Comparison of canine urinary bacterial population in calcium oxalate urolithiasis and healthy conditions

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A Thesis Submitted in Partial Fulfillment of the Requirements
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Department of Veterinary Surgery
FACULTY OF VETERINARY SCIENCE
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การเปรียบเทียบชนิดของแบคทีเรียในกระเพาะปัสสาวะของสุนัขที่เป็นนิ่วประเภทแคลเซียมออกซาเลตและในสุนัขปกติ

น.ส.ณิชมน รักประกอบกิจ

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

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โรคนิ่วในระบบทางเดินปัสสาวะเป็นหนึ่งในโรคของทางเดินปัสสาวะส่วนล่างของสุนัขที่พบได้บ่อย โดยที่ชนิดของนิ่วที่พบได้บ่อยที่สุดในสุนัขคือนิ่วชนิดสตรูไวท์ และพบบ่อยเป็นอันดับสองคือแคลเซียมออกซาเลต ซึ่งจากรายงานพบว่าแนวโน้มของการเกิดนิ่วประเภทแคลเซียมออกซาเลตมีแนวโน้มที่จะเพิ่มขึ้นเรื่อยๆ โดยปัจจุบันยังไม่มีคำตอบที่แน่ชัดถึงสาเหตุของการเกิดนิ่วชนิดแคลเซียมออกซาเลตและการเกิดนิ่วซ้ำหลังการผ่าตัด ซึ่งมีงานวิจัยพบว่าการเปลี่ยนแปลงไมโครไบโอมในทางเดินอาหารเป็นหนึ่งในสาเหตุที่ทำให้เกิดนิ่วแคลเซียมออกซาเลตในกระเพาะปัสสาวะได้ ดังนั้นงานวิจัยนี้มีวัตถุประสงค์เพื่อระบุชนิดของไมโครไบโอมในกระเพาะปัสสาวะของสุนัขที่เป็นนิ่วแคลเซียมออกซาเลต และเพื่อเปรียบเทียบความแตกต่างของไมโครไบโอมในกระเพาะปัสสาวะของสุนัขสุขภาพดีเทียบกับสุนัขที่เป็นนิ่วแคลเซียมออกซาเลต โดยการศึกษานี้แบ่งสุนัขออกเป็นสี่กลุ่มการทดลอง ได้แก่กลุ่มสุนัขสุขภาพดีเพศผู้และเพศเมีย กลุ่มสุนัขที่เป็นนิ่วแคลเซียมออกซาเลตเพศผู้และเพศเมีย โดยเก็บปัสสาวะจากกระเพาะปัสสาวะของสุนัขทั้งสี่กลุ่มโดยวิธีการเจาะเก็บจากกระเพาะปัสสาวะ หลังจากนั้นนำปัสสาวะที่ได้ไปตรวจวิเคราะห์ เพาะเชื้อแบคทีเรีย และนำผนังชั้นในของกระเพาะปัสสาวะไปทำการตรวจหาเชื้อแบคทีเรียเพิ่มเติมในสุนัขที่ทำการผ่าตัดนิ่วในกระเพาะปัสสาวะ เพื่อเป็นการยืนยันว่าไม่มีการติดเชื้อในระบบทางเดินปัสสาวะ และส่งวิเคราะห์ชนิดนิ่วที่ Minnesota Urolith Center (ศูนย์วิจัยนิ่วรัฐมินนิโซตา) หลังจากนั้นนำปัสสาวะไปทำการสกัดดีเอ็นเอ นำไปวิเคราะห์ลำดับเบสเพื่อระบุชนิดของแบคทีเรียทั้งหมดด้วย next generation sequencer (miseq) และนำข้อมูลที่ได้มาแปลผล จากผลการวิเคราะห์พบว่าดัชนีความหลากหลายของแบคทีเรียที่พบในปัสสาวะของสุนัขแข็งแรงและสุนัขที่เป็นนิ่วมีความแตกต่างกันอย่างมีนัยสำคัญทางสถิติทั้งความหนาแน่นและความสม่ำเสมอ แต่ไม่มีความแตกต่างระหว่างสุนัขเพศผู้และเพศเมีย ทั้งยังมีการพบแบคทีเรียที่แตกต่างกันในกลุ่มการทดลอง ปัสสาวะของสุนัขที่เป็นนิ่วชนิดแคลเซียมออกซาเลตพบความเกี่ยวเนื่องกับกลุ่ม oxalotrophic bacteria โดยพบแบคทีเรียลำดับ *Alphaproteobacteria* และ วงศ์ *Caulobacteraceae* รวมถึง วงศ์ *Oxalobacteraceae* สกุล *Ralstonia* มากกว่าอย่างมีนัยสำคัญทางสถิติ ซึ่งแบคทีเรียเหล่านี้มีความเกี่ยวเนื่องกับแบคทีเรีย oxalotrophic ซึ่งเป็นแบคทีเรียในทางเดินอาหารที่มีการใช้ออกซาเลตเป็นแหล่งพลังงานและมีความสำคัญในการสร้างนิ่วแคลเซียมออกซาเลต จากการทดลองดังกล่าว จึงสามารถสรุปได้ว่าทั้งสองกลุ่มการทดลองมีความหลากหลายของแบคทีเรียที่แตกต่างกัน และมีชนิดของไมโครไบโอมที่แตกต่างกันอย่างมีนัยสำคัญทางสถิติ ทำให้อาจจะเป็นประโยชน์ในการอธิบายพยาธิกำเนิด วินิจฉัยเบื้องต้น และเป็นพื้นฐานของการศึกษาการป้องกันและการรักษาได้ในอนาคต

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# # 6175303331 : MAJOR VETERINARY SURGERY
KEYWORD: 16S rRNA sequencing Calcium oxalate Microbiome Urinary bladder Urolith

Nichamon Rakprakobkij : Comparison of canine urinary bacterial population in calcium oxalate urolithiasis and healthy conditions. Advisor: Nicole Mehl, Ph.D. Co-advisor: Assoc. Prof. Dr. SUNCHAI PAYUNGPORN

Urolithiasis is one of the most common lower urinary tract diseases in dogs. Struvite is the most canine uroliths reported, and the second reported is calcium oxalate (CaOx). However, the proportion of CaOx urolithiasis tends to be increased. CaOx urolithiasis has high incidence of recurrence after the treatment and the pathogenesis of the stone formation is uncleared. Recent studies revealed that oxalate degrader bacteria in the gastrointestinal tract has important roles in CaOx stone formation but no research has been done to study the composition and the role of microbiome in the urinary tract of dogs with CaOx urolithiasis. This study has purpose to characterize the urinary bacterial population in urinary bladder of dogs with CaOx urolithiasis and to compare the difference of microbiome in urinary bladder of dogs with healthy condition and CaOx urolithiasis. Urine samples were collected by cystocentesis from 10 healthy female, 9 healthy male, 6 CaOx urolithiasis female, and 7 CaOx urolithiasis male dogs undergoing neutering and cystotomy procedures. Urine samples were used for urinalysis, urine culture, and *16S rRNA amplicon* sequencing by using next generation sequencer (miseq). The V3-V4 region of the 16S rRNA bacterial gene was amplified and compared for operational taxonomic units (OTU) data and relative abundance for both urine samples. We found that the urinary microbiota from CaOx urolithiasis dog had significantly higher abundance and evenness compared to the healthy dogs (p-value = 0.0035), with no significant found difference between sex. We also found significant higher amount of the microbes in CaOx urolithiasis group than another group which are family *Caulobacteraceae*, class *Alphaproteobacteria*, and family *Oxalobacteraceae* genus *Ralstonia*. These microbes are related with the oxalotrophic bacteria, oxalate degrader microorganism lives in gastrointestinal tract that has a role in CaOx stone formation. In conclusion, while recognizing the limitations of the diagnosis and treatment, this finding may lead to more accurate diagnosis, alternative treatment, or even though prevention methods in the future.

| Field of Study: | Veterinary Surgery | Student's Signature .............................. |
|---|---|---|
| Academic Year: | 2020 | Advisor's Signature ............................. |
| | | Co-advisor's Signature ........................ |

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จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

## TABLE OF CONTENTS

Page


.......... iii

ABSTRACT (THAI) .......... iii

.......... iv

ABSTRACT (ENGLISH) .......... iv

ACKNOWLEDGEMENTS .......... v

TABLE OF CONTENTS .......... vi

LIST OF TABLES .......... viii

LIST OF FIGURES .......... ix

CHAPTER I INTRODUCTION .......... 1

1.1 Importance and Rationale .......... 1

1.2 Objectives of the Study .......... 2

1.3 Research frame .......... 2

1.4 Advantages of study .......... 3

CHAPTER II LITERATURES REVIEW .......... 4

2.1 Urolithiasis in dogs .......... 4

2.2 Calcium oxalate Urolithiasis .......... 5

2.3 Microbiome and Microbiota .......... 6

2.4 Urinary microbiota .......... 7

2.5 Microbiomes and Urolithiasis .......... 10

CHAPTER III MATERIALS AND METHODS .......... 12

3.1 Animals .......... 12


จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

3.2 Diagnosis of Calcium Oxalate Urolithiasis.......... 13

3.3 Sample collection.......... 14

3.4 DNA Extraction.......... 15

3.5 16S amplification by PCR (Polymerized Chain Reaction).......... 16

3.6 Statistical analysis.......... 16

CHAPTER IV RESULTS.......... 18

4.1 Animals.......... 18

4.2 Diversity and richness of the canine urinary microbiota.......... 19

4.3 Composition of the canine urinary microbiome in healthy and calcium oxalate urolithiasis dogs.......... 22

4.4 Comparison of urinary microbiome to healthy and calcium oxalate uroliothiasis dogs.......... 24

CHAPTER V DISCUSSION.......... 27

REFERENCES.......... 32

VITA.......... 37

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

# LIST OF TABLES

**Page**

Table 1. Microbiome composition of urine in healthy human both men and women according to Aragón et al., (2018) ........................................................................ 9

Table 2. Population study demographic and the results from urine routine urinalysis in each sample. ........................................................................................................... 18

Table 3. OTU table represents samples and family taxonomic units......................... 23

# LIST OF FIGURES

Page

Figure 1. Lateral view of abdominal radiograph in calcium oxalate urolithiasis dogs................................................................13

Figure 2. Block plots showing alpha diversity index of various taxa detected in a urine sample from healthy and calcium oxalate urolithiasis dogs...........................20

Figure 3. This dot plots revealed Bray-curtis index which compare microbial communities in healthy dogs and calcium oxalate urolithiasis dogs...............................21

Figure 4. Stacked bar charts showing OTU bar plots of relative abundance of microbial DNA detected via *16S rRNA amplicon* sequencing and annotated to the taxonomic level of genus in urine samples............................................................23

Figure 5. the LEfSe algorithm revealed the UM that is differentially represented in calcium oxalate cases versus controls....................................................... 25

Figure 6. This graph shows the amount of these microbes in both control and CaOx urolithiasis groups.......................................................................26

จุฬาลงกรณ์มหาวิทยาลัย
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# CHAPTER I

# INTRODUCTION

## 1.1 Importance and Rationale

Urolithiasis is one of most common lower urinary tract diseases in dogs. Struvite is the most canine uroliths reported (40%), and the second reported is calcium oxalate (CaOx). However, the proportion of CaOx urolithiasis tends to increase since 2009 to 2015 (Hunprasit, 2017). Another study of 2020 Global Urolith Data from Minnesota Urolith Center, University of Minnesota. Urolith Center found that CaOx urolithiasis was the second most common urolith in canine in the world while it is the most common urolith in Asia. Study of the dogs in Spain and Portugal from 2004- 2006 showed that CaOx urolithiasis was the most common (38.1%), followed by Struvite (32.9%) (Vrabelova et al., 2011). From all of the studies above, the trend of CaOx uroliths in dogs seems to increase. Furthermore, CaOx urolithiasis has high incidence of recurrence after the treatment and the pathogenesis of the stone formation is uncleared (Mittal et al., 2003).

In a healthy condition of mammals, the hosts' body has variety of microorganisms such as fungi, viruses, protozoa, and bacteria, which shares most of the proportion. There are many studies about microbiome in different organs especially gastrointestinal and reproductive tract. Previously, the urinary bladder in healthy humans and dogs have been considered free of bacteria. However, recent studies of both human and dog urinary microbiome has been described as it plays a protective role in the urinary bladder (Antunes-Lopes et al., 2018). Wolfe and Brubaker, 2015, have shown the multiple species of normal flora in the organ, these normal flora have the effect on stabilization of the environment in specific organ to prevent other pathogenic organisms to grow and cause the disease. Some of the study also shows some contrast of the microbiome within the urinary tract that might help to prevent the pathogenic organisms (Whiteside et al., 2015). On the other hand, it is still unclear in terms of the role and benefit of the microbiome in the urinary tract, so we would like to investigate furthermore if there is an alteration of

the normal environment of the urinary bladder and in the patient with urinary bladder disease such as urolithiasis.

Further studies are still needed to understand the actual cause and pathology of CaOx urolithiasis. Microbiome could be one of the causes of CaOx due to Allison's study in year 1985 which pointed out a bacteria called *Oxalobactor formigenes*. *Oxalobactor formigenes* in the guts was proved to play a key role in CaOx urolithiasis. They found that this oxalate degrading bacteria decreases the absorption of oxalate in the guts which is the initial substance to form CaOx (Allison et al., 1985). However, no studies have been done on the importance of microbiome in the urinary bladder whether it plays an important role on the formation of CaOx uroliths or not.

**1.2 Objectives of the Study**

1. To characterize the urinary microbiome in urinary bladder of dogs with calcium oxalate urolithiasis.
2. To compare the difference of microbiome in urinary bladder of dogs with healthy condition and calcium oxalate urolithiasis

**1.3 Research frame**

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Twenty four dogs were enrolled in this study. The dogs were divided based on sex, history taking, urinalysis, crystal microscopic examination, and radiography into four groups: healthy control male group (n=9), healthy control female group (n=10), CaOx urolithiasis male group (n=7), and CaOx urolithiasis female group (n=6). Dogs that had been treat with antibiotics, probiotics, or corticosteroids within the previous 30 days, received intravenous or subcutaneous fluids therapy were ruled out from the study. In the healthy conditions groups, urine samples were collected by cystocentesis from 9 male and 10 female dogs that come to Chulalongkorn animal hospital for castration and OVH. The urine samples, calculi, and urinary bladder mucosa were collected from 7 male and 6 female calcium oxalate

urolithiasis dogs while performed cystotomy to remove the calculi. Urine samples were separated for routine urinalysis, routine urine culture, and 16S rRNA amplicon sequencing by using next-generation sequencing and analysed by using QIIME2 informatics analysis. Calculi samples were collected for identification by Urolith service from Hill's Pet Nutrition, Inc. ®, Minnesota.

**1.4 Advantages of study**

One of the most common lower urinary tract diseases in dogs is urolithiasis while CaOx uroliths seems to be increased each year. However, the cause of CaOx stone formation and the prevention of the recurrence after treatment is still not clearly understood. Thus, this study has a benefit in study and characterize the microbiome in canine urinary bladder with calcium oxalate urolith and the difference of urinary microbiome may play an important role in the future to improve the diagnosis, treatment, and prevention of urolithiasis.

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# CHAPTER II

# LITERATURES REVIEW

## 2.1 Urolithiasis in dogs

Urolithiasis is a formation of crystalline and occasionally non crystalline solid substances called uroliths within the urinary tract (Bartges et al., 2015; Koehler et al., 2009). The disease has high incidence of recurrence after the treatment due to the unclear pathogenesis of the stone formation. The incidence of canine urolithiasis was found between 0.5 to 1% of canine population, and 18% to 20.61% in dogs with lower urinary tract diseases (Claudia et al., 2018; Hesse A, 1990). Urolithiasis is a multifactorial disorder with many risk factors included sex, breed, age, diet, anatomical and functional urinary alterations, metabolic disorders, genetic predisposition, and bacterial urinary tract infection (BUTI) (Osborne et al., 1999). From the multiple risk factors of uroliths, it could be composed of one or more mineral composition.

There are several theories of the formation of bladder stones. Precipitation-Crystallization Theory is the most commonly accepted theory. According to this theory, one or more stone-forming crystalline compounds are present in elevated levels in the urine. Dietary factors or some previous disease in the bladder, especially a bacterial infection can be the cause of the elevation of crystalline in urine. Moreover, the cause of the condition may be due to a problem with the metabolism of the body. The urine becomes saturated and cannot hold any more of the compound when the amount of this compound higher a threshold level. The precipitation of excess compounds forms tiny sharp crystals which can irritate the bladder lining. Thus, the production of mucus due to the irritation causing the crystals and mucus stick together, forming clusters that gradually enlarge and harden into stones.

In 2020, Struvite was the most common urolith reported (40%), followed by calcium oxalate (35%), other compounds including urate, cysteine, calcium phosphate, and silica were less common. There were a significant decrease proportion of struvite submissions with increase in calcium oxalate uroliths submissions in many countries including Thailand (Osborne et al., 1999; Piscavet et al., 2007; Houston et al., 2009; Del et al., 2010; Hunprasit., 2019). Urolithisis clinical sign was lower urinary tract disease, red urine may observed. Abnormalities were not usually detected on physical examination. The patient's rectal temperature, pulse rate, and respiratory rate were normal. Evaluation of a serum biochemistry profile might revealed no abnormalities. The culture of the urine or urine sediment were not evaluation the bacteria.

**2.2 Calcium oxalate Urolithiasis**

There is a continuously increasing of calcium oxalate stones widespread presence over the last three decades. The exact mechanism of CaOx stone formation is unknown and is likely a combination of genetic, dietary, and environmental factors. The reasons of the increasing of the prevalence may be from the changes of dietary composition to prevent struvite (the most common type of stones), feeding more dry foods compared to moist foods, changes in breed popularity, etc. Predisposing factors are the age, sex, and breeds. Breeds that have high risk of developing calcium oxalate such as miniature schnauzer, Yorkshire terrier, Miniature poodle and Keeshounds. Calcium oxalate calculi are most commonly found in the bladder compared to other parts of the urinary tract. Normally, the recurrent rate in one year following surgery is about 36% (Lulich et al., 1995). Management should be in good care to reduce the recurrent rate. Dietary management, providing ad libitum water, and constant postoperative monitor. Ideally, urine specific gravity should be maintained at <1.020 with alkaline urine pH 6.8-7 to decrease the oxalate crystal formation (Dana et al., 2004). Recently, *Oxalobacter formigenes* was found in the

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gastrointestinal tract which has roles in degradation of oxalate. The absence of *O. formigenes* population in the gut microbiome environment increase the chance to develope hyperoxaluria or recurrent kidney stone disease. (Allison et al., 1985; Mittal et al., 2003)

However, the calcium oxalate dihydrate crystalluria was observed. Survey abdominal radiographs of the abdomen obtained with the primary objective evaluating the patient for the presence of uroliths revealed multiple urocystoliths.

Once uroliths were confirmed, radiography helped to characterize their locations, number, size, density, and shape. This information is invaluable in helping to predict the mineral composition of the uroliths (Lulich et al., 1999). To confirm the calcium oxalate urolith, the urolith composition need to be assessed by various assays. After sectioning the layer, each layer was analyzed by optical crystallography under the polarized light microscopy. In some case, x-ray microanalysis was used with scanning electron microscopy or Fourier transformation infrared spectroscopy. The classification of the mineral type was used by percentage of the mineralization.

In case that the uroliths comprised of calcium oxalate monohydrate or calcium oxalate dehydrate or both was classified as calcium oxalate (Doreen et al, 2017).

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**2.3 Microbiome and Microbiota**

Each particular environment contains the microbiota that are viewed as specific microorganisms. All the microorganisms can be represented as the microbiota including bacteria, viruses, and fungi. These microbiota species are reviewed by utilizing molecular techniques such as 16S ribosomal RNA (rRNA). There are localized differences in the microbiota from organ in the body of each individual mammal. The microbiome generally refers to the genetic content of all the

microbiota, the products of the microbiota and the surrounding environmental conditions (Whiteside et al., 2015).

## 2.4 Urinary microbiota

Urinary microbiota is defined as the microorganism in the urinary bladder and urinary microbiome represents their genomes. In the past, urinary bladder was considered as a sterile organ but the discover of microbiota made the clinicians and scientists to reassess the understanding of health and disease urinary bladder. Urinary microbiota was first discovered in female human bladder using broad-range *16S ribosomal RNA* (rRNA) gene sequence analysis. This new discovery makes the standard urine culture protocol not enough to assess the condition of urinary bladder. Due to the limitation that the bacteria cannot be detected by the standard protocol, they require special concerns such as special nutrient. Some of the microbiota grow slowly and cannot tolerate oxygen or have very small population. A new technique called expanded quantitative urine culture (EQUC) protocol which uses 100 times of the urine volume and a variety of media and atmospheric conditions to detect the bacteria that standard protocol cannot detect. (Price et al., 2016) From this technique, the researchers found that there are the different of species and number of bacteria between healthy and diseased urinary bladder which is important for the prevention and treatment of lower urinary tract disorders. (Aragón et al., 2018)

Many studies were performed in human between healthy and diseased groups to earn more knowledge of the role of urinary microbiota. However, the difference between healthy and diseased groups that were found in a result of higher population of *Gardnerella spp., Actinomyces spp., Aerococcus spp.* and lower population of *Lactobacillus spp.* in urgency urinary incontinence (UUI) compared to healthy group, higher number of *Lactobacillus gasseri* population in UUI compared to healthy group, lower number of *Lactobacillus spp.* population in post-treatment

urinary tract infection group compared to healthy group, higher number of *Streptococcus spp.* in urine of urothelial cell carcinoma group compared to healthy group, higher number of *Escherichia coli* in seminal fluid and prostatic secretion but lower number in urine samples of prostate cancer group compares to healthy group, higher number of *Enterococcus spp.* in seminal fluid of prostate cancer compares to healthy group, higher bacterial diversity in chronic prostatitis group with higher number of *Clostridia spp.* and *Bacteroides spp.* compare to healthy group, bacterial diversity of lower severity score is more similar to healthy group, higher number of *Lactobacillus spp.* but lower number of other species in interstitial cystitis group compared to healthy group, significant prevalence of fungi (*Candida* and *Saccharomyces sp.*) in bladder pain syndrome/Interstitial cystitis with flares group compare to bladder pain syndrome/Interstitial cystitis without flares group, higher number of *Lactobacillus* and *Corynebacterium spp.* in healthy group compare to Neurogenic bladder dysfunction group (higher *Klebsiella, Enterococcus* and *Escherichia spp.)* and higher *Sneathia, Gemella, Aerococcus, Anaerococcus, Prevotella* and *Veillonella spp.* in sexually transmitted infections group compare to healthy group, etc. (Wolfe and Brubaker, 2015; Aragón et al., 2018). All of these studies imply that there are both direct and indirect roles of microbiota to urinary disease which can be the cause or the effect from the diseases. The knowledge of the relation between microbiota and urinary disease is important for the diagnosis, prognosis, treatment and prevention of the disease. Table 1 shows Microbiome composition of urine in healthy human both men and women. (Aragón et al., 2018) (Table 1)

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Table 1. Microbiome composition of urine in healthy human both men and women. (Aragón et al., 2018)

| Study population | Main bacterial taxa | Sample collection | Technique used | Ref |
|---|---|---|---|---|
| Healthy men aged ~ 18 yr (n=9) | Lactobacillus, Corynebacterium, Escherichia, and Streptococcus | FC urine | 16s rRNA GS | [22] |
| Healthy men (n=22) aged ≥ 18 yr median 28 yr | Lactobacillus, Sneathia, Veillonella, Corynebacterium, Prevotella, Streptococcus, Ureaplasma, Mycoplasma, Anaerococcus, Atopobium, Aerococcus, Staphylococcus, Gamella, Enterococcus, and Finegoldia | FC urine | 16s rRNA GS | [23] |
| Healthy females aged 26-67 yr (n=8) | Lactobacillus, Prevotella, Gardnerella, Peptoniphilus, Dialister, Finegoldia, Anaerococcus, Allisonella, Streptococcus, and Staphylococcus | CC MSU | 16s rRNA GS | [24] |
| Healthy males aged 24-50 yr (n=11)<br>Healthy females aged 22-51 yr (n=15) | Lactobacillus, Klebsiella, Corynebacterium, Staphylococcus, Streptococcus, Aerococcus, Gardnerella, Prevotella, Escherichia, and Enterococcus | MSU | 16s rRNA GS | [25] |
| Healthy males aged 14-17 yr (n=18) | Corynebacterium, Lactobacillus, Staphylococcus, Gardnerella, Streptococcus, Anaerococcus, Veillonella, Prevotella, and Escherichia | FC urine | 16s rRNA GS | [26] |
| Healthy women (n=12) age NA | Lactobacillus, Actinobaculum, Aerococcus, Anaerococcus, Atopobium, Burkholderia, Corynebacterium, Gardnerella, Prevotella, Ralstonia, Sneathia, Staphylococcus, Streptococcus, and Veillonella | CC MSU, SPA, and TUC | 16s rRNA GS | [17] |
| Healthy men aged 39-86 yr (n=6)<br>Healthy women aged 26-90 yr (n=10) | Male and female samples: Firmicutes; Female samples: Actinobacteria, Bacteroidetes | CC MSU | 16s rRNA GS | [18] |
| Healthy women (n=24) age NA | Lactobacillus, Corynebacterium, Streptococcus, Actinomyces, Staphylococcus, Aerococcus, Gardnerella, Bifidobacterium, and Actinobaculum | TUC | 16s rRNA GS and/or EUCT | [21] |
| Healthy women aged 35-65 yr (n=58) | Lactobacillus, Gardnerella, Corynebacterium, Enterobacteriaceae, Anaerococcus, Bifidobacterium, Streptococcus, Staphylococcus, Sneathia, Peptoniphilus, Atopobium, Rhodanobacter, Trueperella, Alloscardovia, and Veillonella | TUC | 16s rRNA GS and/or EQUC | [19] |
| Healthy women aged 35-65 yr (n=60) | Lactobacillus, Gardnerella, Staphylococcus, Streptococcus, Enterococcus, Bifidobacterium, Atopobium, and Enterobacteriaceae | TUC | 16s rRNA GS and/or EQUC | [20] |
| Healthy women (n=10) | Anoxybacillus, Lactobacillus, Prevotella, Gardnerella, Arthrobacter, Escherichia, and Shigella | TUC | 16s rRNA GS | [27] |

NA = not available; EUCT = enhanced urine culture technique; EQUC = expanded quantitative urine cultures; GS = gene sequencing; FC = first catch; CC = clean catch; MSU = midstream urine; SPA = suprapubic aspirate; TUC = transurethral catheter

In year 2017, Burton et al. found that the dogs also have microbiota like in human which could be detected via 16S rRNA amplicon sequencing. Five most abundant microbiota in urine sample (greater than 1% mean relative abundance) are *Pseudomonas sp*. followed by *Sphingobium sp., Acinetobacter johnsonii*, unclassified microbes (UC) in the families *Bradyrhizobiaceae* and UC in the families *Xanthomonadaceae*, respectively. Other microbiota can be found with less abundance including microbiota that are greater than 0.1% mean relative abundance (*Delftia sp*., UC order *Streptophyta*, *Sphingomonas sp., Brevundimonas diminuta*, UC family *Caulobacteraceae, Propionibacterium acnes, Pedobacter sp., Staphylococcus sp*., and *Bacteroides sp.*) and microbiota that are extremely low mean relative abundance (UC family *Pseudomonadaceae, Streptococcus sp*., UC family *Sphingomonadaceae, Agrobacterium sp., Acinetobacter sp.*, and UC family *Methylobacteriaceae*). The number and diversity of urinary microbiota are significantly different from genital and rectal microbiota except *Pseudomonas sp.* and *Acinetobacter sp.* that can be found from both urine and genital samples. There is no difference in urinary microbiota between sexes. However, the roles of these microbiota are still unknown.

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**2.5 Microbiomes and Urolithiasis**

Urolithiasis is one of a common disorder found over in 0.4–2.0% of dogs receiving medical care (Bovee and McGuire, 1984). The disease has high incidence of recurrence after treatment due to the unclear pathogenesis of the stone formation. Calcium oxalate stones are the second most common type of stones in companion animals with 35% of stones submitted from dogs (Minnesota Urolith Center, 2020). The reasons for increasing of the prevalence of stones may be from the changes in dietary composition to prevent struvite (the most common type of stones), feeding more dry foods than moist foods, changes in breed popularity, etc. Predisposing factors are old, male especially neutered male and breeds. Breeds that have high risk of developing calcium oxalate include the Miniature Schnauzer, Yorkshire Terrier,

Miniature Poodle and Keeshounds. Recently, *Oxalobacter formigenes* was found in the gastrointestinal tract which has roles in degradation of oxalate. *Oxalobacteraceae* is a family within the order *Burkholderiales* in the subclass of *Betaproteobacteria* which found in diverse environmental habitats like water, soil, and plant; some species are mild plant pathogens or are claimed to be normal flora or opportunistic pathogens in human (Baldani et al., 2014). The absence of *O. formigenes* could be one of the risk factor for development of hyperoxaluria or recurrent kidney stone disease. (Allison et al., 1985; Mittal et al., 2003). This bacteria plays an important role in calcium oxalate stone formation. There are variety of factors, including calcium in diets, presence of unabsorbed fatty acids, and oxalate-metabolizing microflora of the gut, influence the level of free oxalate in the gastrointestinal tract available for absorption. There was a study that revealed that the intestinal colonization of *O. formigenes* reduced the risk of recurrent stone formation by 70% by in humans and administration of *O. formigenes* or its oxalate-metabolizing enzymes reduced hyperoxaluria in rats and humans. (Sidhu et al., 2001; Hoppe et al., 2006). There is study described about the association between the colonization of *O. formigenes* in gut and CaOx urolith formation in dogs, *O. formigenes* is positively associated with healthy dogs. The prevalence of gut colonization with *O. formigenes* were significantly different between CaOx dogs and healthy dogs, indicated that absence of *O. formigenes* in gut is a risk factor for CaOx urolithiasis (Gnanandarajah et al., 2012).

Therefore, no studies have been done to study the composition and the role of microbiome in the urinary tract of dogs with CaOx urolithiasis.

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# CHAPTER III

# MATERIALS AND METHODS

## 3.1 Animals

Urine samples were collected from both healthy and diseased animals, fully informed consent was obtained from the owners of all dogs participating in the study. In the healthy condition population, urine samples were collected by cystocentesis from 9 male and 10 female dogs that came to the Small Animal Hospital, Faculty of Veterinary Science, Chulalongkorn University for castration and ovariohysterectomy (OVH) without any evidence of systemic infection and history of clinical signs associated with urinary disease. The urine samples, calculi, and urinary bladder mucosa were collected from 7 male and 6 female dogs with calcium oxalate urolithiasis, which were diagnosed by history taking, urinalysis, crystal microscopic examination, and radiography. Dogs that had been treated with antibiotics, probiotics, or corticosteroids within the previous 30 days, received intravenous or subcutaneous fluids therapy within the previous 24 hours were excluded from the study. Urinalysis findings of pyuria, bacteriuria, or bacterial growth on routine urine culture after sampling were also excluded from the study. Full physical examination, blood collection for complete blood count (CBC) and blood chemistry, thoracic radiograph, and electrocardiogram (ECG) were performed in all dogs in the study. To minimize the discomfort, all procedures were performed under general anesthesia by using Acepromazine and Morphine as a premedication then induction by Propofol and maintenance with inhalation anesthetic drug (Isoflurane). All animal and bacterial usages in the study were approved by the Faculty of Veterinary Science-Animal Care and use Committee (FVS-ACUC 1931075), and the Faculty of Veterinary Science Institutional Biosafety committee (CU-VET-IBC 1931050) respectively.

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**3.2 Diagnosis of Calcium Oxalate Urolithiasis**

Clinical signs associated with urinary bladder urolithiasis in dogs usually caused by the formation of macroscopic uroliths in the lower urinary tract that interfere the flow of urine and/or irritate the mucosal surface results in dysuria, hematuria, and stranguria. The dogs with the clinical signs were diagnosed by physical examination, urinalysis, crystal microscopic examination, and radiography. For physical examination, abdominal palpation was performed to detect urocystoliths. Sometimes, when the bladder is palpated, the bladder wall may be thickened and a sensation or large uroliths may be noted. Urinalysis results of dogs with calcium oxalate (CaOx) showed the presence of acidic urine along with or without numerous calcium oxalate crystals. Survey abdominal radiographs are also helpful for identifying radiopaque uroliths. Radiographs are more accurate to predict the stone size and amount. Struvite stones are less radiopaque compared to calcium oxalate (CaOx) and tend to be larger, whereas CaOx typically accumulates as many small, irregular stones (Figure 1). However, calcium oxalate stones are still necessary to be confirmed by urinalysis and stone analysis, only urines collected from dog confirmed with calcium oxalate stone were included in the research.

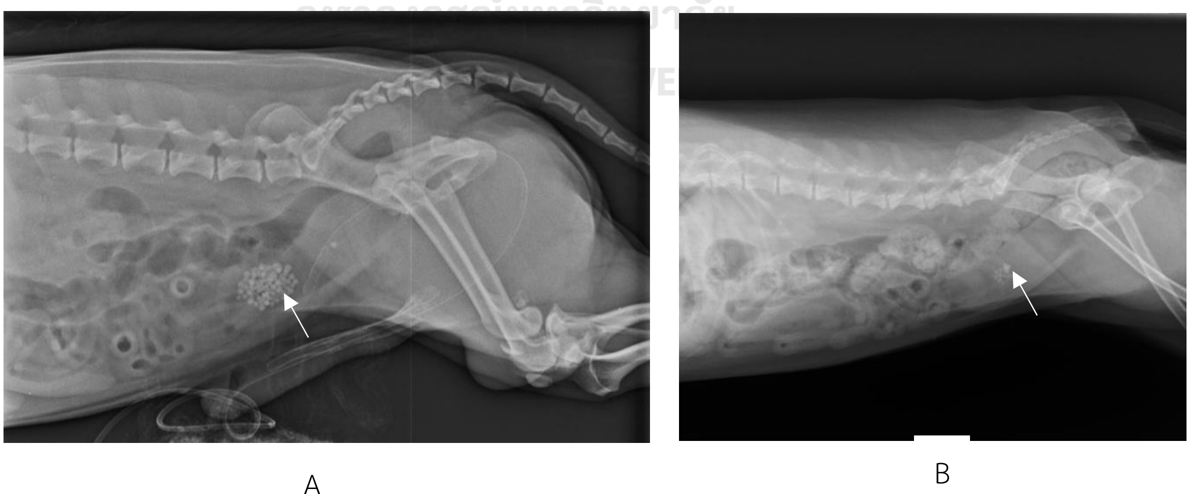


Figure 1. Small and irregular stone on lateral view of abdominal radiograph in calcium oxalate urolithiasis both male (A) and female (B) dogs.

### 3.3 Sample collection

Urine was collected via cystocentesis using a 22 ga. needle from all dogs in sterile field; samples were separated for routine urinalysis (1 mL), routine urine culture (1 mL), and 16S rRNA amplicon sequencing (10 mL). Urine for 16S rRNA amplification was centrifuged by refrigerated laboratory centrifuge at 6000 rpm for 15 minutes at 4 degrees Celsius. The supernatant was disposed and the remaining pellet was placed into 2.0 mL sterile round bottom tubes and stored at -80 °C until DNA extraction.

Calculi samples were collected for identification by Urolith service from Hill's Pet Nutrition, Inc. ®, Minnesota. All stones and plugs sent to the Minnesota Urolith Center were analyzed by state-of-the-art technology to give an accurate analysis of the stone or plug. The urolith center perform quantitative mineral analysis utilizing Optical Crystallography and Infrared Spectroscopy (ATR and FTIR). For identification of unusual minerals, they were accessed to equipment and specialists on campus in techniques such as XRD, EDS, micro-CT, and other specialized methodology. The results arrived in 2-4 weeks from Minnesota Urolith Center.

Urinary bladder mucosal samples at the size of 0.5 cm x 0.5 cm were collected in the modified Stuart transport medium tube. The samples were used for minimum inhibitory concentration (MIC) determination by using VITEK and bacterial identification by using MALDI-TOF which is the routine bacterial identification and sensitivity for cystotomy.

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**3.4 DNA Extraction**

DNA was extracted and purified using GenUP® gDNA kit (Biotechrabbit, Germany). The protocol for extraction of total DNA from the samples was revised concerning the quantification of starting material, the volume of Proteinase K and lysis buffers and the number of washing steps. In particular, when using frozen pellet, thaw the sample then transfer 40 mg of the pellet in a 1.5 ml microcentrifuge tube, and homogenized with 400 μl of lysis buffer LG and 25 μl proteinase K. The homogenate pellet was incubated at 50 °C until the tissue was completely lysed approximately 30 – 45 minutes with shaking platform. Centrifuged at 10,000 × *g* (12,000 rpm) for 30 s to pellet unlysed material. Then the supernatant was transferred into a new 1.5 ml tube, the 200 μl Buffer Binding BD was added to the lysed sample then mixed by vortex. The mixture was then pipetted to the Mini Filter placed in a collection tube. The solution was completely passed through the Mini Filter by centrifuge at 10,000 × *g* (12,000 rpm) for 2 min then discard the collection tube with the filtrate. Added 700 ul buffer wash C to the mini filter placed into a new collection tube then centrifuge at 10,000 × *g* (12,000 rpm) for 1 min. Discarded the filtrate and re-used the collection tube then repeat buffer wash C protocol again before removed residual ethanol by centrifuged at 10,000 × *g* (12,000 rpm) for 1 min again. Discarded the collection tube and placed the mini filter into an elution tube. Added 30 μl Buffer elution to the center of the Mini Filter then incubated at room temperature for 1 min and centrifuged at 6000 × *g* (8000 rpm) for 1 minute before discarded the mini filter. Extracted DNA samples in the Elution tube were used immediately or stored at −20°C until further use. DNA concentration and purity of each sample was determined with Implen NanoPhotometer using 260 nm absorbance. Extracted urine DNA was processed in *16S rRNA* sequencing by using next-generation sequencing (Miseq) and amplified by using universal primer and informatics analysis.

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### 3.5 16S amplification by PCR (Polymerized Chain Reaction)

Due to the low quantities of concentration of the extracted urine DNA, and reliable microbiome profiling was not achievable by only a single round of Polymerase Chain Reaction (PCR). The PCR was used to amplify specific, target DNA fragments with gene specific primers.

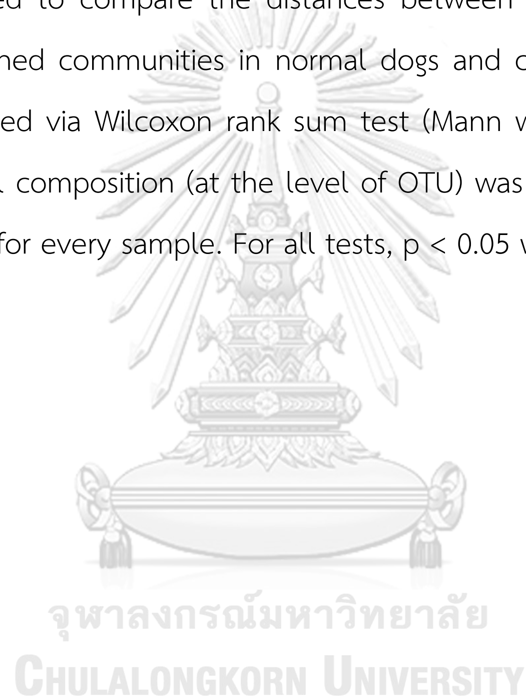
We performed a PCR amplification protocol for the V3-V4 region of the bacterial 16S rRNA gene with some modifications. The first PCR using primer sequences: 515F:5'-GTGCCAGCMGCCGCGGTAA-3' and 806R: 5'- GGACTACHVGGGTWTCTAAT-3' (size approximately 380 bp). The second PCR contained Illumina adaptor, dual multiplexing index (8 ×12), and the specific TruSeq adaptor sequences (Illumina, USA). Finally, the total length of the DNA library was approximately 450 bp which can be examined by 2% agarose gel electrophoresis and purified by QIAquick gel extraction kit (Qiagen, Germany), followed by next-generation sequencing (Miseq) of the third-round products. The amount of purified product concentrations were measured by using the KAPA absolute quantification kit (KAPA Bioscience, Germany). The samples were pooled at equal concentration to 2 nM before being denatured and loaded into a MiSeq reagent nano kit v2 cartridge with a final concentration of 6 pM. The library was sequenced for paired-end 2 ×250 cycles in Illumina MiSeq platform (Illumina, USA) with 20% PhiX control.

### 3.6 Statistical analysis

Data was compiled and analyzed by IBM® SPSS® software platform. The classification of healthy and CaOx patients was described in descriptive analysis. Extracted urine DNA was processed in *16S rRNA* sequencing by universal primer and informatics analysis. The FASTQ sequences were multiplexed by MiSeq reporter software (version 2.6.2.3) Subsequently, the sequences were fully processed by using QIIME2 pipeline (version 2021.4) (Bolyen et al., 2019). The paired-end reads were

merged and then filtered based on quality score (Q30). Then filtered reads were deduplicated and clustered with 97% similarity by VSEARCH (Rognes et al., 2016). The chimeric reads were also filtered out by the UCHIME algorithm (Edgar et al., 2011). Finally, these passed reads were classified by comparing the sequence against the Greengenes Database version 13.5 (DeSantis, et al., 2006).

Data was implemented in QIIMES2 to determine the effect of the calcium oxalate calculi on diversity, Chao1, and Shannon diversity indices. Bray-Curtis distances was used to compare the distances between microbial profiles in both groups. The matched communities in normal dogs and calcium oxalate urolithiasis dogs was performed via Wilcoxon rank sum test (Mann whitney U test) using SPSS program. Microbial composition (at the level of OTU) was revealed using QIIMES2 in the OTU bar plot for every sample. For all tests, $p < 0.05$ was considered statistically significant.



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# CHAPTER IV
# RESULTS

## 4.1 Animals

Thirty-two dogs that came in the Surgery unit of the Small Animal Teaching Hospital, Faculty of Veterinary Science, Chulalongkorn University, Bangkok, from February 2020 to March 2021, were enrolled in this study. The dogs were separated into four groups, healthy male (n=9), healthy female (n=10), calcium oxalate urolithiasis male (n=7), calcium oxalate urolithiasis female dogs (n=6). Fifthteen samples of healthy group and ten samples of CaOx urolithiasis group were excluded from further analysis due to bacterial growth on routine culture and the type of the stones. The mean age of included healthy dogs was 5.1 years (range 1 to 12) with varies of breeds and mean age of urolithiasis dogs was 8.84 years (range 2 to 12). (Table 2)

Table 2. Population study demographic and the results from urine routine urinalysis in each sample.

| Sex | Age (years) | Breed | pH | Specific Gravity | Crystal |
|---|---|---|---|---|---|
| Healthy Male | 2 | German Shepherd | 6 | 1.046 | |
| (9) | 6 | Australian Shepherd | 6 | > 1.050 | |
| | 2 | Mixed Breed | 7 | 1.044 | |
| | 4 | Shetland Sheepdog | 7 | > 1.050 | |
| | 4 | Mixed Breed | 6 | > 1.050 | |
| | 12 | Chihuahua | 7 | 1.026 | |
| | 5 | Poodle x Chihuahua | 7 | 1.042 | |
| | 5 | Mixed Breed | 7 | 1.036 | |
| | 1 | Mixed Breed | 6 | 1.044 | |

| | | | | | |
|---|---|---|---|---|---|
| Healthy Female | 7 | Chihuahua | 5 | > 1.050 | |
| (10) | 3 | Rottweiler | 6 | 1.020 | |
| | 4 | French Bulldog | 6 | > 1.050 | |
| | 4 | Chihuahua | 8 | > 1.050 | |
| | 2 | French Bulldog | 5 | 1.036 | |
| | 9 | American Pit Bull | 6 | 1.050 | |
| | 3 | Labrador Retriever | 5 | 1.022 | |
| | 12 | Chihuahua | 7 | 1.044 | |
| | 8 | West Highland White | 6 | 1.026 | |
| | 4 | Terrier | 5 | > 1.050 | |
| CaOx Urolihiasis | 10 | Shih Tzu | 5 | 1.020 | CaOx |
| Male (7) | 12 | Mixed Breed | 6 | 1.022 | - |
| | 9 | Shih Tzu | 5 | 1.020 | - |
| | 11 | Pomeranian | 7 | 1.024 | - |
| | 11 | Chihuahua | 6 | 1.028 | - |
| | 9 | Mixed Breed | 5 | 1.026 | CaOx |
| | 12 | Labrador Retriever | 5 | 1.034 | - |
| CaOx Urolihiasis | 2 | Chihuahua | 6 | 1.026 | CaOx |
| Female (6) | 9 | Shih Tzu | 5 | 1.022 | CaOx |
| | 4 | Beagle | 6 | 1.020 | - |
| | 11 | Mixed Breed | 6 | 1.026 | CaOx |
| | 11 | Poodle | 7 | 1.032 | - |
| | 4 | Beagle | 6 | 1.024 | - |

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**4.2 Diversity and richness of the canine urinary microbiota**

$\alpha$-diversity is an indicator of the combined richness and evenness of distribution among the various taxa detected in a sample. Two commonly used metrics of $\alpha$-diversity were compared between groups, yielding slightly different

results. Comparison of Shannon diversity index, a traditional measure of α-diversity which describes both abundance and evenness but places more weight on the evenness of taxa, revealed a main effect of the condition with a significant interaction ($p = 0.035$) between healthy control dogs and calcium oxalate urolithiasis dogs. There is no significant effect of sex on the Shannon diversity index was detected ($p = 0.45$). Therefore, from Chao1 indices which is richness estimator, no detected significant effect of condition on the Chao1 index ($p = 0.13$). Similar to the Shannon diversity index, there was no main effect of sex on the Chao1 index. (Figure 2)

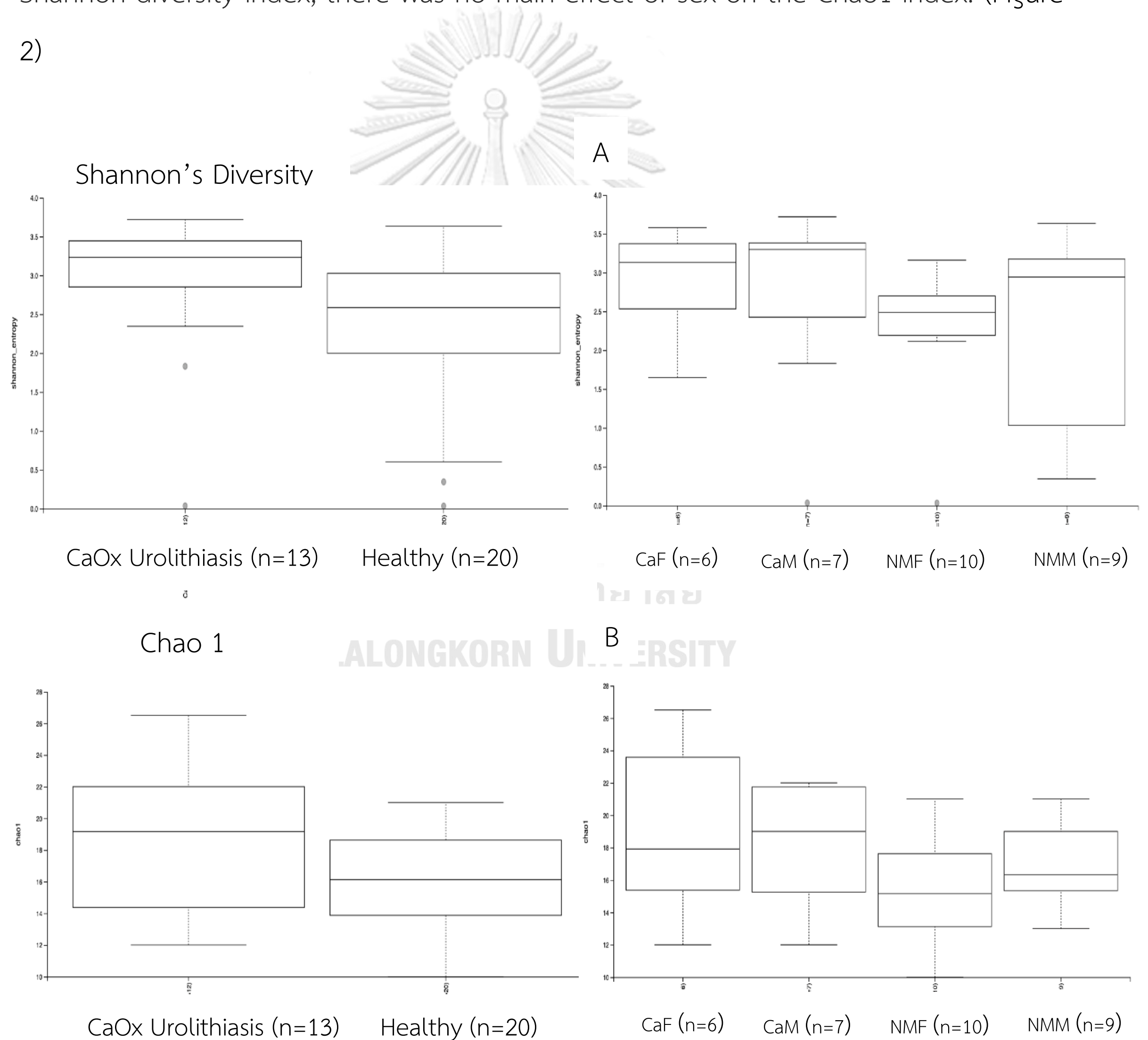

Figure 2. Block plots showing diversity index of various taxa detected in a urine sample from healthy and calcium oxalate urolithiasis dogs. Shannon's diversity indices (A) revealed a significant higher of abundance and evenness ($p = 0.035$) in calcium oxalate urolithiasis dogs than in healthy control dogs. There is no significant effect of sex on the Shannon diversity index was detected ($p = 0.45$). Chao1 indices (B) detected no significant effect of condition and no difference of sex in richness.

β-diversity measures the change in diversity of species from one environment to another. Although a strong trend was observed in Bray-curtis index that healthy dogs formed a cluster when compared to calcium oxalate urolithiasis dogs, no significant differences in microbial communities were observed. (Figure 3)

Bray-curtis index

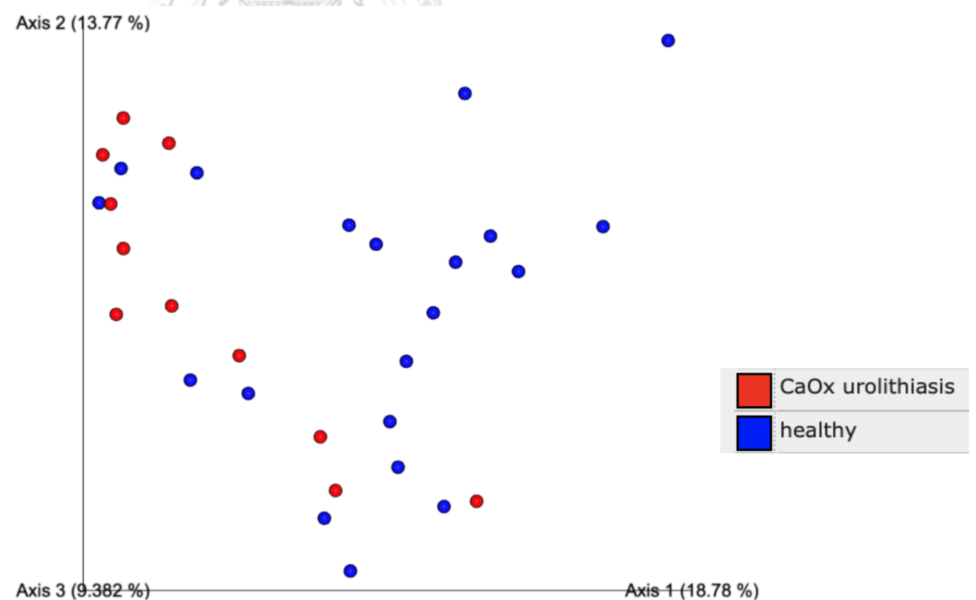


Figure 3. This dot plots revealed Bray-curtis index with no significant differences in microbial communities in healthy dogs and calcium oxalate urolithiasis dogs.

**4.3 Composition of the canine urinary microbiome in healthy and calcium oxalate urolithiasis dogs**

In calcium oxalate urine samples, only two dominant operational taxonomic unit (OTUs), all within the phylum *Proteobacteria*, were detected at greater than 10% mean relative abundance (Figure 4). These included unclassified (UC) microbes in the family *Caulobacteraceae* (mean ± SD of 14.7 ± 15.0%) and *Bradyrhizobium spp.* (mean ± SD of 12.31 ± 11%) which detected in 11 and 13 samples from 13 samples respectively. Additional OTUs that were revealed greater than 5% mean relative abundance in calcium oxalate urine samples are mostly in phylum *Proteobacteria and Bacteroidetes,* including UC family *Enterobacteriacea, Sediminibacterium spp., Phyllobacterium spp., Sphingomonas spp., and Achromobacter spp.* In healthy urine samples, four OTUs, were detected higher than 8% mean relative abundance, which are UC microbes in the family *Enterobacteriacea* (mean ± SD of 9.6 ± 24.0%), *Stenotrophomonas spp.* (mean ± SD of 8.9 ± 19.6%), *Enhydrobacter spp.* (mean ± SD of 8.8 ± 21.0%), and Bradyrhizobium spp. (mean ± SD of 8.28 ± 9.5%). Of the 4 OTUs listed above, the microbes were detected in 18, 13, 13, and 15 urine samples out of 19 samples. The OTUs detected in healthy urine more than 5% are *Phyllobacterium spp*. (mean ± SD of 6.93 ± 11.1%), *Sediminibacterium spp*. (mean ± SD of 6.37 ± 7.0%), *Staphylococcus spp*. (mean ± SD of 5.78 ± 11.4%), and *Prevotella spp*. (mean ± SD of 5.08 ± 22.0%), which is mostly in phylum *Proteobacteria and Bacteroidetes.* There are still many OTUs were detected in all urine samples with extremely low mean relative abundance. (Table 2)

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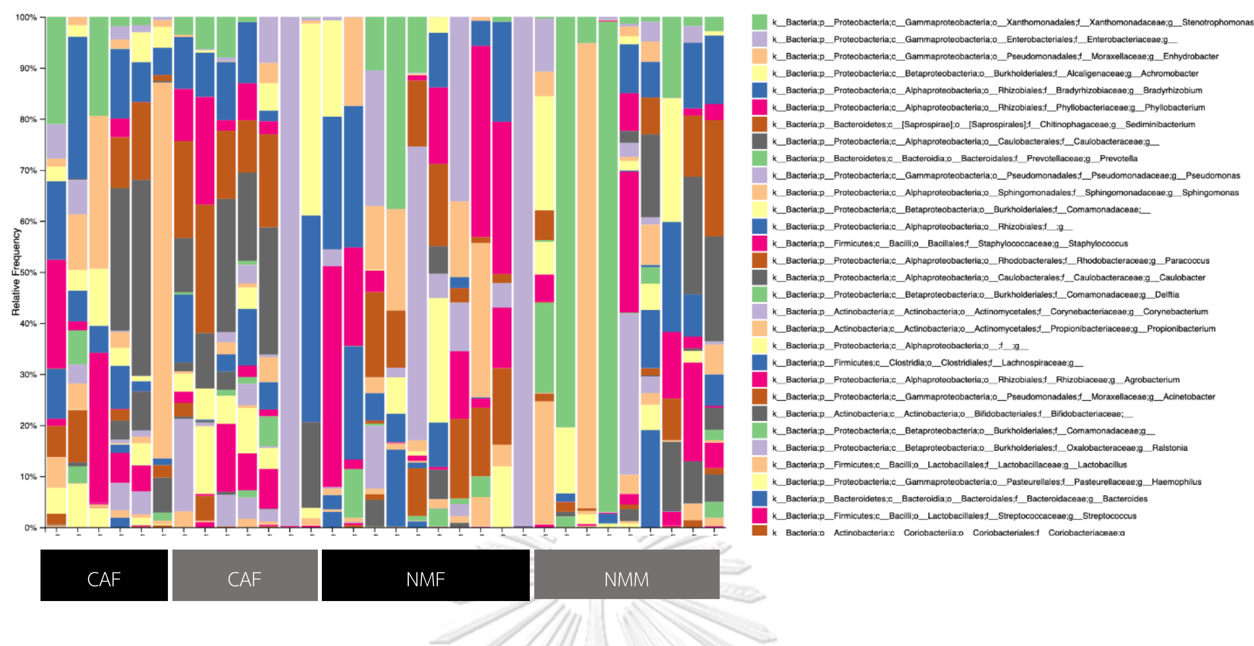


Figure 4. Stacked bar charts showing OTU bar plots of relative abundance of microbial DNA detected via 16S rRNA amplicon sequencing and annotated to the taxonomic level of genus in urine samples.

Table 3. OTU table contains the number of sequences that are observed for each taxonomic unit in each sample. Columns usually represent samples and rows represent family taxonomic units.

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| OTU (Family) | Mean ± SD (%) | | | |
|---|---|---|---|---|
| | CAF | CAM | NMF | NMM |
| **Phylum Proteobacteria** | | | | |
| Caulobacteraceae | 13.1 ± 20.5 | 16 ± 9.7 | 1.2 ± 3.7 | 8.2 ±10.3 |
| Bradyrhizobiaceae | 12.5 ± 8.6 | 12.1 ± 13.4 | 10.1 ± 11.4 | 6.6 ± 7.6 |
| Enterobacteriaceae | 1.9 ± 2.6 | 15.8 ± 37.2 | 7.1 ± 14.0 | 11.9 ± 31.1 |
| Phyllobacteriaceae | 9.1 ± 12.9 | 6.2 ± 7.6 | 11.9 ± 14.3 | 2.5 ± 4.4 |
| Sphingomonadaceae | 14.6 ± 29.2 | 1.2 ± 1.9 | 3.9 ± 9.9 | 1.6 ± 2.9 |
| Alcaligenaceae | 4.4 ± 3.9 | 6.2 ± 14.0 | 2.5 ± 6.2 | 6.2 ± 9.8 |

| | | | | |
|---|---|---|---|---|
| Xanthomonadaceae | 7.4 ± 10.0 | 2.7 ± 3.3 | 6.7 ± 12.5 | 10.9 ± 24.9 |
| Rhizobiaceae | 1.8 ± 2.8 | 4.4 ± 5.1 | 0 | 2.9 ± 6.0 |
| Moraxellaceae | 8.5 ± 11.3 | 1.8 ± 2.1 | 8.4 ± 8.3 | 10.7 ± 28.4 |
| Oxalobacteraceae | 1.6 ± 2.5 | 4.4 ± 6.5 | 0 | 0.3 ± 1.0 |
| Comamonadaceae | 3.3 ± 5.4 | 3.2 ± 3.0 | 5.8 ± 8.9 | 3.8 ± 7.3 |
| Pasteurellaceae | 2.3 ± 3.3 | 0 | 0 | 0.6 ± 1.5 |
| Pseudomonadaceae | 1.2 ± 2.7 | 0.9 ± 1.4 | 9.1 ± 18.4 | 0.5 ± 0.9 |
| Rhodobacteraceae | 1.4 ± 2.5 | 0 | 5.0 ± 7.3 | 0 ± 0.1 |
| **Phylum Bacteroidetes** | | | | |
| Chitinophagaceae | 4.4 ± 6.6 | 12.3 ± 9.6 | 7.0 ± 7.1 | 5.8 ± 7.3 |
| Bacteroidaceae | 0.3 ± 0.8 | 0 | 0.5 ± 1.0 | 1.9 ± 6.0 |
| Prevotellaceae | 0 | 0.2 ± 0.3 | 0 | 9.6 ± 30.4 |
| **Phylum Actinobacteria** | | | | |
| Propionibacteriaceae | 2.2 ± 2.7 | 0.4 ± 0.5 | 2.0 ± 1.8 | 0.5 ± 1.2 |
| Corynebacteriaceae | 0.9 ± 1.4 | 0.7 ± 1.5 | 1.7 ± 4.1 | 3.2 ± 10.0 |
| Bifidobacteriaceae | 1.3 ± 2.7 | 0.2 ± 0.2 | 0.7 ± 1.7 | 1.6 ± 2.9 |
| Coriobacteriaceae | 0.1 ± 0.2 | 0.0 ± 0.1 | 0.1 ± 0.1 | 0.1 ± 0.4 |
| **Phylum Firmicutes** | | | | |
| Lactobacillaceae | 0.6 ± 0.5 | 0.8 ± 1.2 | 0.1 ± 0.1 | 3.2 ± 7.5 |
| Staphylococcaceae | 0.6 ± 0.8 | 0.5 ± 0.9 | 8.2 ± 14.1 | 3.6 ± 8.6 |
| Streptococcaceae | 0.0 ± 0.1 | 0.2 ± 0.4 | 0.1 ± 0.1 | 0.1 ± 0.2 |
| Lachnospiraceae | 0.3 ± 0.7 | 0 | 2.1 ± 4.9 | 1.3 ± 3.6 |

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### 4.4 Comparison of urinary microbiome to healthy and calcium oxalate uroliothiasis dogs

The most common taxa observed in the UM of healthy dogs was UC microbes in the family *Enterobacteriacea*, detected in the majority of the healthy group. Microbes in family *Enterobacteriacea* was the dominant OTU detected in 10

out of 10 and 8 out of 9 female and male healthy dogs, respectively. Notably, the calcium oxalate urolithiasis dogs yielded substantial relative abundances of microbes in the family *Caulobacteraceae.*

We therefore used the linear discriminant analysis effect size (LEfSe) algorithm to identify the specific UM that are differentially represented in calcium oxalate cases versus controls (Figure 5). The LEfSe algorithm is used for biomarker discovery from data to identify differentially expressed taxonomic divisions. From the results, we found that 3 taxa were overrepresented in urolithiasis dogs over controls which are microbes in the family *Caulobacteraceae*, microbes in the class *Alphaproteobacteria* and family *Oxalobacteraceae* (genus *Ralstonia*).

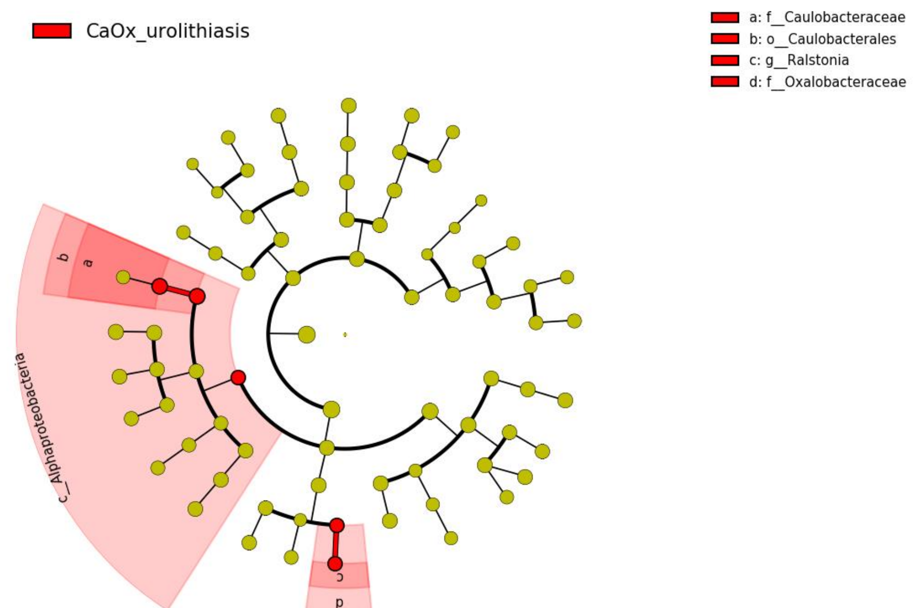


Figure 5. the LEfSe algorithm revealed the UM that is differentially represented in calcium oxalate cases versus controls. The red dots show the UM that is significantly different in both groups and found higher in CaOx urolithiasis group.

To more accurately assess the difference of these microbes between two groups, testing via Wilcoxon rank sum test detected significantly differences of the microbes between both groups which are family *Caulobacteraceae* (p-value = 0.006), class *Alphaproteobacteria* (p-value = 0.005), and family *Oxalobacteraceae* genus *Ralstonia* (p-value = 0.005) (Figure 6). These microbes were detected higher in calcium oxalate urine than in control group.

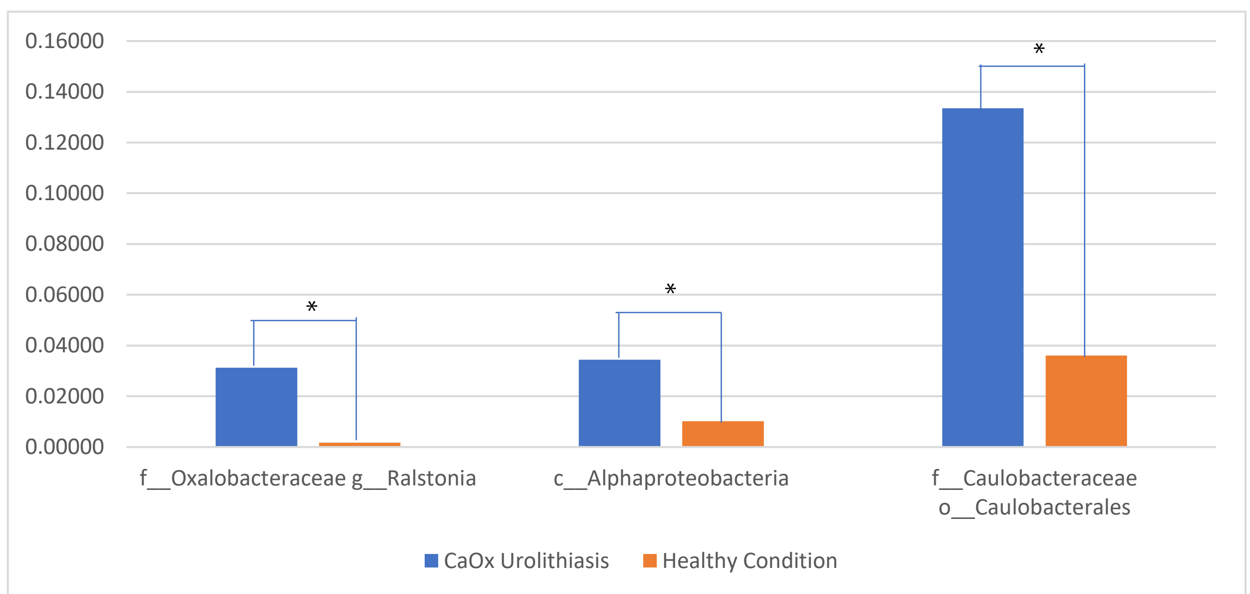


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Figure 6. This graph shows the amount of these microbes in both control and CaOx urolithiasis groups. These microbes were detected significantly higher in calcium oxalate urine samples than another group. *$p < 0.05$

# CHAPTER V
# DISCUSSION

Apart from routine preventative health care, cystitis is one of the most common reasons dog owners seek veterinary care. The incidence of canine urolithiasis was found between 0.5 to 1% of the canine population, and 18% to 20.61% in dogs with lower urinary tract diseases (Claudia et al., 2018; Hesse A, 1990). Previously, the urinary bladder had been known as sterile environment. While routine urine culture methods continue to be the standard for the diagnosis of clinically relevant UTI and also used to observed urine profiles in urolithiasis in dogs. Thus, we were able to characterize the UM in healthy dogs using a method of detection more sensitive compared to routine bacterial culture. The 16S rRNA sequence analysis revealed that urine deemed no growth by the standard protocol contained bacteria that could be cultured but not by the standard bacterial culture protocol. To our knowledge, there is a document which presents a variety of microorganisms in the urine of dogs using the 16S rRNA sequencing. The urine samples revealed a large number of OTUs, described as the sample richness and evenness although there had no bacterial growth in normal bacterial culture routine protocol (Burton et al., 2017). To date, none of the documents in the urinary system has so far identified a particular consistent biomarker or pathogenic target for therapy in calcium oxalate urolithiasis in dogs. In the gastrointestinal tract, *Oxalobacter formigenes* has roles in degradation of oxalate. The absence of *O. formigenes* population in the gut microbiome environment increases the chance to develop hyperoxaluria or recurrent kidney stone disease (Allison et al., 1985; Mittal et al., 2003).

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In calcium oxalate urolithiasis dogs, two dominant OTUs, all within the phylum *Proteobacteria*, were detected at greater than 10% mean relative abundance. These Gram-negative bacteria included unclassified (UC) microbes in the family *Caulobacteraceae* and *Bradyrhizobium spp.* Only four OTUs, were detected higher than 8% mean relative abundance in healthy condition urine, which are UC microbes in the family *Enterobacteriacea* (mean ± SD of 9.6 ± 24.0%), *Stenotrophomonas spp.* (mean ± SD of 8.9 ± 19.6%), *Enhydrobacter spp.* (mean ± SD of 8.8 ± 21.0%), and Bradyrhizobium spp. (mean ± SD of 8.28 ± 9.5%). Therefore, compared with the prior study in 2017, there are 2 taxa dominated in the urinary bladder of healthy dogs which are Family *Bradyrhizobiaceae* and Family *Xanthomonadaceae* that same as in our study.

The Shannon's index was often used to account for the diversity of species' abundance and evenness. The shannon's diversity index showed significantly higher in the group of calcium oxalate urolithiasis dogs, compared to the healthy dogs. It could be described that there is more species in the community, more uniform of the distribution of various individuals, and also the higher of index indicated a good variety of the community.

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We found no statistical difference between males and females in the richness, diversity, or composition of the urinary microbiota in both control and calcium oxalate urolithiasis groups. This was also described in one previous study which shown no different of UM between sex, even though the large majority of calcium oxalate urolithiasis is found in male dogs (Kopecny et al., 2021). This differs from the literature in human, which the UM is difference by sex. In women, the predominant microbes are *Lactobacillus* and *Gardnerella,* following by *Streptococcus*, *Staphylococcus*, and *Corynebacterium.* (Pearce et al., 2014; Thomas-

White et al., 2016). In men, *Lactobacillus*, *Streptococcus*, *Ureaplasma*, *Mycoplasma*, and *Sneathia* is the predominant one (Dong et al., 2011; Nelson et al., 2012). Although the UM did not differ between the sexes in the study, anatomic characteristics assigned that the male urethra favors the permanence of small uroliths, unlike females which can expel some small uroliths while urination, but both sexes are equally prone to form uroliths.

There are the significantly difference of urinary microbiome between calcium oxalate urolithiasis group and control group which are the higher OTU of family *Caulobacteraceae* (p-value $< 0.01$, conf.level $= 0.95$), class *Alphaproteobacteria* (p-value $< 0.01$, conf.level $= 0.95$), and family *Oxalobacteraceae* genus *Ralstonia* (p-value $< 0.01$, conf.level $= 0.95$) in calcium oxalate urolithiasis group.

Oxalate-metabolizing bacteria in the gut has an important role in limiting oxalate absorption, reduce oxalate levels in urine, and relate with the formation of calcium oxalate stone (Robijn et al., 2011). These bacteria are called oxalotrophic bacteria, because they utilize oxalate as a source for their energy (Sahin et al., 2003). These bacteria can be classified into generalists group which is bacteria that fermented both oxalate and other substrates and specialists group, use only oxalate as main source (Sahin et al., 2003).

*Oxalobacter formigenes* is one of the bacteria in family *Oxalobacteraceae*, which belongs to the specialist group of oxalotrophic bacteria, was first reported in humans with this bacteria exclusively metabolizing oxalate for its energy and, therefore, is considered an efficient oxalate degrader in the gastrointestinal

tract. (Allison et al., 1985). There was a study that revealed that the intestinal colonization of *O. formigenes* reduced the risk of recurrent stone formation by 70% by in humans and administration of *O. formigenes*or its oxalate-metabolizing enzymes reduced hyperoxaluria in rats and humans. (Sidhu et al., 2001; Hoppe et al., 2006). In dogs, there are studies described about the association between the colonization of *O. formigenes* in gut and CaOx urolith formation in dogs. The prevalence of gut colonization with *O. formigenes* were significantly different between CaOx dogs and healthy dogs, indicated that absence of *O. formigenes* in gut is a risk factor for CaOx urolithiasis (Gnanandarajah et al., 2012). In our study, we found significantly different in the prevalence of bacteria in family *Oxalobacteraceae* which is higher in calcium oxalate urolithiasis dogs compared to healthy dogs. Thus, further study is recommended to reveal the importance of the bacteria in urinary bladder in calcium oxalate urolithiasis dogs.

Oxalyl coenzyme A decarboxylase (OXC) is one of key enzymes that is important in the catabolism of oxalate. Thus, another bacterial population found higher in urine of calcium oxalate urolithiasis dog is bacteria in class *Alphaproteobacteria* which contains widely spread and mainly distributed of OXC sequences and mostly enriched in the gut and vagina of human (Jiang et al., 2020 ). Moreover, the significantly higher of family *Caulobacteraceae* in calcium oxalate urine samples could be a reason from some of bacteria from this family requires micromolar concentrations of calcium for normal growth and development whereas most bacteria require only nanomolar concentrations of calcium (Herrmann et al., 2017).

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These data revealed the differences of microbiome in both control and calcium oxalate urolithiasis groups which could possibly be the consequence from the CaOx urolith forming in the bladder. The dissimilarity between groups could lead to the early diagnosis, alternative managing methods or the possible solutions of preventing calcium oxalate stone cases in dogs.

In conclusion, while recognizing the limitations of the diagnosis and treatment, this finding may lead to more accurate diagnosis, treatment, or prevention methods in the future.

Moreover, should this analysis proceed for further research, it might help to decrease the raising problems of calcium oxalate stone in dogs.

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