<sup>9,21</sup>. All the sequences were compared to the Silva rRNA database using BLASTN<sup>1,14</sup>. The sequences that matched to 16S

rRNA genes with an *E*-value less than 0.01 were classified as partial 16S rRNA sequences. Non-16S rRNA sequences comprised less than 1% of the total sequences. Taxonomic assignment of the sequence reads was performed using NCBI Taxonomy databases. Using the BLASTN program, the five most similar sequences for each sequence were selected according to their bit scores and *E*-values. The Needleman-Wunsch global alignment algorithm was used to perform optimal alignment of the two sequences along their entire duration. A pairwise global alignment was performed on the selected candidate hits to identify the best-aligned hit<sup>12</sup>. The taxonomy of the sequence with the highest similarity was assigned to the sequence read. Statistical analysis was performed using SPSS Statistics 20.0.0 (SPSS Inc., USA). A *p*-value of <0.05 was considered significant. For microbiota analysis, an unpaired *t*-test was used to compare normally distributed data and Man Whitney U-test was used for nonparametric data.

Pyrosequencing by Roche-454-GS-FLX Titanium of 16S rRNA amplicons from all dogs yielded 31,902 bacterial tag-encoded FLX amplicon pyrosequencing (bTEFAP) reads. To compare the bacterial diversity and species richness in the healthy dogs and dogs with pyometra, OTUs were analyzed for each sample at 97% similarity cutoff (Table 2). A mean of

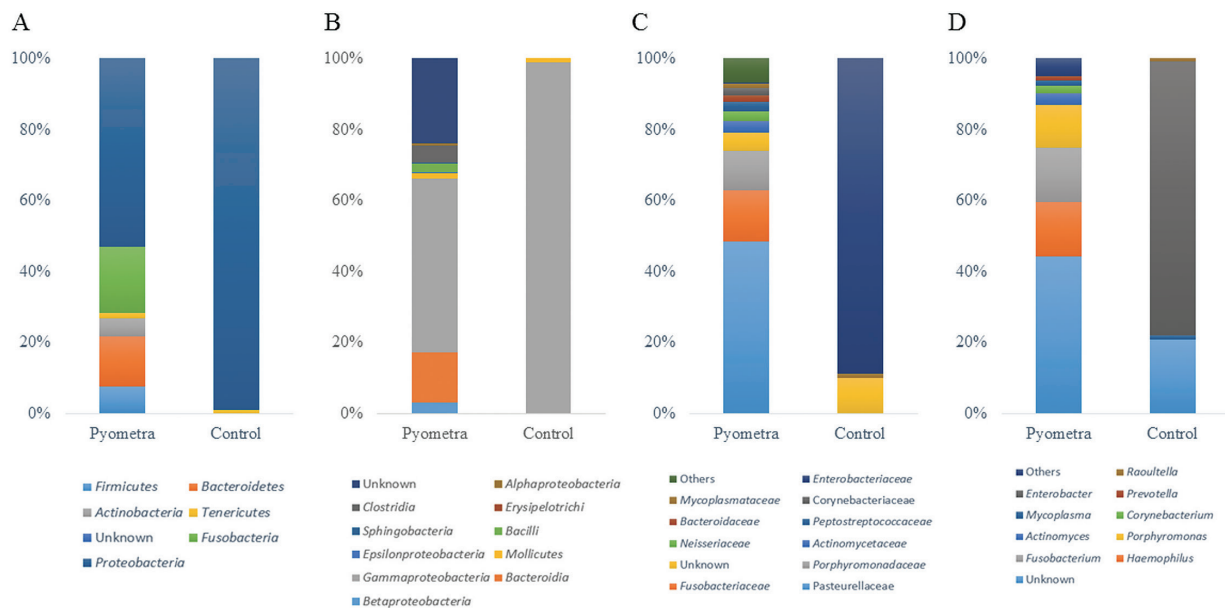


**Fig. 1. Rarefaction curves for 16S rRNA gene sequences of the control (n = 5) and pyometra groups (n = 5) with an OTU definition at 97% identity cutoff.** Letters C and P denote the control and pyometra dogs, respectively, and numbers denote replicates.

2971.20 ± 1486.87 reads was analyzed and 12 OTUs were identified in the healthy group. On the other hand, a mean of 3409.20 ± 1843.37 sequences was analyzed and 153 OTUs were identified in dogs with pyometra. At 95% identity level, the total number of OTUs were 8 and 201 for healthy dogs and dogs with pyometra, respectively. To calculate the diversity of microbial communities at the genus level, Shannon and Simpson's diversity indices were used (Table 2). The mean Shannon index value, which indicates bacterial diversity (species richness and evenness), was higher in pyometra group (2.32 ± 0.36) than the control group (0.06 ± 0.09). A higher Shannon index indicates a higher diversity of microbiota in the sample. Moreover, the mean estimate for species richness, ACE, was significantly higher in dogs with pyometra group (33.27 ± 5.06) than the control group (0.80 ± 1.79). In addition, the Simpson's diversity index values were 0.82 ± 0.25 for the control group and 0.25 ± 0.05 for the pyometra group. Simpson's diversity index indicates that diversity decreases as the index value becomes closer to one and diversity becomes infinite as the value approaches zero<sup>16</sup>. As observed in the diversity analysis, the average number of observed OTUs was significantly higher in the pyometra samples than in the control group. Rarefaction curves of each samples of healthy dogs and dogs with pyometra were provided at an OTU definition of 97% identity

(Fig. 1). The rarefaction curves plateaued after sampling more than 1000 sequence reads in the pyometra group indicating that sampling was sufficient for microbial diversity analysis.

A taxon-dependent analysis was performed to describe the microbiome of dogs with and without pyometra. The relative abundance of uterine and vaginal microbiome at phylum, class, family and genus levels are shown in Fig. 2. Taxonomic classification of the sequences for individual sample analysis is shown in Fig. 3. At the phylum level, the sequences making up the vaginal microbiota of healthy dogs were mainly classified under the phylum *Proteobacteria* (98.97%). On the other hand, the relative abundance of the sequences of uterine samples from pyometra group consisted mostly of *Proteobacteria* (53%), *Fusobacteria* (18.72%) and *Bacteroidetes* (14.32%) (Fig. 2A). At the class level, majority of the sequences in healthy dogs examined were classified as *Gammaproteobacteria* (98.97%). However, majority of the sequences at the class level making up the uterine microbiota of dogs with pyometra consisted of class *Gammaproteobacteria* (49.07%), *Bacteroidia* (14.26%), *Erysipelotrichi* and unclassified bacteria accounted for 23.82% of the total sequences (Fig. 2B). At the family level, the bacterial sequences from healthy group comprised predominantly of *Enterobacteriaceae* (88.92%), unclassified bacteria (10.05%) and *Mycoplasmataceae* (1.03%) while the relative



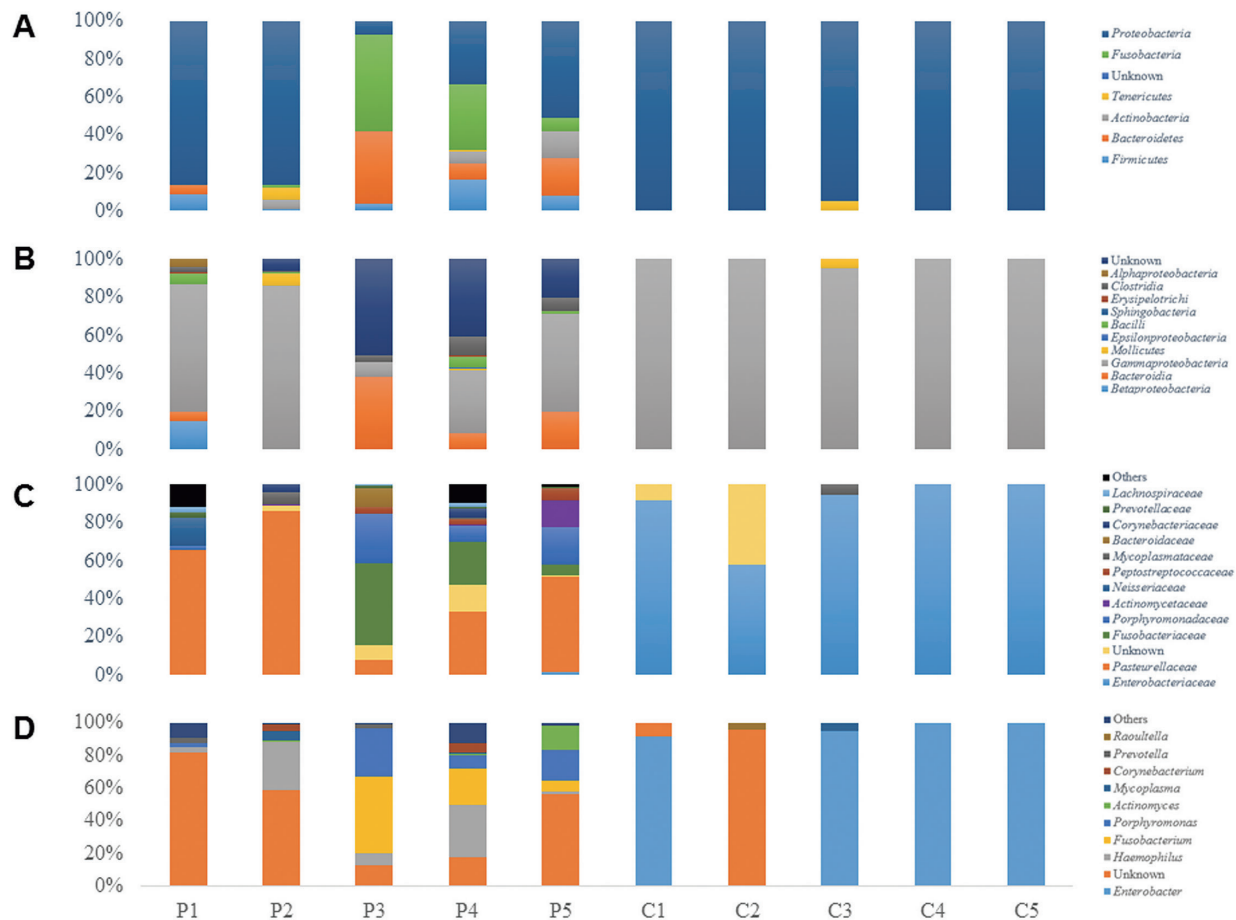
**Fig. 2. Group-based taxonomic classification of the 16S rRNA gene sequences at the phylum (A), class (B), family (C) and genus levels (D) of the control and pyometra samples.**

distribution of the sequences in the pyometra group were mainly classified as *Pasteurellaceae* (48.53%), *Fusobacteriaceae* (14.25%) and *Porphyromonadaceae* (11.23%) (Fig. 2C). At the genus level, the bacterial sequences from the healthy group comprised predominantly of *Enterobacter* (77.33%). By comparison, the sequences analyzed from the pyometra group at the genus level consisted predominantly of unclassified bacteria (41.3%), *Haemophilus* (14.3%), *Fusobacterium* (14.3%) and *Porphyromonas* (11.2%) (Fig. 2D).

In this study, we aimed to compare the reproductive system bacterial communities of healthy dogs and dogs with pyometra using 16S rRNA gene sequencing. The results of this study showed that there were significant differences between the vaginal microbiota of healthy bitches and uterine microbiota of bitches with pyometra. At 97% similarity cutoff level, we identified 12 and 153 OTUs from healthy and pyometra group, respectively, suggesting that uterus of dogs with pyometra have higher bacterial diversity than dogs with normal uterus. The low bacterial diversity in the control group was also reflected in the Shannon diversity index ( $0.34 \pm 0.44$ ). The increased in species richness and species diversity

in the pyometra group may be attributed to bacterial infection in the uterus. In contrast with our results, a recent study of characterization of bacterial community in bovine pyometra revealed a reduced bacterial diversity in the uterus of cows<sup>10</sup>. This suggests that there is a high bacterial diversity involved in the pathogenesis of pyometra in dogs.

The recent advent of omics technology and advanced sequencing techniques expanded our catalog of bacterial species that cannot be cultured in the laboratory. However, there were lack of information on the microbial community of female dog reproductive system microbiome. To the best of our knowledge, our study is the first attempt to investigate the microbial community in uterus of bitches with pyometra using high throughput next generation sequencing. The microbial community composition between control and pyometra group showed significant differences in the enrichment of taxa in bitches with pyometra these bacteria belonged to the following group: *Proteobacteria*, *Gammaproteobacteria*, *Pasteurellaceae*, *Porphyromonadaceae*, *Fusobacteriaceae*, *Fusobacterium*, *Haemophilus* and *Porphyromonas*. These bacterial group may



**Fig. 3. Individual sample-based taxonomic classification of the 16S rRNA gene sequences at the phylum (A), class (B), family (C) and genus levels (D) of the control (n = 5) and pyometra group (n = 5). Letters C and P in the x-axis denote the control and pyometra group, respectively, and numbers denote replicates.**

harbor species that may be involved in the pathogenesis of pyometra that have not been previously identified using traditional culture techniques.

Microbiological aspects of canine pyometra has been evaluated previously using culture-based techniques. Coggan *et al.*<sup>2)</sup> reported the microbial isolates from the intrauterine contents of 100 dogs with pyometra. According to their results, the most important pathogen of pyometra in bitches is *E. coli* which accounted for 76.6% of the total isolates from dogs with pyometra. Other bacteria that have been isolated were *Klebsiella pneumoniae* subsp. *pneumoniae*, *Citrobacter diversus*, *Pseudomonas aeruginosa*, *Staphylococcus kloosii*, *Salmonella* spp., *Proteus mirabilis*, *Streptococcus* sp., *Morganella morgani* *Klebsiella*

*pneumoniae* subsp. *azanae*, *Staphylococcus schleiferi* subsp. *coagulans*, *Staphylococcus intermedius*, *Staphylococcus epidermidis*, *Streptococcus canis*, and *Corynebacterium jeikeium*. In addition, vaginal microbiota of spayed dogs with or without recurrent urinary tract infection (UTI) were investigated using culture dependent approach in a previous study by Hutchins *et al.*<sup>7)</sup>. They showed that the most common bacterial isolated obtained from the vaginal tract of dogs were *E. coli* and *Staphylococcus pseudintermedius*. These suggest that *E. coli* is an important microorganism that can be isolated from dogs infected with pyometra and may have an important role in the transmission of the disease to other animals<sup>2)</sup>. Moreover, it can be speculated that UTI is

associated with pyometra and it enhances the growth of *E. coli* in the uterus.

However, in the present study very few sequences were classified to the *Enterobacteriaceae* family which harbors *E. coli*. Furthermore, relative abundance of the sequences belonging to the family *Pasteurellaceae*, *Porphyromonadaceae*, and *Fusobacteriaceae* were identified in large quantities suggesting that they may play a role in the pathogenesis of pyometra. For example, the genera *Haemophilus*, *Porphyromonas* and *Fusobacterium* could be potential detrimental bacteria in dogs with pyometra. These group of bacteria have not been previously associated with pyometra in dogs and may have been underestimated in culture studies. Moreover, it should be noted that majority of the OTUs at the genus level in pyometra samples were unclassified suggesting that high bacterial diversity involved in pathogenesis of pyometra in dogs are yet to be discovered. In human uterine microbiome studies, *Bacteroides xylinivorans*, *Bacteroides thetaiotaomicron*, *Bacteroides fragilis* and *Pelomonas* were the most predominant bacterial species in the human uterus<sup>22)</sup>. In the present study, the bacterial families that could be associated in pyometra were members of the phyla *Fusobacteria*, *Proteobacteria* and *Bacteroidetes*, which are also the prominent phyla observed in human uterine microbiome<sup>22)</sup>.

To the best of our knowledge, our study is the first attempt to investigate the microbial community in uterus of dogs with pyometra using high throughput next generation sequencing of the 16S rRNA gene. In comparison to the microbial community profiles identified using culture-based studies, the present study identified bacteria that have not been previously reported in bitches with pyometra. In addition, results of this study showed that there was an increase in the alpha diversity in the uterus of bitches with pyometra as compared to the control. During the estrus period, the vaginal microbiome showed that there was a low bacterial species richness and diversity in the

healthy group. In addition, we detected the three most abundant families in the pyometra group that might exacerbate the pathogenesis of pyometra: *Pasteurellaceae*, *Fusobacteriaceae* and *Porphyromonadaceae*. While further studies are required using metagenomic sequencing to determine the functional bacterial genes involved in pyometra pathogenesis, findings of this study would provide us with fundamental knowledge to better understand pathogenesis of pyometra. In addition, further clinical and experimental investigations along with metagenomics studies will help us to elucidate the pathogenesis of canine pyometra.

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