ROLE OF OXALATE METABOLIZING BACTERIA IN CALCIUM OXALATE UROLITHIASIS IN DOGS

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ABSTRACT

The incidence of calcium oxalate (CaOx) urolithiasis in dogs, cats and humans has increased steadily over the last two decades. Supersaturation of calcium and oxalate ions in the urine leads to CaOx urinary stone formation in dogs, cats and humans. Therefore, reducing urine concentrations of these stone components are essential to prevent reformation. Oxalate-metabolizing enteric bacteria minimize the freely available oxalate in the gut and reduce the enteric absorption. We hypothesize that decreased colonization of enteric oxalate-degrading bacteria is a risk factor for CaOx urolithiasis in dogs.

Fecal samples from dogs with CaOx uroliths, clinically healthy breed matched-dogs, and healthy non-stone forming breed dogs were screened for the presence of *Oxalobacter formigenes*, *Lactobacillus acidophilus*, and *Bifidobacterium animalis* by PCR to detect the species-specific oxalyl CoA decarboxylase (*oxc*) genes. Further, hind-gut microbiota of the dogs with CaOx stone and healthy dogs was compared by analyzing the sequences of V3-hypervariable region of 16S rDNA libraries. Finally, the *E. coli* Nissle 1917 (EcN) probiotic strain was engineered to heterologously co-express the *oxc*, *frc* and *OxlT* of *O. formigenes* or OXDC of *B. subtilis.*

Presence of *Oxalobacter formigenes*, *Lactobacillus acidophilus*, and *Bifidobacterium animalis* was significantly higher in healthy non-stone forming breed dogs than in the dogs with CaOx stones. Based on the 16S rRNA sequences, in total, 1,223 operational taxonomic units (OTUs) were identified at 97% similarity. Principal coordinate analysis, based on the fecal bacterial diversity, revealed that the healthy dogs

were clustered together whereas the dogs with CaOx uroliths were dispersed with no apparent pattern. Engineered EcN strains that expressed OXDC of *B. subtilis* metabolized oxalate *in vitro*. In the absence of the expression of OxlT of *O. formigenes* a second version of engineered EcN failed to metabolize oxalate *in vitro*.

Reduced presence of enteric oxalate-metabolizing bacteria is a risk factor for CaOx urolithiasis and fecal microbiota of healthy dogs is distinct from that of dogs affected with CaOx stones. Therefore, enteric colonization by oxalate-metabolizing bacteria may have a preventive effect on CaOx urolithiasis and present a novel therapeutic approach to prevent urinary stones in dogs, cats and humans.

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LITERATURE REVIEW

1. Prevalence of calcium oxalate urolithiasis

Urolithiasis is one of the important lower urinary tract diseases in dogs and cats. The estimated incidence rate of urolithiasis in dogs is between 0.2% - 3% (163). Urolithiasis is a process of formation of stones in the lower urinary tract. Based on mineral composition, the uroliths are classified as calcium oxalate, calcium phosphate, struvite (magnesium ammonium phosphate), purine, cystine, and silica uroliths (164). Among these, calcium oxalate (CaOx) uroliths are the most commonly reported type of stone in dogs, cats and humans (128, 165).

Most of the reports on the incidence of CaOx uroliths are based on the number of stones submitted to a urolith centre for analysis. The stones are identified by optical crystallography, X-ray diffraction and infrared spectroscopy (131). Over the past two decades, prevalence of CaOx stones has increased in dogs and cats whereas the prevalence of struvite uroliths has declined reciprocally in North America and Europe (93, 94, 127, 128, 138, 164, 165, 173, 212, 238, 252). In 1980, 78% of the analyzed canine uroliths were struvite while only 5% of the stones were CaOx. In 2003, 41% of the analyzed uroliths were CaOx. Even though no further increase was observed in CaOx urolith prevalence over the last five years, the prevalence still remains high (40%) (165). Some of the factors that contributed to the change in CaOx and struvite incidence patterns are the introduction of diets aimed to dissolve and/or prevent struvites, increased longevity of dogs and owner preference for breeds that are genetically prone to form CaOx stones (128, 138, 166, 214). In the past, urine acidifiers were introduced into pet

feed to prevent struvite stones (165, 209). Breeds that predisposed to form CaOx stones are the most commonly preferred breeds in the USA (128). As stones are formed more frequently in older dogs it is reasonable to expect that the observed increase in CaOx stone incidence is due to increase in life span of domestic dogs (128).

In 1981, 2% of the analyzed feline stones were CaOx while in 2002 it reached about 55% (165). However in cats, over the last five years a progressive decrease in occurrence of CaOx stones was observed but the incidence of struvite stones has increased (28, 165). During the last few years, feline adult maintenance diet has been manipulated to control CaOx urolithiasis (133, 165). Specifically, diets were modified to reduce urine acidity and increase moisture content (133).

In humans, occurrence of CaOx nephroliths also has increased over the years and the contributing factors are increased intake of animal protein, decreased urine citrate excretion and other causes related to changes in lifestyle (39, 98, 159, 213, 228, 259).

In dogs and cats, CaOx stones are found in lower urinary tract, mostly in bladder, while in human CaOx stones are found more frequently in renal pelvis and ureter (164, 186). Therefore, most of the studies on CaOx stone formation in humans were focused on nephrolithiasis while in dogs and cats studies were focused towards urolithiasis. In recent years, occurrence of CaOx uroliths within the upper urinary tract has increased in dogs and cats (165).

CaOx urolith causes clinical discomfort to the patient by blocking the urinary tract, eroding the uroepithelium causing inflammation and leading to secondary bacterial infection. Medical dissolution is a challenge in CaOx stones and thus leads to surgical

interventions (32, 167). Small CaOx stones can usually be voided by urohydropropulsion (132, 137). Larger stones are either removed by lithrotripsy and cystotomy (43, 70, 131). Despite the removal of CaOx stones, dogs, cats and human show recurrent episodes of CaOx stones (3, 4, 25). High recurrence rate and challenges in successful medical dissolution warranted the necessity to identify the underlying aetiological factors for CaOx urolithiasis. Studies have identified several predisposing factors associated to CaOx urolithiasis. The major factors are increased urine CaOx supersaturation, reduced inhibitors of the CaOx crystal growth and prolonged transit of urine time through the urinary tract (195).

2. Risk factors associated with CaOx urolithiasis

Increased urinary calcium (Ca^{2+}) and oxalate concentration is the driving force for CaOx urolithiasis in dogs, cats and humans (167, 171, 217). The threshold of supersaturation of Ca^{2+} and oxalate ions in the urine that cause crystallization cannot be simply predicted by measuring the concentration of these ions alone in urine. Evaluating "Relative Supersaturation of Urine CaOx" (RSS_{CaOx}) and "Activity Product Ratio" (APR) are the most commonly used methods to predict the likelihood of CaOx stone formation (18). RSS_{CaOx} is calculated by comparing the activity of product (AP) of CaOx in the urine of affected and healthy individuals. Urine samples of individuals are distinct not only in ions but also in macromolecules, other salts and pH. During RSS_{CaOx}, computational analysis, only a few ions (Na, K, citrate and Mg) are evaluated but macromolecules (inhibitors and promoters) and other salts in the urine are not taken into consideration (18). APR values are calculated by comparing the AP of CaOx in urine

before and after incubation with pure CaOx seed crystals. APR analysis indirectly accounts for the other factors, as the same urine is compared, but it is not an exact measurement of supersaturation of CaOx (18). Therefore, risk factor studies based on RSS_{CaOx} and APR should be interpreted cautiously. RSS_{CaOx} in urine of the stone forming dogs is significantly higher than in that of healthy dogs (217).

Under normal physiological conditions, Ca^{2+} levels in the blood and urine are highly regulated by parathyroid hormone (PTH) to maintain Ca homeostasis. Circulating Ca^{2+} is filtered via the glomeruli and reabsorbed by proximal tubules, loops of Henle and distal tubules of the kidney. Therefore, less than 1.5% of the filtered Ca is excreted in urine. PTH regulates reabsorption of Ca^{2+} at the distal tubules. PTH also regulates the activation of 1,25-di-hydroxy-vitamin D3 (1, 25-hydrocholeocalciferol) through hydroxylation. Absorption of dietary Ca is under the regulation of 1, 25-Hydrocholeocalciferol. PTH directly acts on osteoblast and osteoclast cells of bone to mobilize Ca. Studies have shown that CaOx stone-forming dogs excrete significantly higher levels of Ca^{2+} than the healthy non-stone forming dogs (134, 217). Healthy dogs, on average, excrete 0.51 ± 0.28 mg/kg Ca^{2+} per day whereas Miniature schnauzers (a CaOx stone-forming breed) excrete 2.54 ± 1.20 mg/kg of Ca^{2+} per day (134). In humans, hyper calciuric patient excrete more than 250 - 300 mg of Ca^{2+} per day (169). Increased serum and urine Ca^{2+} levels were observed in dogs with pathological conditions such as primary hyperparathyroidism, vitamin D intoxication, osteolytic neoplasia and hyperthyroidism (105). However, CaOx stones were reported only during primary hyperparathyroidism due to parathyroid adenoma (105). Increased enteric Ca^{2+}

absorption and reduced renal tubular reabsorption also lead to hypercalciuria without altering the serum Ca^{2+} levels (167). In humans, 30 - 50% of the CaOx stone formers suffer from idiopathic hypercalciuria (169).

Urine oxalate concentration was comparable between healthy and stone-forming dogs (134, 135, 217). Extensive epidemiological studies in humans confirmed that subtle increase in the urine oxalate concentration significantly enhanced the risk of CaOx stone formation (39). Oxalate is generated endogenously as a metabolic end product of glycine, hydroxyproline, serine, glyoxylate, glycollate and ascorbic acid metabolism. Oxalate is excreted via the urine and mammals are incapable of metabolizing oxalate. Absorbed oxalate from the intestine also is excreted through the urine. In dog, oxalate is filtered by glomeruli, actively secreted by renal tubules and passively reabsorbed by renal tubules (31, 167). In dogs, average oxalate concentration of the urine is 0.35 mM (0.90 mg/Kg/day), (218).

Multiple risk factors contribute to supersaturation of Ca^{2+} and oxalate ions and/or precipitation of the CaOx ions in the urine. Combinations of familial, congenital and acquired pathophysiological-abnormalities contribute to CaOx urolithiasis in dogs and cats. Reports on contextual risk factors involved in CaOx stone formation in cats and dogs are limited. Case control studies have identified breed, age and gender as the common risk factors associated with CaOx urolithiasis in dogs (94, 127, 138, 173, 212).

2.1 Breeds

Miniature Schnauzer, Bichon Frise, Lhaso Apso, Cairn Terrier, Yorkshire Terriers, Maltese, Pekingese and Miniature, Shi Tzu, Toy poodle and Chihuahua,

Dachshund are highly prone to develop CaOx uroliths (93, 121, 127, 128, 138, 139, 238). Although it is rare, larger dog breeds also form CaOx uroliths (121). Ragdoll, Burmese, Himalayan, Siamese, Scottish Fold and Persian cats are at a relatively higher risk of forming CaOx stone (93, 120). In humans, familial predisposition has been reported in CaOx stone formation. Breed predispositions lead to a hypothesis that CaOx urolithiasis is an inherited disease and its expression is influenced by gender, age and diet (139). However, genetic basis for CaOx urolithiasis has not been proved in dogs. Apparent anatomical and physiological characteristics of certain breeds can also predispose them to develop CaOx stones. Miniature Schnauzers urinate less frequently and void less urine than the non-stone forming Labrador Retrievers but specific gravity of the urine is not significantly different between the two breeds (215). Prolonged retention time of the urine in the bladder may promote the crystallization and aggregation of Ca and Oxalate ions. As this study was performed in younger dogs it is not known whether the same physiology is applicable to the age group at high risk in Miniature schnauzers. It was also found that Miniature schnauzers excreted more urinary calcium than the non-stone forming breeds (134, 216). However, in these studies hypercalcaemia was not observed in Miniature schnauzers (134, 216). Studies have also reported that Ca content of the diet significantly alters the Ca excretion via urine in Miniature schnauzers (134). Based on the above observation it is speculated that Miniature schnauzers are hyperabsorbers of Ca. In addition urine pH in Miniature schnauzers was maintained above 6.2 thus increasing the risk of calcium phosphate crystal formation (216). Familial link between stone formers in dogs is yet to be proven.

2.2 Gender and age

Males and older dogs (peak 8-9 years) fall into high-risk category of CaOx stone-formers (93, 121, 127, 128, 138, 167, 252). A similar trend is observed in cats. CaOx uroliths were more frequently present in male and older cats with the age range of 7 – 10 years (93, 120). In humans, the age group with peak incidence is 40-59 years, but some studies have reported the highly susceptible age range as 31 - 40 years (228). The occurrence of CaOx stone is higher among neutered dogs compared to intact dogs (121, 252). Epidemiological reports claim that 95% of the cats diagnosed with CaOx stones were neutered.

In humans, males are more prone to kidney stone formation than females (98, 159, 213). Epidemiological studies in the US indicate that 12 % of men and 5 % of women are prone to get kidney stones during their lifetime (152, 204). In humans, testosterone levels in plasma were found to be higher in stone formers than in non-stone formers (241). Studies in rat models found that testosterone enhanced the activity of glycolate oxidase, causing increased endogenous oxalate production and urine excretion while oestrogen reduced urinary oxalate excretion (56). Expression of osteopontin, an CaOx crystallization inhibitor protein, is also altered by sex hormones in humans (257). These studies indicate that increased level of plasma testosterone is a risk factor for CaOx urolithiasis. Influence of the sex hormones on urinary oxalate excretion is not known in dogs and cats.

2.3 Geographical region

Limited information is available for dogs and cats in relation to geographical region as a risk factor. Urban dogs are at high risk compared to rural dogs, perhaps due to non-biological reasons such as availability of veterinary care and discrepancies in reported case and number of stones submitted for the analysis (121, 252). Further, small breeds are most commonly found in urban areas (128). Interestingly, more CaOx cases are reported by the higher income category and struvite stones are largely reported by low-income households (252). Thus, socio-economical status of the owner should be considered as a risk factor. Since the income of the owner is a key factor in decision making on type of diet, regular veterinary care, and follow up on treatments (252, 253). These reciprocal observations lead to a focus on diet as a major risk factor for CaOx urolithiasis.

2.4 Urine volume

Ample intake of water increases the urine volume and thus reduces the concentration of ions in the urine and prevents crystallization. Increasing the moisture content of the diet is one way of increasing the urine volume. Dogs maintained on high moisture content diets were less prone to form CaOx stone (122, 253). Studies have also showed that increased moisture content of diet has significantly reduced the relative supersaturation of CaOx in the urine of Miniature schnauzers (215). On the other hand, moisture content of the diet had no significant effect on voided urine volume or the relative supersaturation of CaOx in the urine of Labrador retriever, a non-stone forming

large breed (215). Cats fed with high moisture content diet were at lower risk to form CaOx stones compared to cats on low moisture content diet. The case control study claims that high protein diet lowers the risk of CaOx urolithiasis in dogs and cats and lower protein consumption was associated with high water intake (122).

2.5 pH of the urine

Components in the diet that reduce the pH of the urine also indirectly contribute to the CaOx urolithiasis (122, 258). Solubility of salts is dependent on the pH of urine. The upper limits of CaOx concentrations in which these ions remain in solution mainly depend on pH of the urine. In urine with pH 7, CaOx remains in solution up to 5 mg/l. Acidic urine promotes CaOx crystallization. The pH of the urine is 6.7 - 6.9 and 6.0 – 6.2 in dogs when fed with regular diet and acidifier diet, respectively (122). The pH of urine fluctuates throughout the day, usually showing an elevated pH 2-5 h after feeding. In dogs, aciduria is often accompanied with hypercalciuria and hypocitraturia (122, 206). Studies have shown that cats fed with urinary acidifiers excreted elevated levels of calcium in their urine. Changes in the pH of the urine had no effect on renal oxalate excretion in dogs (31). Smith *et al*, reported that relative supersaturation of CaOx in the urine of cats on diet designed to control struvite stones was greater than one and the urinary pH was < 6.5 (209).

2.6 Composition of the diet

Dietary Ca is one of the major sources of urinary calcium in dogs (135). Ca content of the diet also influences the intestinal oxalate absorption. Ca^{2+} and oxalate ions within the GI tract form an insoluble complex that is excreted via feces. Therefore Ca^{2+}

content of the diet limits the bioavailability of the oxalate for enteric absorption (88, 96). In commercial pet food, average Ca content is 180 – 750 mg/100 kcal (217, 218). Relative supersaturation of the CaOx is significantly increased in the urine of dogs fed with high/medium oxalate and low Ca diet compared to low Ca and oxalate diet (215). Studies also found that decreased calcium intake has increased the risk of CaOx stone formation in humans, dogs and cats (23, 39, 122). Even though Ca and oxalate contents of the diet appear to be a major risk factor, the absorption of Ca and oxalate is complicated and shows wide variations among individuals (122).

Studies found that dietary sodium alters water intake, urine volume and Ca excretion (122). Dogs consuming high sodium diets were less likely to form CaOx stones (122). The relative supersaturation of CaOx in the urine was reduced when dogs consumed Na-supplemented diet, but no change in volume of voided urine was observed (136, 215). In humans intake of a high sodium diet increased calcium excretion via the urine (40, 160). Due to the discrepancies in findings between different studies, manipulation of dietary Na levels in the diet needs to be carried out with caution.

Dogs consuming potassium, phosphorous and Mg-containing diets were less likely to form CaOx stones (122, 138). Presence of Mg in the urine reduces the risk of forming CaOx stones. It is believed that Mg and oxalate form soluble complexes in the urine, thus preventing the formation of insoluble CaOx stones. However increased consumption of Mg will increase urinary calcium excretion (122). These observations clearly indicate that it is important to design a balanced diet, which should not facilitate one type of urolith when preventing another type.

In humans, studies have shown that hypocitraturia is a risk factor for CaOx formation. Dietary citrate is absorbed from the intestine and metabolized into bicarbonate. Bicarbonate ions cause alkalinisation, resulting in increased urine pH. The alkalinisation also causes elevated mitochondrial citrate production in the renal cells, decreased renal absorption of citrate and increased calcium reabsorption in the proximal tubules. Further, in the alkalized urine Ca^{2+} binds to citrate and forms soluble salts. In dogs, there were no significant differences in the urinary citrate concentration between stone formers and non-stone formers (135). Stevenson *et al* reported that dietary potassium citrate supplement did not alter the urinary concentration of Mg, Ox, Ca and citrate and urine relative supersaturation of the CaOx (218). It is worth noting that in the same study, the relative supersaturation of the CaOx in three Miniature schnauzers that were fed with dietary potassium citrate supplements was less than one (218). However these dogs also showed a non-significant increase in urine citrate levels (218). Citrate supplement did not alter the pH of the urine significantly except later in the day (218). Unlike in humans, based on the above findings citrate supplementation is not a promising prophylactic dietary supplement to minimize CaOx stone formation in dogs (172).

Carbohydrate diet increases the risk of CaOx stone formation in dogs (122). Transient increase in Ca excretion is observed in dogs after an intake of Carbohydrate diet (47). Increase in consumption of the carbohydrate lead to increase in postprandial insulin and thus impair Ca reabsorption in proximal renal tubules (122). Overweight dogs are more prone to form CaOx stone (121, 253). Similar trend was observed in humans (41).

In summary, organic and mineral compositions of the diet affect the urinary Ca^{2+} and oxalate levels in dogs, cats and humans.

2.7 Inhibitors and promoters in the urine

Crystallization inhibitors and promoters are present in the urine and play a vital role in development of urolithiasis (34, 103). The upper limit of Ca^{2+} and oxalate concentrations in the urine in which these ions remain in solution depends on other salts and macromolecules. Macromolecules, inorganic substances and salts in the urine interfere with crystallization and aggregation of the Ca^{2+} and oxalate ions (34, 103). Most of the crystallization inhibitors are glycoproteins and glycosaminogylcans (34, 103). Tamm-Horsfall protein inhibits the aggregation of CaOx crystals while desialylated Tamm-Horsfall protein promotes the aggregation of CaOx crystals (235). Nephrocalcin inhibits the growth of CaOx crystals in dogs (30, 157). Hyaluronic acid, present in the surface of tubular epithelial cell, promotes the retention of CaOx crystals within the renal tubules (119). Ceruloplasmin is reported to promote CaOx crystal formation (255). CaOx crystallization is also inhibited in the presence of pyrophosphate, Mg and citrate (61). Alkalized urine Ca^{2+} binds to citrate and forms soluble salts. Several proteins were identified from canine CaOx uroliths but their precise role in CaOx urolithiasis is not known (62).

In conclusion, based on various epidemiological studies, multiple factors alter the Ca and oxalate ions in the urine or enhance the crystallization of CaOx. Measures to prevent Ca and oxalate ion supersaturation need to be promptly implemented to control the increased occurrence of CaOx stones. Controlling the saturation of calcium is

physiologically critical since Ca^{2+} homeostasis is highly regulated by parathyroid hormone (PTH). Therefore, manipulation of Ca^{2+} ion needs to be carried out cautiously. Oxalate, on the other hand, is a toxic metabolic end product in mammals. Therefore control measures to reduce urine oxalate levels are less likely to cause any inadvertent side effects in the animal. These facts indicate the importance of manipulating urinary oxalate levels to prevent CaOx urolithiasis.

3. Importance of oxalate

In theory, supersaturation of calcium and oxalate ions in urine is a predisposing factor for CaOx urolithiasis. In normal human urine, the Ca:Oxalate ratio is greater than 5:1 and the stoichiometric relationship between Ca and oxalate in CaOx crystals is 1:1 (126). When Ca and oxalate ions are in equimolar concentration CaOx crystalline mass is produced (126). Even a smaller increment in oxalate concentration presents a greater risk for CaOx stone formation compared to an equivalent increase in Ca^{2+} concentration (134, 182, 183). Studies have found that urine oxalate concentrations between stone formers and non-stone formers in dogs were comparable (134, 135, 217). In human patients with CaOx uroliths, only a mild hyperoxaluria was observed except in cases with a primary hyperoxaluric condition (182). Subtle changes in urine oxalate ion concentration were often considered insignificant but such differences are now known to be critical in the pathogenesis of CaOx urolithiasis.

3.1 Source of oxalate

Oxalic acid and/or oxalate salts are widely present in plants, animals and microbes as a metabolic end product. Oxalate salts confer protection to plants by forming

an exoskeleton and precipitating the excessively absorbed Ca^{2+} by the roots. The peculiar odor of oxalic acid makes plants a less desirable fodder for animal consumption (185).

3.1.a Endogenous oxalate

In animals, oxalate is generated endogenously mainly in liver as a metabolic end product of glycine, serine, alanine, hydroxyproline, glyoxylate, glycollate and ascorbic acid metabolism (57, 86, 87). In humans, 40 to 50% of the urinary oxalate arises from hepatic synthesis of oxalate through glyoxylate metabolism (86). Overall breakdown of ascorbic acid contributes to another 30 to 40% of urine oxalate (249). In humans, inborn errors of glyoxylate metabolism lead to accumulation of oxalate in plasma and thus in urine (referred to as primary hyperoxaluria). Individuals who are unable to express functional liver-specific alanine:glyoxylate aminotransferase (Primary hyperoxaluria Type 1) and D-glycerate dehydrogenase (Primary hyperoxaluria Type II) that are essential in glyoxylate metabolism suffer from primary hyperoxaluria (87). In dogs, inherited primary hyperoxaluric conditions have not been reported. In one study, inherited hyperoxalauria was observed among related young kittens within 5 -9 months of age range (148). The observed hyperoxaluria was associated with reduced expression of D-glycerate dehydrogenase (Primary hyperoxaluria type 2) (148). Among afflicted young kittens, urinary oxalate excretion was higher than that of unrelated adult cats (148). Other types of enzyme deficiencies causing inherited hyperoxaluria have not been reported in animals.

Pyridoxine deficiency (Vitamin B6) contributes to generation of endogenous oxalate (65). Pyridoxine is a cofactor for alanine:glyoxylate aminotransferase. In the

absence of pyridoxine, glyoxylate cannot be converted into glycine. The average amount of pyridoxine in commercial pet food is 160 mg/100 kcal (218). In cats, under experimental conditions pyridoxine deficiency has led to hyperoxaluria and oxalate nephrosis (16, 65). However, there are no reports on the association between pyridoxine deficiency and CaOx urolithiasis in client-owned cats and dogs.

Increased intake of ascorbic acid and oxalate-containing food also can lead to enhanced enteric absorption of oxalates (145, 233). Ascorbate can also spontaneously convert into oxalate at alkaline pH (249).

3.1.b Exogenous oxalate

Dietary oxalate is the major exogenous source of oxalate in animals. Oxalate concentration of urine gradually declines in individuals who consume an oxalate-free diet, demonstrating that dietary oxalate is absorbed and excreted via urine (88, 162). Consumption of low-oxalate diet has reduced urinary oxalate excretion by 36% in idiopathic hyperoxaluric patients (126). Consumption of 10 – 250 mg /2500 kcal of oxalate per day would contribute a 24.4±15.5% - 41.5±9.1% increase in excreted urine oxalate in humans (88). In humans oxalate excretion was reduced upon consumption of a low-oxalate diet (160, 172). The aforementioned findings clearly indicate that dietary oxalate significantly contributes to the oxalate concentration of urine.

The average oxalate content of commercial dog food is 4 – 25 mg/100 kcal (215, 216). Precise oxalate content of the commercial diets in USA is not available but such studies are underway. Commercially manufactured pet foods contain various types of plant materials. Vegetables, legumes and vegetable-based fermentable fibers (beet pulp

and soybean fiber) that are rich in oxalates are used in canine diets (17). It is essential to identify the oxalate-containing sources in commercial pet diets to reduce oxalate intake. Human foods that contain oxalic acid (chocolate, tea, rhubarb, spinach, beetroot, parsley, okra, soybeans, yams, wheat bran, nuts, peanuts and black pepper) are not recommended for dogs that are at high risk to form CaOx stones (138). Designing oxalate-free diets requires detailed identification and quantification of each ingredient used in the pet food processing. However, effective implementation of such measures may be difficult to achieve.

In dogs, relative supersaturation of CaOx was increased in urine with increased consumption of oxalate-containing food without significantly increasing urinary oxalate excretion (215). Dogs, fed a low calcium and high oxalate diet were at high risk to form CaOx stones (215). Presumably, a high Ca:Oxalate ratio in the diet prevents gut oxalate absorption by forming insoluble CaOx salt. It was also noticed that oxalic acid content of the diet did not influence the urine volume in dogs (215).

In addition to oxalate, oxalate precursors, such as L-glycerate, hydroxypyruvate, glyoxylate, glycolate and hydroxyproline, are also present in pet food. Hydroxyproline is a major component in collagen, which is abundant in red meat (24). In human hyperoxaluric patients, oxalate excretion was reduced by 29% upon consumption of a low animal protein diet (160). A high consumption of sugar (fructose and sucrose) also was associated with occurrence of CaOx stones in humans (24). Since carbohydrates are considered to be the major carbon donors for glyoxylate synthesis in the liver, high sugars can result in elevated oxalate production (24, 86).

3.2 Absorption of oxalate

In humans, on average 10% of the dietary oxalate is absorbed in intestines (88). A radioactive oxalate ($^{13}C_2$) absorption test identified that some individuals with normal GIT function were capable of absorbing more (>15%) oxalate (hyper absorbers) (83, 107, 237). Often these individuals were from the recurrent CaOx stone formers group (83, 107, 237). However, concentrations of urine oxalate between normal absorbers and hyper absorbers were not statistically different (107). In dogs, similar oxalate absorption studies have not been reported.

Absorption of dietary oxalate also depends on other factors, including other nutrients present in the food and environment (i.e. pH) of the GIT. Dietary Ca and Mg ions bind to oxalate and reduce the absorption of free oxalate ion. Dietary lipids on the other hand enhance gut oxalate absorption (158, 248).

Free oxalate ions in the intestinal tract are absorbed by carrier-mediated active transepithelial transport and paracellular transport into the plasma and excreted via urine (21, 79, 106). Paracellular oxalate transport has not been experimentally demonstrated. However, based on indirect evidence such as the transit time of food in GIT, histology of the mucosa and time taken for oxalate absorption, it is speculated that in anterior portions of the intestine oxalate is transported paracellularly (237). Majority of the oxalate absorption occurs within 6 to 8 hours after consumption of an oxalate-containing diet (107, 237). The junctional resistance between epithelial cells is comparatively lower in the anterior segment of the intestine and thus facilitates oxalate absorption (107, 237).

Pathological conditions, such as chronic pancreatic and biliary tract disease, steatorrhea (malabsorption of fatty acid and bile salts), and disorders of the small intestine (i.e. Crohn's disease) enhance the paracellular enteric oxalate absorption in large intestine (48). In addition, following jejunal ileal bypass, bariatric surgery and small intestine resection, increased enteric oxalate absorption has been observed in humans (113). During fat malabsorption, availability of free oxalate ions is increased in the GIT as Ca^{2+} binds to the fatty acids (113). Absorptive hyperoxaluria was also observed in cystic fibrosis patients with CaOx stones (92).

Studies in rat and rabbit models confirmed the energy-dependent transepithelial oxalate absorption in colon (63, 79). Apical uptake is mediated through an anion exchanger belonging to the SLC26 (solute link career) gene family (63, 79). Potential trans-epithelial oxalate transporters such as SLC26A1 (SAT1), SLC26A3 (DRA), and SLC26A6 (PAT1) are expressed in intestine of the mice, rats and humans (75, 180, 194, 207). SLC26A3 is involved in apical oxalate absorption in the ileum of the mice (77). Apical oxalate absorption is followed by sodium-dependent basolateral oxalate efflux (80).

3.3 Excretion of oxalate

Mammals are generally protected from oxalate toxicity from the endogenous sources. Studies confirmed that oxalate homeostasis is maintained through oxalate excretion via kidney and intestine (20). In dog, oxalate is filtered in glomeruli, actively secreted by renal tubules and passively reabsorbed by renal tubules (31, 167). In humans, oxalate transporters SLC26A6 and SLC26A1 are expressed in kidney on apical and basal

membranes of proximal tubules. In dogs, presence of oxalate transporters and its involvement in oxalate excretion has not been confirmed yet.

In mice, rats and rabbits net secretion of oxalate was observed in small intestine and proximal colon while net oxalate absorption occurred at the level of distal colon (64, 78, 80, 81, 97). In mice, SLC26A6 mediates apical oxalate efflux in duodenum and ileum under normal physiological conditions (64, 97). SLC26A6 protein is expressed on basolateral membrane of distal ileum, cecum and proximal colon in mice (46). Reduced cecal oxalate content in *Slc26*a1$^{-/-}$ mice compared to wild type mice indicates that *Slc26*a1 facilitates oxalate excretion into cecal lumen (46). The exact mode of oxalate transport and expression/distribution of oxalate transporters in the GIT have not been studied in dogs and cats. It is speculated that regulation of the enteric oxalate secretion is mediated through neuro-hormonal mechanisms in conjunction with the acid-base regulation of enteric cells (77). However, precise mechanisms involved in regulation of oxalate homeostasis need further investigation.

Unlike in plants, oxalate salts cause deleterious effects in animals and according to existing evidence mammals cannot metabolize oxalate (185). However, one report has confirmed the expression of oxalate-metabolizing enzymes in liver of guinea pigs (155).

4. Oxalate metabolizing bacteria

Microbes residing in the intestinal tract express vital enzymes to metabolize complex organic compounds such as oxalate that cannot be processed by mammals (175, 250). Oxalate-metabolizing activity has been identified among the commensal organisms of the mammalian GIT. Enteric microbes metabolize oxalate and limit enteric oxalate

absorption (6). It has been speculated that 50% - 80% of dietary oxalate is degraded by the oxalate metabolizing bacteria in the gut (91). Exploring the oxalate-metabolizing bacteria in the GIT in dogs would provide more insights into the pathophysiological process of CaOx stone formation in humans, dogs and cats.

In nature, oxalate oxidase (OxOx), oxalate decarboxylase (OXDC), oxalyl CoA decarboxylase (OXC) and oxalate oxidoreductase (OOR) are the four known oxalate-metabolizing enzymes that are present in microbes and plants. Bacteria and fungi express OXDC, OXC and OOR, while OxOx is expressed primarily in plants. OxOx enzyme was also isolated from *Pseudomonas spp* OX53 and *Ceriporiopsis subvermispora* (55, 177). OOR has been identified in *Moorella thermoacetica,* an anaerobic bacteria found in the GIT of animals. OOR oxidizes oxalate and generates HCO_3 or two electrons and CO_2 (174).

Oxalate decarboxylase (OXDC) catabolizes oxalate to formate and carbon dioxide. This catalytic reaction is oxygen-dependent and requires Mn^{2+} as a cofactor (225). Fungi and bacteria possess the gene encoding OXDC (143, 224). Reports have also confirmed the expression of OXDC in liver of the guinea pig (155). Among bacteria, OXDC is expressed by *B. subtilis.* OXDC expression in *B. subtilis* is induced by acidic pH (pH 5) (224, 225). A wide range of fungi expresses OXDC and the fungal OXDC's are known to be induced by oxalate (143).

OXC converts oxalyl CoA to formyl CoA and CO_2. To complete the oxalate metabolism by OXC, activity of formyl CoA transferase enzyme (FRC) is essential. FRC transfers CoA from formyl CoA to oxalate and forms Oxalyl CoA. Genes encoding OXC

and FRC are present in a wide range of commensal microbes. Among them, *Oxalobacter formigenes* is unique as it solely relies on oxalate for its energy requirement (7). In addition to OXC, the bacterium expresses formyl CoA transferase (FRC) and both are essential to metabolize oxalate to formate and CO_2 (14, 15, 140). Oxalate and formate are transported across the cell membrane through an oxalate/formate antiporter (OxlT) (10). Proton motive force generated through oxalate and formate exchange is a driving force for ATP generation. Eight molecules of oxalate are utilized to produce one molecule of ATP (35, 36). Since the synthesis of ATP is oxalate-dependent in *O. formigenes*, the genes coding for *oxc, frc* and *OxlT* are constitutively expressed in the bacterium although they are not present in a single operon (10, 35, 36). However, it is not known whether these genes are constitutively expressed in other oxalate metabolizing bacteria. Regulation of the *oxc, frc* and *OxlT* in *O. formigenes* expression has not been extensively studied. Despite their role in a single function (oxalate metabolism), these genes are located in separate operons, driven by independent promoters and rho-independent terminator sequences (14, 15, 202). *O. formigenes* is classified into two strains based on the nucleic acid sequences in the C-terminus of the *oxc* gene and fatty acid composition of the organism (7, 197, 199).

O. formigenes has been isolated from humans, cattle, sheep, pigs and rats (6, 42, 45). Enumeration based on culture methods found that in healthy individuals, 4.0×10^5 – 2.3×10^8 CFU of *O. formigenes* were present in a gram of feces (6). However, based on PCR, 5.2×10^3 – 1.04×10^9 cells of *O. formigenes* were found in a gram of feces and the limit of detection by the PCR was 5×10^3 cells (176). Even though *oxc* gene sequences

were detected in fecal samples of dogs by PCR no enumeration data was available (242, 243). Oxalate-metabolizing activity by *Lactobacillus spp* isolated from animal feces, dairy products and pharmaceutical probiotic preparation has been observed (12, 13, 19, 27, 123, 154, 232). Homologues of *oxc* and *frc* of *O. formigenes* have been identified in the acid-inducible operon of *L. acidophilus*, *L. gasseri* and *L. reuteri* (8, 13, 123, 142). In the presence of oxalate in acidic (pH 5.5) environment, *oxc* and *frc* are co-transcribed in *L. acidophilus* and *L. gasseri* (123). The OXC and FRC enzymatic activities of the recombinant *L. acidophilus* were biochemically evaluated by capillary electrophoresis (232). Oxalate metabolism by the bacterium was assessed *in vitro* by the amount of the oxalate depleted in the oxalate-supplemented culture medium or in a simulated colon model (123). The *in vivo* oxalate degradation studies were based on comparative urine oxalate excretion following the administration of probiotics containing *Lactobacillus spp* (27).

Fecal isolates of *L. reuteri* and *L. animalis* from cats and *L. salivarius* from dogs failed to metabolize oxalate *in vitro* (154). *L. animalis* and *L. murinus* strains that were isolated from feces of dogs metabolized oxalate *in vitro* while only *L. animalis* metabolized oxalate *in vivo* (mouse model) (154). Human isolates of *L. gasseri*, *L. reuteri* and *L. acidophilus* NCFM and the dog isolate of *L. acidophilus* were capable of metabolizing oxalate (12, 123, 245). Human isolates of *L. helveticus, L. johnsonii* were incapable of metabolizing oxalate (12).

Probiotic isolates of *L. plantarum, L. johnsonii, L. paracasei, L. delbrueckii subsp. lactis, L. delbrueckii subsp. bulgaricus, L. brevis, L. reuteri and L. helveticus*

failed to metabolize oxalate *in vitro* (27, 232). *In silico* screening revealed that *oxc* and *frc* genes were not identified in the genome of the *L. plantarum, L. salivarius L. johnsonii, L. delbrueckii subsp. bulgaricus, L. casei* and *L. brevis* (232). However, mild oxalate degradation was observed *in vitro* by few strains of *L. plantarum* (27). *L. plantarum* isolated from dogs also showed oxalate degrading activity (245). Oxalate degradation was not observed in *L. reuteri* despite the presence of *oxc* and *frc* in its genome (232).

Homologues of *oxc* and *frc* of *O. formigenes* have been identified in the genome of the *B. animalis* subsp. *lactis, B. gallicum, B. dentium, and B. pseudocatenulatum* (58, 231). *Oxc* and *frc* of *B. animalis* subsp. *lactis* are not part of a polycistronic operon (231). In acidic pH (4.5) conditions and in the presence of other carbon sources (eg. sucrose), *B. animalis* subsp. *lactis* efficiently metabolized oxalate *in vitro* (231). Activity of the recombinant OXC enzyme of *B. lactis* has been biochemically evaluated (58). Oxalate-degrading activity was not observed in probiotic and human isolates of *B. adolescentis, B. bifidum, B. breve, B. longum,* and *B. catenulatum* (231).

Murphy *et al* reported that fecal isolates *B. longum and B. acidophilis* from cats and isolates of *B. globosum* and *B. animalis* from dogs failed to metabolize oxalate *in vitro* while *B. infantis* isolated from humans metabolized oxalate *in vitro* (27, 154).

Based on *in vitro* and *in vivo* studies, oxalate degradation by *Lactobacillus* and *Bifidobacteria* appears to be species and strain dependent (12, 60, 154, 232). Among the *Lactobacillus spp., L. acidophilus* metabolized oxalate efficiently (12, 232).

Enterococcus faecalis metabolized oxalate in an anaerobic medium with limited carbon source (0.02% yeast extract) other than oxalate (84). Among the human isolates of *Enterococcus faecalis*, only oxalate-metabolizing *Enterococcus faecalis* strains expressed the intra-cellular OXC and FRC proteins (84). Failure to express OXC and FRC by non oxalate-metabolizing *Enterococcus faecalis* strains indicates that expression of these proteins is inducible under nutritionally deprived conditions to utilize oxalate (84). Hokama *et al* also observed expression of an additional 40 kD protein (based on SDS PAGE) by the oxalate-metabolizing *Enterococcus faecalis* strains but the protein was not identified (84). It is worth noting that the molecular weight of the OxIT protein of *O. formigenes* is 38 kD therefore it is possible the unidentified 40 kD protein could be a OxIT homologue. The precise mechanism of oxalate metabolism in *Enterococcus faecalis* is yet to be studied (84). Oxalate-metabolizing *Enterococcus faecalis* and *Enterococcus faecium* strains have been isolated from feces of dogs (181).

Hokama *et al* also identified an oxalate metabolizing *Providencia rettgeri* strain isolated from human feces and expression of the oxalate metabolizing enzymes OXC and FRC was confirmed in this strain (85). However, expression of OxIT was not observed in the oxalate metabolizing strains of the bacterium (85). Also, expression of OXC and FRC were lost in the oxalate-metabolizing strains when the organisms were alternated between oxalate-free and oxalate-supplemented medium (85). These observations indicate that expression of the oxalate-metabolizing enzymes is not entirely oxalate-dependent. The biochemical mechanisms involved in the regulation of oxalate-metabolizing genes need to be elucidated to understand the environmental factors influencing the expression of

these genes. As *Providencia rettgeri* is a pathogenic bacterium, its role in prevention of enteric oxalate absorption in healthy individuals is questionable.

Homologues of *oxc and frc* of *O. formigenes* were identified within the bile-acid-inducible operon y*fdXWUVE* of *E. coli* (146, 147, 227). Expression of *yfdX, yfdW, yfdU, yfdV* and *yfdE* were observed in *E. coli* MG1655 mutant that survived in acidic environment (146). Genes of *yfdU* and *yfdW* are the homologues of *oxc* and *frc*, respectively (227), (73). YhjX protein of *E. coli* has been annotated as OxlT based on sequence similarity to OxlT of *O. formigenes* (227). However, oxalate-metabolizing activity has not been studied in the *E. coli* MG1655 mutant and other commensal *E. coli*. *E. coli* has been used as a negative control in studies in *Lactobacillus* oxalate degradation *in vitro* therefore leading to the speculation that *E. coli* does not degrade oxalate (232).

Several other commensal bacterial isolates, including *Leuconostoc mesenteroides, Lactococcus garvieae* and *Lactococcus subsp. lactis,* from dogs, metabolized oxalate *in vitro*, in the presence of high sugars (181). Isolates of *Leuconostoc lactis* from dog also exhibited oxalate degrading activity *in vitro* (245). *In vitro* oxalate-metabolizing activity was also detected in *Eubacterium lentium* (human isolate), *Streptococcus thermophilus* (probiotic isolate) and *Pseudomonas oxaliticus* (environmental isolate) (27, 95, 178).

In summary, gastrointestinal tract of dogs, cats and humans are colonized with oxalate metabolizing bacteria that could play a role in reducing absorption of enteric oxalate ions.

5. Effect of oxalate metabolizing bacteria on CaOx stone formation

An association between the presence of oxalate metabolizing bacteria and absence of CaOx stone formation has been observed in humans and lab animals (203). In this context, most of the studies were focused on clinical application of *O. formigenes,* the efficient oxalate-metabolizing bacteria known to date (76, 82, 90, 91, 201, 205).

Presence of *O. formigenes* in a biological sample is mainly detected by the oxalate depletion in an oxalate-supplemented anaerobic culture media and/or by detection of *oxc* gene by PCR (42, 197, 199, 200). Selective culture media for *O. formigenes* has not been established yet. Colonies of *O. formigenes* are confirmed by the presence of a zone of clearance in a turbid (CaOx) agar medium within roll tube cultures (42). Because the optimum growth conditions vary depending on the strains the culture-based screening of *O. formigenes* in biological samples is challenging (42, 52). Recently, PCR-based methods have been widely used to detect the *O. formigenes*-specific fragments of *oxc* gene (109, 176, 199, 200, 242). *In silico* analysis confirmed the specificity of the primers designed to detect the *oxc* gene of *O. formigenes* (109, 176, 197). However, unavailability of the sequences for all the enteric bacteria and the possibility of horizontal gene transfer indicate that PCR-based results should be interpreted with caution.

According to a study, the overall prevalence of *O. formigenes* in healthy humans was 37 to 77% (101, 102, 108, 116, 118, 199, 203, 205, 229). In populations affected with CaOx urolithiasis, the prevalence was 17 - 46% (114, 205, 229). Urinary oxalate excretion in CaOx urolithiasis patients who were colonized with *O. formigenes* was

significantly less than that of non-colonized patients (114, 205, 229). Case-control studies in humans observed that colonization of *O. formigenes* decreased the risk of CaOx formation by 70% (101).

Recently, the *oxc* gene of *O. formigenes* has also been found in feces of cats and dogs by quantitative PCR (242, 244). Based on screening by PCR, Weese *et al* reported that prevalence of *O. formigenes* was 86 % (24/28) and 34 % (22/65) in random fecal samples obtained from healthy cats and dog, respectively (242, 244). However, no studies were performed in dogs or cats to investigate the association of CaOx stone formation and presence of *O. formigenes*.

In studies involving humans and dogs, wide variability was observed in the detection of *oxc* gene of *O. formigenes* within fecal samples from the same individual (176, 243). Therefore, *O. formigenes* prevalence data based on single fecal sample may not reflect the accurate enumeration of colonized *O. formigenes*.

Secretion of oxalate via the enterocytes (into gut lumen) was enhanced by the presence of *O. formigenes* (76). *O. formigenes* increased the colonic secretion of oxalate by generating a trans-epithelial oxalate gradient through which oxalate ions in the plasma of a hyperoxalemic patient efflux via epithelial cells into the lumen of cecum and colon (76, 82). Based on the above mechanism, most of the *O. formigenes* probiotic therapy studies were focused on primary hyperoxaluric patients. Colonization of *O. formigenes* in mouse models with type 1 primary hyperoxaluria has reduced the plasma and urine oxalate concentration by 50% (82). Another study showed that administration of *O. formigenes* ($>10^7$ CFU) for four weeks reduced the urinary oxalate excretion by 50% in

hyperoxaluric human patients (91). However, stable colonization was observed only in few of the patients (91). Hoppe *et al* observed that oral administration of frozen *O. formigenes* paste and enteric-coated capsule containing viable *O. formigenes* did not cause any harmful side effects in humans (89). Oral administration of enteric-coated tablets containing viable *O. formigenes* reduced the urinary oxalate excretion by 39 - 92% in four out of six healthy individuals but the colonization was transient (89). A recent study claimed that oral administration of *O. formigenes* did not significantly alter the urine oxalate excretion in primary hyperoxaluric patients (90). Recently, Hoppe *et al* observed a 45% reduction in plasma oxalate levels upon oral administration of *O. formigenes* to two infants (11 months old) suffering from infantile oxalosis (90). These contradictory observations indicate that efficacy of *O. formigenes* therapy needs to be evaluated on a case-by-case basis.

Restriction on dietary oxalate intake had an impact on *O. formigenes* colonization (42, 67). Jiang *et al* observed that a 15-fold increase in dietary oxalate resulted in 12-fold increase in the gut population of *O. formigenes* (96). Controlled diet experimental studies found that individuals colonized with *O. formigenes* excrete 19.5% less oxalate than the non-colonized control individuals (96). Interestingly, Knight *et al* observed that none of the enteric oxalate hyper-absorbers were colonised with *O. formigenes* (107). Administration of *O. formigenes* via oesophageal gavage to rats with diet-induced hyperoxaluria significantly reduced the urinary oxalate excretion (198). In addition, treatment with enteric-coated capsules containing OXC and FRC of *O. formigenes* and

cofactors (oxalyl-CoA, $MgCl_2$, and thiamine diphosphate (TPP)) significantly reduced the urinary oxalate excretion in rats (203).

Frequent antibiotic treatment alters the enteric microbial flora in animals (42, 52, 101, 102, 153). Studies have shown that development of hyperoxaluria in cystic fibrosis patients was associated with loss of *O. formigenes* (201). Comparative prevalence of *O. formigenes* among children (100%), adults (40-60%) and cystic fibrosis patients (< 16%) led to the speculation that use of antibiotics is associated with reduction in colonization of *O. formigenes* (92, 199, 201, 203). In support of this observation, Hoppe *et al* reported that hyperoxaluria observed in cystic fibrosis patients with CaOx stones was due to increased absorption of enteric oxalate (92). Based on epidemiological reports, prevalence of *O. formigenes* was 55% among individuals who never used antibiotics (102). The prevalence of *O. formigenes* among individuals treated with antibiotics more than 5 years previously, within 5 years and less than a year was 36%, 27% and 17% respectively (102). Daniel *et al* reported that isolates of *O. formigenes* showed resistance to a wide range of antibiotics including erythromycin, vancomycin, rifampin, streptomycin, penicillin, carbenicillin, ampicillin, cephalothin, and neomycin (42). In their studies, Duncan *et al* observed that *O. formigenes* was resistant to clarithromycin and doxycycline (52). Based on epidemiological reports, in subjects who used erythromycin, clarithromycin, azithromycin, tetracycline, minocycline, doxycycline, or metronidazole the *O. formigenes* colonisation was reduced (102).

Reports indicate that following gastric bypass surgery CaOx stone formation is common in humans. It has been hypothesised that loss of *O. formigenes* colonisation

contributes to the CaOx stone formation in the gastric bypass patients. However, another study showed that only 16% of morbidly obese patients were colonized with *O. formigenes* suggesting other obesity-related causes may be involved in impaired colonization (50).

Urinary oxalate excretion was significantly higher in individuals colonized with *O. formigenes* compared to non-colonized individuals (205). However, according to a large-scale epidemiological study, urine oxalate excretion was comparable between *O. formigenes* colonized and non-colonized individuals (101).

Oral administration of probiotics (Oxadrop) composed of *Lactobacillus acidophilus, Lactobacillus brevis, Streptococcus thermophillus, Bifidobacterium infantis* reduced urinary oxalate excretion although the finding was not reproducible in different studies (27, 68, 125, 126). Rats fed with *L. casei* formed less CaOx crystals in the kidney than the control rats (117). Consumption of probiotic strains of *L. casei* and *B. breve* reduced the urine oxalate excretion by more than 25% in stone-formed non-hyperoxaluric humans (60). In another study, use of probiotic VSL#3 reduced enteric oxalate absorption by 30% in oxalate hyperabsorbers but no significant reduction was observed among normal absorbers (161). VSL#3 was composed of *S. thermophilus, B. breve, B. longum and B. infantis, L. acidophilus, L. plantarum, L. paracasei and L. delbrueckii subsp. bulgaricus* (161). Due to these contradictory findings, the role of oxalate degrading lactic acid bacteria in the prevention of CaOx urolithiasis is still controversial. The association between enteric oxalate-metabolizing *Enterococcus faecalis* strains and CaOx urolithiasis has not been explored in animals or humans.

Recombinant OXDC enzyme of *B. subtilis* is commercially available ("Oxazyme") (38). Toxicity trials of the product were carried out in dogs and rats and significant adverse effects were not observed (38). However, the oxalate-metabolizing efficacy of the aforementioned product is not reported yet. All the above studies were focused only on few oxalate-metabolizing bacteria but other commensal bacteria also may metabolize oxalate.

6. Exploring potential oxalate metabolizing bacteria

The gastrointestinal tract of mammals is colonized with a vast number of bacterial species (189). In general, herbivores harbour the most diverse bacterial community compared to carnivores and omnivores. Bacterial diversity is less in carnivores compared to the omnivores (100, 124). Differences in bacterial phylotypes were also observed among hindgut fermenters, foregut fermenters and monogastrics (100, 124). In terms of physico-chemical and microbiological characteristics, the gastrointestinal tract is not a uniform environment. Each segment of the intestine harbours unique microbiota (221). Even though microbial fermentation does not significantly contribute to the host energy requirements in a monogastric animal like dog the process is essential to maintain a healthy environment in the GIT (223). In a given animal species, diversity of the microbial community is continuously altered by a variety of factors including diet, availability of the nutrients and space, use of antibiotic, pathogenic bacteria and metabolic diseases (100, 124, 230). A vast array of microbial metabolic activities in GIT determines the enteric absorption and secretion of the metabolites. Recent studies have

also proved that microbial composition plays a critical role in altering the metabolic status of the host (51, 230).

Recent studies have demonstrated that oxalate-metabolizing bacteria are pivotal to prevent the absorption of toxic oxalate ions. Further, oxalate-metabolizing activity by the bacteria within GIT also enhanced the enteric oxalate excretion in hyperoxaluric patient. However, no high throughput oxalate metabolic bacterial screening methods were employed to identify potential oxalate-degraders. Routine identification and enumeration of the bacteria were carried out by culturing the environmental sample in suitable nutrient media. Extensive culture-dependent methods have been adopted to understand the bacterial diversity in GIT of the dogs (26, 149). Relying solely on culture methods to identify oxalate-metabolizing bacteria may be misleading, since specific nutrient and growth requirements are unknown for most of the microbes in GIT. Enteric microbes acquire nutrients from the host diet and metabolites from the host cells and co-existing microbial flora Therefore, fulfilling all the obligatory nutritional requirements is impractical *in vitro* culture systems.

A revolution in rapid high-throughput nucleic acid sequencing technology and advancement in computational tools to handle metadata have provided the means to identify bacterial phylotypes in a wide range of environmental samples (29, 37, 71, 184, 210). In-depth analysis of hypervariable regions of the bacterial 16S rDNA, using aforementioned technology allows investigators to phylotype the bacterial community and explore the bacterial diversity in a given sample (33, 144, 168, 251, 254). Further, metagenomics approaches would expedite the discovery of functional oxalate-

metabolizing genes by analysing transcripts in feces or gut contents. Therefore nucleic acid sequencing methods warranted feasibility analysis for large scale screening of potential bacterial genes involved in the oxalate metabolic pathways

7. Limitations in existing oxalate metabolizing probiotics

In humans, studies on oxalate metabolizing bacteria suggested that use of *O. formigenes* as probiotic therapy will minimize the risk of CaOx stone formation. As *O. formigenes* is solely dependent on oxalate for its energy needs, sustainable colonization by this organism is dependent upon a continuous supply of oxalate (42, 96). Feeding oxalate-containing diets to dogs that are at high risk for forming CaOx stone is not feasible. As an alternative, oxalate metabolizing lactic acid bacterial probiotics can be used. However, *in vitro* experiments showed that expression of the oxalate-metabolizing enzyme is inducible under acidic environments (pH 4.5 - 5.5) in these lactic acid bacterial species. Further, oxalate metabolizing activity of other probiotic bacteria is not as efficient as that of *O. formigenes* and results of studies that used such bacteria in *in vivo* experiments was not reproducible. Therefore, successful engineering of a probiotic strain to imitate the oxalate-metabolizing activity of *O. formigenes* would be a superior approach.

Recently, several attempts have been made to heterologously express the genes involved in oxalate metabolism in probiotic bacterial species.

FRC, OXC and OxlT of *O. formigenes* were individually heterologously expressed in *E. coli* (DE3) (140, 187, 202). However, no published data is available on co-expression of all three genes in a heterologous host bacterium.

Duong *et al*, constructed an expression vector containing *oxc* and *frc* genes of the *L. acidophilus* NCFM under the control of constitutively active phosphoglycerate mutase promoter (P_{pgm}) (53). An enhancement in oxalate degradation was observed in *L. gasseri* that were transformed with the above expression plasmid (53). Further, complemented oxalate degradation was observed in a mutant *L. acidophilus* deficient in oxalate-metabolizing enzymes when transformed with the above expression plasmid (53).

In another study, OXDC of *B. subtilis* was heterologously expressed in *L. plantarum* NC8 and *E. coli* DE3 and the purified recombinant enzyme metabolized oxalate *in vitro* (110). However, oxalate-metabolizing activity of the intact bacterium was not reported.

8. Concluding remarks and rationale

In conclusion, presence of oxalate-metabolizing bacteria is correlated with the absence of CaOx stone formation in humans. Among the CaOx stone formers, urinary oxalate concentration is significantly less in individuals who are colonized with *O. formigenes* than that of non-colonized individuals. Probiotic therapy with oxalate metabolizing bacteria has reduced urinary oxalate excretion mainly in hyperoxaluric humans but this observation was not consistent between studies. All the above studies were focused on very few oxalate-metabolizing bacterial species and potentially confounding effects by other unkown oxalate metabolizing bacteria in CaOx urolithiasis was often neglected.

Briefly, lack of information on oxalate-metabolizing bacteria in CaOx urolithiasis in dog prompted us to investigate the prevalence of the potential oxalate-degrading

bacteria in healthy and CaOx stone forming breeds and to develop methods to engineer efficient oxalate-metabolizing probiotic candidates to overcome the limitation in existing oxalate- metabolizing probiotics. The overall hypothesis of my thesis is that the presence of enteric oxalate-metabolizing bacteria minimizes the risk of CaOx stone formation in dogs. Two major aims of my thesis are to study the association between the presence of oxalate metabolizing bacteria and absence of CaOx stone formation in dogs and to develop methods to engineer probiotic bacteria to metabolize oxalate.

CHAPTER II

Presence of oxalate metabolizing bacteria in the intestinal tract is associated with the absence of calcium oxalate urolith formation in dogs.

Manuscript in preparation for Urological Research

The incidence of calcium oxalate (CaOx) urolithiasis in dogs has increased steadily over the last two decades. A potential mechanism to minimize CaOx urolithiasis is to reduce enteric absorption of dietary oxalate by oxalate-metabolizing enteric bacteria. Enteric colonization of *Oxalobacter formigenes*, an anaerobe which exclusively relies on oxalate metabolism for energy, is correlated with absence of hyperoxaluria or CaOx urolithiasis or both in humans and laboratory animals. We thus hypothesized that decreased enteric colonization of oxalate metabolizing bacteria is a risk factor for CaOx urolithiasis in dogs. Fecal samples from dogs with CaOx uroliths, clinically healthy, age, breed and gender matched-dogs, and healthy non-stone forming breed dogs were screened for the presence of *O. formigenes, L. acidophilus* and *B. animalis* by quantitative PCR to detect the oxalyl CoA decarboxylase (*Oxc*) gene, and by oxalate degrading biochemical activity in fecal cultures. We found that the presence of *Oxc* genes of *O. formigenes*, *L. acidophilus* and *B. animalis* was significantly higher in healthy non-stone forming breed dogs than in the dogs with CaOx stones. Further, dogs with calcium oxalate stones and the stone-forming breed matched-controls showed comparable levels of oxalate degrading activity. We conclude that absence of enteric oxalate metabolic bacteria is a risk factor for CaOx urolithiasis in dogs.

Introduction

Urolithiasis due to formation of calcium oxalate (CaOx) stones is an increasingly common clinical condition in dogs. The prevalence of CaOx stones in samples submitted to the Minnesota Urolith Center increased from 5.3% in 1981 to >41% in 2007 (165). CaOx stones present a unique therapeutic challenge since they cannot be dissolved using medical therapy. Despite an array of advanced methods currently available for prevention, CaOx uroliths often recur within 1 to 3 years, requiring repeated removal to eliminate recurrent clinical signs. Increased oxalate absorption from dietary sources is a risk factor for CaOx urolithiasis (138). A variety of factors, including dietary calcium, presence of unabsorbed fatty acids and oxalate-degrading microbial flora of the gut, influence the level of free oxalate in the gastro-intestinal tract that is available for absorption (5, 48, 122, 239). Among the known culturable oxalate-metabolizing bacteria identified from the mammalian gastrointestinal (GI) tract, only *Oxalobacter formigenes* exclusively depends on oxalate for its energy and, therefore, is considered an efficient oxalate degrader in the GI tract (7, 44). This anaerobe has been cultured from the enteric tract of sheep, pig, rats, and humans (7, 42, 45).

In human and lab animals, enteric colonization of *O. formigenes* is correlated with the absence of hyperoxaluria and calcium oxalate stones (118, 201, 203, 205, 229).The risk of recurrent stone formation is reduced by 70% by intestinal colonization of *O. formigenes* in humans and administration of *O. formigenes* or its oxalate-metabolizing enzymes reduced or reversed hyperoxaluria in rats and humans (52, 89, 90, 101, 198). *O. formigenes* colonization also reduced blood oxalate levels in human and rat by generating

a trans-epithelial oxalate ion concentration gradient which facilitates enteric efflux of oxalate ions from plasma through the enterocytes, contributing to the enteric clearance of plasma oxalate (76, 82).

Weese *et al* reported the presence of *O. formigenes* in healthy dogs and cats through PCR screening but its potential role in the prevention of CaOx urolith formation was unknown (242-244). Oxalate metabolizing lactic acid bacteria also have been isolated from healthy dogs but their prevalence in dogs with CaOx stones has not been explored yet (154, 181, 245). In this study, we hypothesize that decreased enteric colonization of oxalate metabolizing bacteria is a risk factor for CaOx urolithiasis in dogs. We screened for the presence of *O. formigenes, L. acidophilus,* and *B. animalis* in healthy dogs and those affected by CaOx uroliths. Oxalate degradation was assayed in fecal cultures to predict the functional outcome of increased colonization by oxalate-degrading bacteria.

Materials and Methods

Selection of dogs

Fecal samples were obtained from 20 dogs with idiopathic CaOx urolithiasis at the time of urolith removal. Uroliths were quantitatively analyzed and reported to contain ≥90% CaOx monohydrate or dihydrate or both. Twenty clinically healthy dogs of similar breed, age (+/-1 year), and gender to the stone-formers were also sampled (Table 1). Medical history, survey radiography, urinalyses, and biochemical profiles were used to exclude dogs with urolithiasis, calcium oxalate crystalluria, or hypercalcemia. Samples were also collected from 20 dogs of non-stone forming breeds (Table 2). All dogs were

Table 1. List of stone-forming breeds contributing to the study.

Breeds	Age	Gender	Total Number *
Bichon Frise	7	F	2
Chihuahua	11	M	2
Collie	10	M	2
Fox Terrier Wirehair	11	M	2
Jack Russell Terrier	11	M	2
Miniature Schnauzer	10	F	4
Miniature Schnauzer	4-12	M	10
Papillon	7	M	2
Pekingese	6	F	2
Pomeranian	5	M	2
Pomeranian	6	F	2
Shi Tzu	6	F	2
Yorkshire Terrier	6-12	M	6

* Number of dogs was evenly divided in each group in every breed.

Table 2. List of non-stone forming breeds contributing to the study.

Sample type	Breeds	Age	Gender	Total Number
1	German Shepherd Cross	7-12	F	2
		2	M	1
	Saint Bernard	5-10	M	2
	English setter	5	F	1
	Golden Retriever	5-7	M	2
	Labrador Retriever	5-10	M	3
	German short hair pointer	2	F	2
	Dalmatian	10	M	1
	Standard Poodle	4	M	1
	Clumber spaniel	3	M	1
	Border Collie	11	M	1
	Greyhound	3-5	M	3
2	Pit Bull	1-6	M	4
		4-6	F	3
	German Shepherd Cross	8	M	1
		6	F	1

1- Fecal samples

2- Swabs collected from the intestinal tract

client-owned and had no history of antibiotic use two months prior to sample collection, which was performed in compliance with an approved University of Minnesota IACUC protocol.

Ileal, cecal, colonic and rectal samples were obtained from 9 dogs of non-stone forming breeds following humane euthanasia at an animal shelter (Table 2). Samples were obtained within 60 minutes of euthanasia.

Determination of *O. formigenes* status by PCR

Fecal DNA was extracted from 200 mg of fresh feces using the QIAmp DNA stool kit according to the manufacturer's instructions (Qiagen, Alameda, CA). Primer3 software (National Human Genome Research) was used to design *O. formigenes* specific primers (Table 3), which generated a 214 bp fragment of the oxalyl CoA decarboxylase (*oxc*) gene. To calculate *Oxc* gene copy number, plasmid pCR4-TOPO (Invitrogen, Carlsbad, CA) containing a 214 bp fragment of *Oxc* was generated by standard cloning methods, confirmed by Sanger sequencing (Biomedical Genomics Center, University of Minnesota), purified and quantified (www.uri.edu/research/gsc/resources/cndna.html, Rhode Island Genomics and Sequencing Center, University of Rhode Island). Serial dilutions were used to construct a standard curve.

Quantitative PCR (qPCR) was carried out on ABI 7500 Real time PCR system (Applied Biosystems, Carlsbad, CA) using SYBR Green PCR master mix (Applied Biosystem, Carlsbad, CA) with 100 nM primers. Amplification was carried out with activation at 95° C for 10 minutes, followed by 50 cycles of denaturation at 95° C for 23 seconds, annealing at 60° C for 20 seconds and extension at 70° C for 40 seconds.

Table 3. List of primers used for qPCR

Organism	Primer sequence
O. formigenes – M77128 (214 bp)	Forward 5’ GTGTTGTCGGCATTCCTATC 3’
	Reverse 5’ GGGAAGCAGTTGGTGGTT 3’
Bifidobacterium animalis AB163432 (176 bp)	Forward5’ CAAGTTCGTATTGCGACAGC 3’
	Reverse 5’ GCATGTGTTCCTCATGTTCG 3’
Lactobacillus acidophilus AB276023 (160 pb)	Forward 5’ TCAGCATTTGGTTTTGATGG 3’
	Reverse5’ CATAGTGGGCATTGTGATCC 3’

Melting peak of each sample was analyzed to confirm product specificity (83.4° C to 84.5° C).

Determination of *L. acidophilus and* B. *animalis* status by PCR

Fecal DNA extraction, plasmid generation and qPCR were carried out as described above. In these experiments, the samples were processed in a beat beater prior to chemical extraction.

Determination of *O. formigenes* status by culture

O. formigenes culture medium 175-132 supplemented with 10 mM sodium oxalate was prepared as described previously (7). Fecal swab extract was anaerobically inoculated and incubated anaerobically at 37° C for 5-14 days. A pure isolate of *O. formigenes* and cecal swabs from pigs were inoculated as positive controls. Broth cultures were centrifuged at 10,000 rpm for 10 min. Oxalate ion concentration of the supernatant was measured by ion chromatography. Fecal cultures that showed complete oxalate depletion were considered as positive for *O. formigenes* and also confirmed by sequencing the 16S rDNA amplicons (Biomedical Genomics Center, University of Minnesota).

Fecal culture

100 mg of fresh fecal samples were inoculated into the MRS oxalate supplemented medium (MRS-Ox) and incubated anaerobically for 48 hours. Oxalate ion concentration of the culture supernatant was measured by ion chromatography.

Ion chromatography

Samples were diluted 10-fold with de-ionized water and analyzed on a Dionex ICS-2000 ion chromatography system consisting of an AS19 analytical column, ASRS 300 suppressor, AS40 autosampler, and integrated dual piston pump and conductivity detector. The eluant was generated by Reagent Free eluant generator system (Dionex, Sunnyvale, CA), which produced a variable concentration KOH eluant, regulated by Chromeleon control software. The control program used a comprehensive anion elution scheme.

Data analysis

Groups were compared by non-parametric Mann-Whitney tests using GraphPad Prism 4.0 (GraphPad Software, Inc. La Jolla, CA) and the crude odds ratio (OR) was calculated by MedCalc software. Differences between groups were considered significant at $p<0.05$.

Results

Prevalence of *O. formigenes* in dogs

Presence of *O. formigenes* in feces was identified by qPCR amplification of *oxc*. An overall *O. formigenes* prevalence of 50% was observed regardless of CaOx status, and *O. formigenes* abundance was low, in the range of 10^1 - 10^4 organisms per gram of feces (Figure 1). Ideally, presence of viable *O. formigenes* in oxalate-supplemented fecal culture would result in 100% depletion of oxalate as observed in the pure isolate of *O. formigenes* and in pig cecal culture. However, only two fecal cultures showed 100% depletion of oxalate (Figure 2).

Figure 1. Fecal qPCR-based screening for *O. formigenes*.

The *Oxc* gene of *O. formigenes* was quantified in CaOx stone dogs (●, n=20), breed-matched negative controls (○, n=20) and negative non-stone forming breeds (Δ, n=20). Horizontal line is the mean.

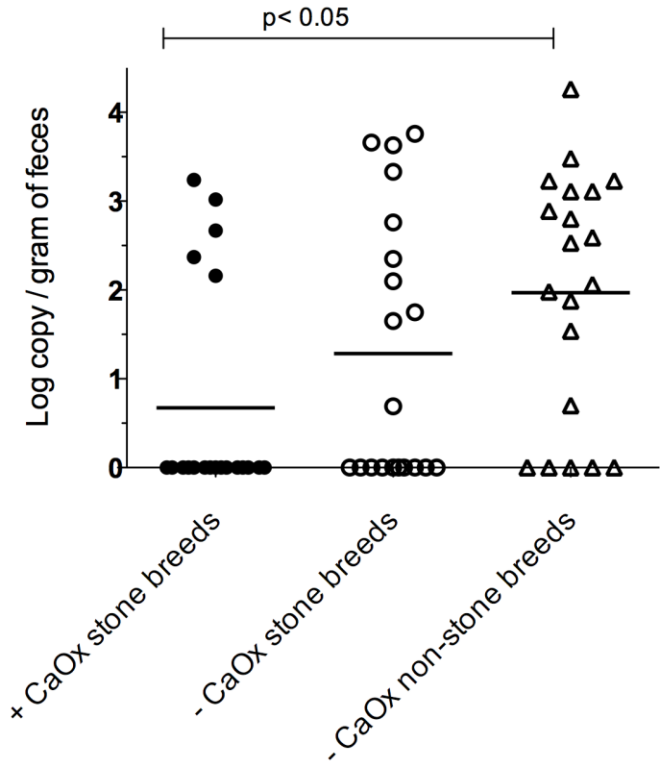
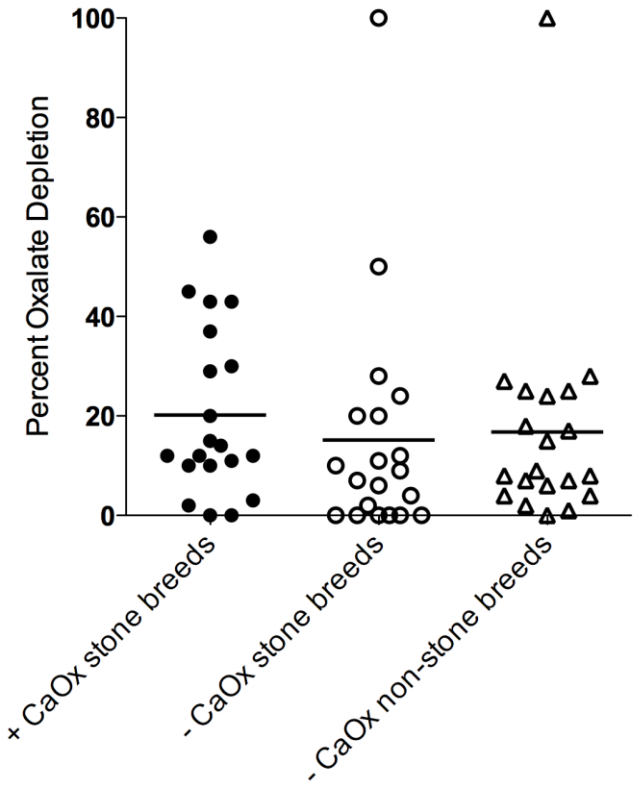


Figure 2. Oxalate degrading activity in fecal cultures.

Fecal samples collected from CaOx stone dogs (●, n=20), matched control dogs (○, n=20) and dogs belonging to non-stone forming breeds (Δ, n=20) were cultured in oxalate-supplemented anaerobic medium for 14 days and oxalate concentrations were analyzed by ion chromatography. Horizontal line is the mean.



To determine if *O. formigenes* was more abundant in situ, samples were collected from ileum, cecum, colon and rectum of additional dogs and oxalate degrading activity was measured. Two out of 9 dogs showed 100% oxalate depletion in cecal swabs. One of the two animals also showed 100% oxalate depletion in the rectal swab sample (Figure 3). Quantitative PCR showed an elevated copy number of *O. formigenes oxc* gene in the 5 cultures that showed 100% oxalate depletion. Presence of *O. formigenes* in the oxalate-depleted cultures was confirmed by 16S rDNA sequencing. However, pure colonies of *O. formigenes* were not isolated from the positive cultures by roll tube culture despite numerous attempts. Our data show that *O. formigenes* is present in the intestinal tract of the dogs. It appears that its culture requirements are more stringent than for porcine *O. formigenes*, which was readily culturable using identical condition.

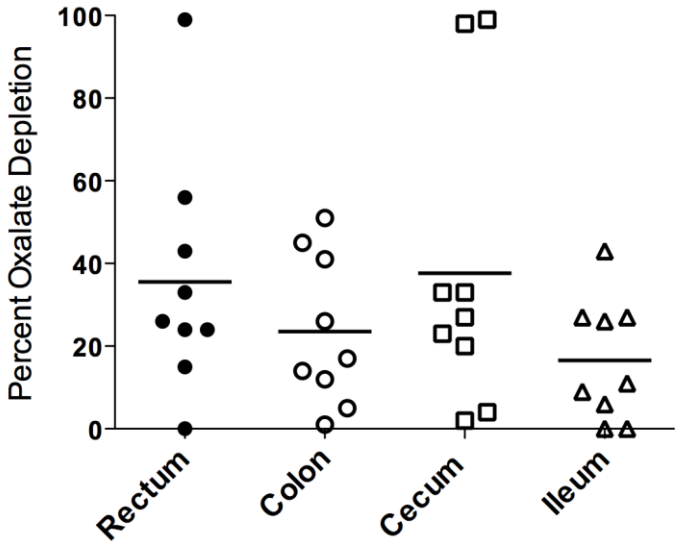
Prevalence of *O. formigenes* and incidence of CaOx uroliths

Prevalence of *O. formigenes* in dogs with CaOx uroliths was 25% (n=20) whereas the prevalence in breed, age and gender-matched clinically healthy dogs was 50% (n=20) (Figure 1). In clinically healthy, non-stone forming large breeds, prevalence of *O. formigenes* was 75% (n=20). The prevalence is higher in non-stone forming healthy large breeds compared to dogs with CaOx uroliths. Although the prevalence of *O. formigenes* was numerically higher in healthy matched-control animals compared to the CaOx dogs, the difference was not statistically significance (Figure 1).

The crude OR between CaOx and healthy matched-control dogs was 3.0 (95% Confidence interval: 0.8 – 11.05). The crude OR between the CaOx dogs and the non-stone forming large breeds was 9.0 (95% confidence interval: 2.21 – 36.59). Based on

Figure 3. Oxalate degrading activity in the intestinal tract.

Gastrointestinal tract contents were collected from euthanized, healthy dogs (n=9), from the following regions: rectum (●), colon (○), cecum (□) and ileum (Δ). The contents were inoculated into oxalate-supplemented anaerobic medium to determine oxalate depletion. Cultures from all four regions showed comparable levels of oxalate depletion, with highest oxalate degrading cultures from cecal and rectal regions. Horizontal line is the mean.



the crude OR, absence of *O. formigenes* enteric colonization increases the likelihood of CaOx stone formation in dogs.

Quantitative PCR results also showed that, among the *O. formigenes* positive samples, mean copy numbers of *oxc* genes were comparable, indicating no difference in colonized *O. formigenes* abundance between groups. There was no significant difference in the oxalate degrading activity between the healthy, matched-controls and urolith dogs (Figure 2). Complete oxalate depletion was only achieved in two fecal samples; one from a matched control and one from non-stone forming large breed. Extensive variation was observed in the oxalate depletion among the fecal cultures, which may indicate the presence of other oxalate-metabolizing bacteria.

Prevalence of *L. acidophilus and B. animalis* in dogs

Presence of *L. acidophilus and B. animalis* in feces was detected by qPCR amplification of *oxc*. In healthy dogs, overall prevalence of *L. acidophilus and B. animalis* was 37.7% and 17.5%, respectively (Figure 4 and 5). Interestingly, none of the CaOx stone formed dogs were colonized with *L. acidophilus and B. animalis* (Figure 4 and 5).

85% of the healthy non-stone forming breed dogs and 75% of healthy stone forming-breed dogs were colonized with at least one of the above bacterium while the same trend was observed only in 25% of CaOx dogs with CaOx stone.

Fecal oxalate-degrading activity was comparable between CaOx stone-formed dogs and healthy controls but a varying degree of oxalate degradation was observed (Figure 6). Further, *L. reuteri, L. acidophilus, B. animalis and B. pseudolongum* were

Figure 4. **Fecal qPCR-based screening for *L. acidophilus*.**

The *oxc* gene of *L. acidophilus* was quantified in CaOx stone dogs (●, n=20), breed-matched negative controls (○, n=20) and negative non-stone forming breeds (Δ, n=20). Horizontal line is the mean.

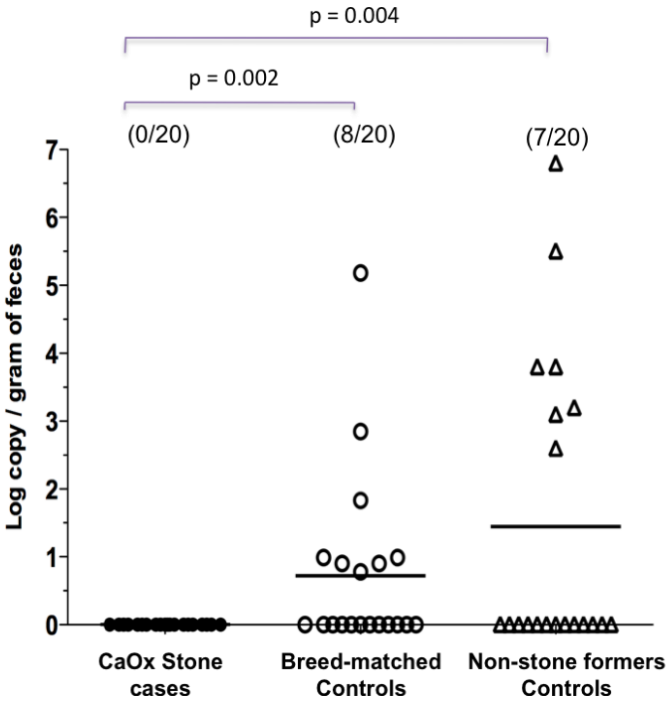


Figure 5. Fecal qPCR-based screening for *B. animalis.*

The *oxc* gene of *B. animalis* was quantified in CaOx stone dogs (●, n=20), breed-matched negative controls (○, n=20) and negative non-stone forming breeds (Δ, n=20). Horizontal line is the mean.

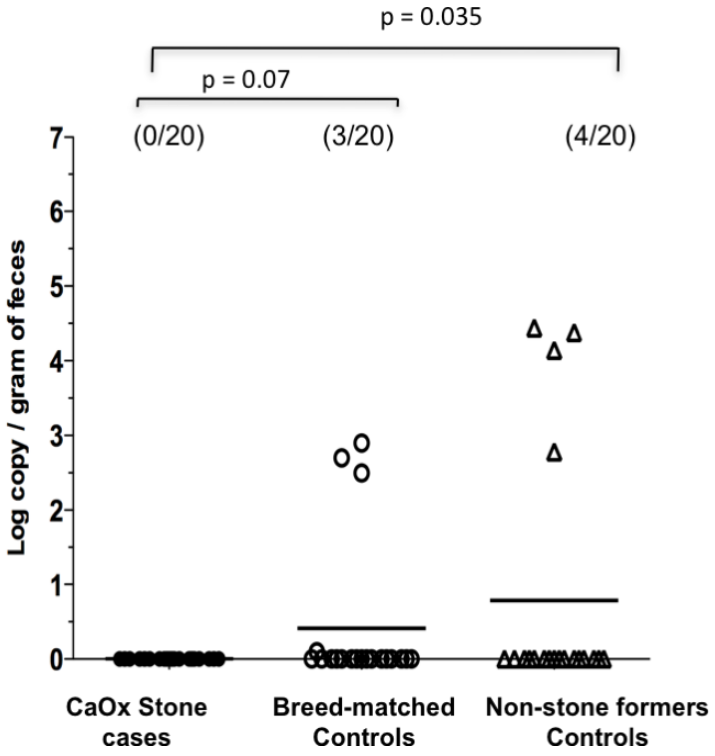
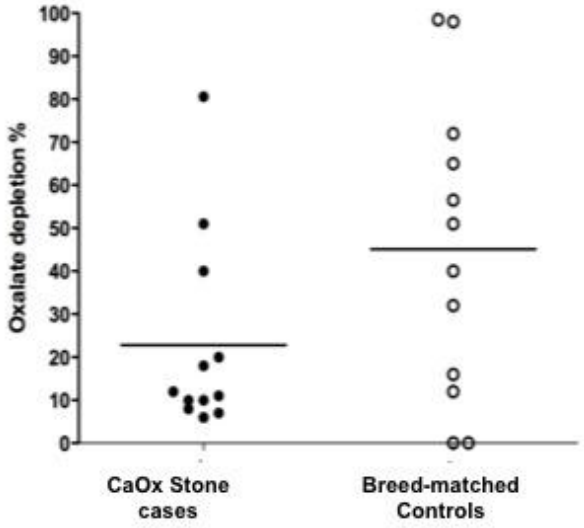


Figure 6. Oxalate-degrading activity in fecal cultures.

Fecal samples collected from CaOx stone dogs (●, n=12), matched control dogs (○, n=12) were cultured in MRS- oxalate-supplemented anaerobic medium for 14 days and oxalate concentrations were analyzed by ion chromatography. Horizontal line is the mean.



isolated as efficient oxalate metabolizers from the fecal samples showed high oxalate degrading activity (Appendix 1).

Discussion

We report here the first association between gut *O. formigenes* colonization and CaOx urolith formation in dogs. Specifically *O. formigenes* is positively associated with healthy dogs, especially in non-stone forming breeds. The differences in the prevalence of *O. formigenes* were significant between CaOx dogs and non-stone forming breed dogs. These findings indicate that absence of gut colonization with *O. formigenes* is a risk factor for CaOx urolithiasis. The overall *oxc* copy numbers were similar and relatively low among all three groups of animals, suggesting a lack of quantitative relationship between the bacterial load and CaOx urolith development.

In humans and other mammals, presence of *O. formigenes* is assessed by both fecal culture and non-culture-based methods. Prevalence of fecal *O. formigenes* in healthy humans is 38% but only 17% of subjects afflicted with CaOx uroliths have *O. formigenes* (101). Although *O. formigenes* mainly colonizes the cecum and colon, fecal samples are considered the best non-invasive clinical sample in live animals. However, a selective media for *O. formigenes* has not been described. Therefore, presence of viable *O. formigenes* is generally detected by the depletion of oxalate ions in an oxalate-supplemented culture medium (7). Based on culture methods, *O. formigenes* in humans is present in a range between $3 \times 10^5 - 3 \times 10^8$ CFU/g of feces, suggesting that 10^5 bacteria per g is the lower detection limit (6). Detection of *Oxc* gene is an indirect way of detecting *O. formigenes* in fecal samples that avoids a stringent anaerobic growth

requirement (176, 197, 199, 200, 202, 242-244). *Oxc* is also present in other oxalate-degrading bacteria, but sequence variations among bacterial genera allowed amplification specifically from *O. formigenes*. The majority of humans who harbor *O. formigenes* had fecal colonization of less than 4×10^4 CFU/gram (176).

Our results show similar levels in positive dogs and demonstrated the reduced sensitivity of culture, as only 2 of 60 were positive. Intra-fecal variation is observed in shedding of *O. formigenes* in humans and dogs (176, 243). However our finding of a 50% prevalence is intermediate between the 37% and 89% rates previously reported in dogs (242, 243). Therefore, we conclude PCR is substantially more sensitive than culture, possibly due to either lack of viable fecal *O. formigenes* or very low levels of *O. formigenes*.

Feces may not be an ideal sample for culture of *O. formigenes,* a strict anaerobe. Therefore, *O. formigenes* culture also was attempted from various areas of the GI tract. In cecal cultures, 22% (2 of 9) of dogs were culture positive for *O. formigenes*. One of the dogs also yielded a positive fecal culture. The findings indicate that cecum, where anaerobic conditions predominate, is a better site from which to obtain viable *O. formigenes*.

The potential use of oxalate-degrading bacteria, particularly *O. formigenes,* as novel therapeutics against CaOx urolithiasis has been explored in humans and laboratory rodents with encouraging outcomes (52, 76, 82, 89, 90, 198). In dogs, such novel therapeutics for CaOx urolithiasis have not been explored, largely due to the lack of preliminary screening studies on gut microflora or oxalate-catabolizing bacteria. The

potential variability among numerous dog breeds also might have contributed to the lack of progress in oxalate-metabolizing bacteria studies. Here, we investigate the risk of CaOx urolithiasis in the absence of *O. formigenes* in the intestinal tract by including dogs with CaOx urolithiasis, matched controls and non-stone forming large breeds. Interestingly, the proportion of dogs positive for fecal *O. formigenes* were significantly higher in non-stone forming large breeds compared to the CaOx urolithiasis dogs. It is reasonable that altered oxalate-metabolizing microbes such as *O. formigenes* provide a mechanism to prevent CaOx stones. While many risk factors, including environment, diet, genetic and metabolic disorders, can contribute to the pathogenesis of CaOx urolithiasis, oxalate-metabolizing microbes should be considered as a factor amenable to therapeutic or dietary manipulation. Apart from *O. formigenes* other oxalate-degrading bacteria including *Bifidobacterium spp and Lactobacillus spp*. in the intestinal tract also can prevent oxalate absorption (2). Among them, *L. acidophilus* and *B. animalis* were the efficient oxalate metabolizers among the species in their respective genus.

In this study, we also screened for the presence of *L. acidophilus* and *B. animalis,* but their prevalence was low (<37.5% compared to *O. formigenes* 50%).

In conclusion, elevated fecal prevalence of *O. formigenes*, *L. acidophilus* and *B. animalis* in healthy stone forming and non-stone forming breed dogs supports a potential preventive role for this oxalate metabolizing bacterium in CaOx urolithiasis. The findings provide a strong rationale to pioneer novel and non-invasive therapeutic approaches to minimize CaOx urolithiasis in dogs by exploiting oxalate-metabolizing bacteria.

CHAPTER III

Comparative fecal microbiota of dogs with CaOx stone and healthy dogs

Manuscript in preparation for Applied Environmental Microbiology

Calcium oxalate urolithiasis (CaOx) is one of the recurrent urinary tract diseases in dogs. Reducing the concentration of calcium and oxalate ions in the urine can minimize the incidence of urolithiasis. Oxalate-metabolizing bacteria act to minimize the enteric absorption of the oxalate ions from the diet, and the intestinal tract of healthy dogs is colonized with microbes that express enzymes to metabolize oxalate. Therefore, the reduced colonization by these oxalate-metabolizing bacteria is a risk factor for CaOx urolithiasis in humans and dogs. However, studies to date have utilized culture-dependent approaches to identify bacteria. We hypothesized that the overall intestinal microbiota of healthy dogs is distinct from that of the dogs affected with CaOx stones, including shifts in oxalate-degrading bacterial species. Fecal samples from healthy (n=5) and CaOx stone dogs (n=5) were obtained and analyzed to obtain a representative composition of the hindgut microbiota. Amplicons of the 16S rDNA V3 hypervariable region were analyzed using pyrosequencing. In total, 1,223 operational taxonomic units (OTUs) were identified at 97% similarity. Only 38% of these OTUs were shared by healthy dogs and dogs with CaOx stones. We also found significant differences in the relative abundance of 36 genera (phylotypes) and 152 OTUs between the two groups of dogs. In conclusion, the fecal microbiota of healthy dogs is distinct from that of dogs affected with CaOx stones.

Introduction

Urolithiasis is one of the common urinary tract diseases in dogs. The incidence of calcium oxalate (CaOx) urolithiasis in dogs has increased steadily over the last two decades. (165). Since medical dissolution of CaOx stones is challenging, CaOx stones are usually removed by surgery and lithotripsy. To prevent recurrent CaOx stone formation it is also necessary to modulate risk factors that favour CaOx urolithiasis. A prerequisite for CaOx urolith formation is over-saturation of urinary calculogenic precursors, oxalate and calcium. Multiple risk factors contribute to the supersaturation of urinary calcium and oxalate ions and stone formation, including diet. Mammals including dogs, except for guinea pig, do not express endogenous oxalate metabolizing enzymes (155). Instead, microbes residing in the intestinal tract express the enzymes required for the metabolism of complex organic compounds that mammals lack (175).

The presence of enteric oxalate-metabolizing bacteria is inversely correlated with incidence of hyperoxaluria or/and CaOx stone formation in humans, lab rodents and dogs (101, 118, 151, 153, 201, 229) and chapter 1 of this thesis). Prior studies in this context were focused on a few bacteria, *Oxalobacter formigenes*, *Bifidobacterium spp* and *Lactobacillus spp*, that are known to efficiently metabolize oxalate in dogs (154, 181, 242, 245). Oxalate-metabolizing bacteria in the gastrointestinal tract minimize freely available oxalate and thus prevent oxalate absorption.

Microbes are continuously under selection pressure due to a variety of factors, including chemical composition of the diet, immune responses due to host-microbe interaction, presence of pathogenic microbes and use of antibiotics (100, 211).

Composition of the diet is one of the key components that determine the microbial diversity and its metabolic capabilities (124, 150, 230, 256). Although microbial metabolic activities do not contribute significantly to the energy requirements of the dog, these microbes are critical to maintain the healthy environment in the gut (124, 223). Microbes with diverse metabolic capabilities colonize the intestine of dogs and many of them express oxalate-metabolizing enzymes. Culture-based approaches do not necessarily capture all of the microbes involved in oxalate-degrading activity. In this study, we hypothesized that the intestinal microbiota of healthy dogs is distinct from that of the dogs with CaOx stones. Recent developments in DNA sequencing technology have greatly enhanced our ability to study microbiomes. In this study we undertook a pilot approach to identify the microbiota of hind-gut based on analysis of the V3 hypervariable region of the bacterial 16S rRNA gene, one of the phylogenetic markers universal to bacterial species, in a small sample set of dogs representing healthy and CaOx stone groups. In total, we identified 1,223 OTUs from 82 genera. We found significant differences in the relative abundance of 36 genera (phylotypes) and 152 operational taxonomic units between the two groups of dogs.

Materials and Methods

Study samples

Fecal samples were collected from healthy dogs (n=5) and dogs with CaOx stones (n=5). Breed, age and gender are listed in Table 1. CaOx stones from dogs were quantitatively analyzed and reported to contain ≥90% CaOx monohydrate and/or CaOx dihydrate. Healthy dogs were scrutinized after survey radiography, urinalyses, and biochemical

Table 1. Description of dogs

ID	Breed	Gender	Age	Disease status
H1_PO7M	Pomeranian	Male	7	Healthy
H2_MS10F	Miniature Schnauzer	Female	10	Healthy
H3_JT11M	Jack Russell Terrier	Male	11	Healthy
H4_ST6F	Shi Tzu	Female	6	Healthy
H5_YT6M	Yorkshire Terrier	Male	6	Healthy
C1_BI7M	Bichon	Male	7	CaOx
C2_WT11M	Welsh Terrier	Male	11	CaOx
C3_MS11M	Miniature Schnauzer	Male	11	CaOx
C4_JT11M	Jack Russell Terrier	Male	11	CaOx
C5_CH10M	Chihuahua	Male	10	CaOx

profiles. Samples were collected from client-owned animals in compliance with an approved University of Minnesota IACUC protocol.

Extraction of DNA and pyrosequencing

Total DNA was extracted from individual fecal samples following previously described methods (104, 150, 260). Microbial diversity of these samples was determined by pyrosequencing of the phylotype marker, amplicons spanning the V3 hypervariable region of 16S rRNA region (150, 156). Fusion primers composed of adaptor sequences followed by a unique molecular identifier (barcode) and target-specific primers were used to amplify this region (170). Adapter sequences at the 5' end of the forward and reverse fusion primers were 5' GCCTCCCTCGCGCCATCAG 3' and 5' GCCTTGCCAGCCCGCTCAG 3' respectively (454 Life Sciences, Branford, CT). V3 region-specific primer sequences were: forward 5' CCTACGGGAGGCAGCAG 3' (341F) and reverse 5' ATTACCGCGGCTGCTGG 3' (534 R') (150, 156). V3 amplicons were generated by PCR using 50 ng of fecal DNA as template in 50 μl reaction containing the following: High fidelity *Taq* polymerase (25 ng/μl) (Roche Applied Science, Indianapolis, IN), fast start high fidelity buffer with $MgCl_2$ (1.8 mM) (Roche Applied Science, Indianapolis, IN), dNTP (200 μM) (Roche Applied Science, Indianapolis, IN), and primers (0.4 μM each) (Integrated DNA Technologies, Coralville, IA). Amplification was carried out with activation at 95° C for 2 minutes, followed by 20 cycles of denaturing at 95° C for 30 seconds, annealing at 60° C for 30 seconds and extension at 72° C for 30 seconds and final elongation at 72° for 7 minutes (150). Amplicons were resolved by agarose gel electrophoresis and visualized by ethidium

bromide staining. The resolved DNA bands of appropriate size in agarose gels were extracted and purified using Qiagen PCR clean up kit (Qiagen, Valencia, CA). Purified amplicons were analyzed and quantified by Agilent 2100 bioanalyzer using DNA1000 kit (Agilent Technologies, Waldbronn, Germany). Equal amount of amplicons (5 ng/μl) from each samples were pooled. In total, 50 ng of V3 amplicons were sequenced on the 454-Genome Sequencer FLX sequencer by employing GS-FLX chemistry (454 Life Sciences, Branford, CT) at the Advanced Genetic Analysis Centre, University of Minnesota.

Data analysis

Assigning operational taxonomic units

An in-house program was used to trim and sort the sequences (Minnesota Supercomputing Institute, University of Minnesota). Mothur 1.17.0 was used to quality-trim and analyze the filtered sequences using the Galaxy web interface at MSI, University of Minnesota, unless stated otherwise (22, 66, 190-192). Unique sequences from each sample were selected and aligned using the Needleman-Wunsch algorithm and Silva 16S rRNA non-redundant database as template. Uncorrected pairwise distance matrices were calculated between aligned sequences with a 0.031 cut off level. Sequences with ≥97% identity were clustered to generate Operational Taxonomic Units (OTUs). Representative sequences (most closely related to other sequences in the cluster) for each OTU were generated for analysis. Richness and diversity indices were calculated using the entire sequence dataset. OTU sequences were classified using naïve Bayesian RDP classifier using the RDP database to assign the nearest matched genus at 50% confidence threshold

(240). Differential relative abundances of OTU and phylotypes between healthy and CaOx samples were statistically analyzed by web-based statistical tool http://metastats.cbcb.umd.edu/ (non-parametric t-test) and $p < 0.05$ (false positive) and $q < 0.05$ (false discovery rate) were considered significant (247).

UniFrac analyses

Individuals were compared based on the bacterial community composition (Beta diversity) using an unweighted UniFrac distance metric. Unique sequences from all the samples were selected by de-replicating sequences using the Tornado tool (http://tornado.igb.uiuc.edu/) and aligned by CLUSTALW and distance matrix was created (208, 226). Based on the distance information single rooted tree was built using "Neighbour" command (59). Unique sequence frequency and tree data were used to generate the UniFrac metric (http://bmf.colorado.edu/fastunifrac/). Principal coordinate analysis (PCoA) (unweighted) was performed using the UniFrac metric (74, 129, 130).

Results

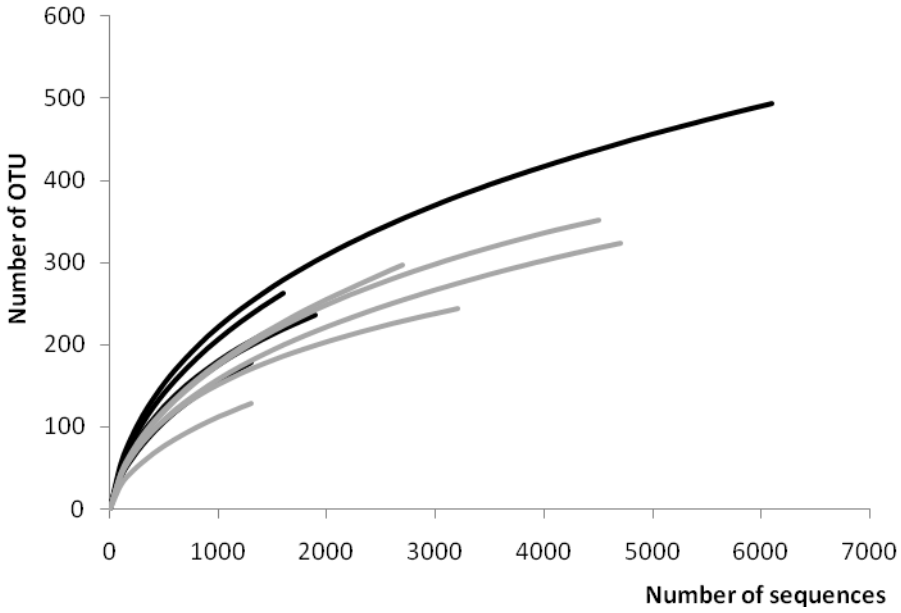
In total 47,531 reads were obtained with an average quality score of 30. Among the 47,531 sequences, 61% (29,239) were qualified for further analysis and the average length of a sequence was 140 bp. In total 12,671 (1313 – 6128 per sample) and 16,568 (1364 – 4747 per sample) sequences were analyzed from healthy and CaOx dogs, respectively. Bacterial diversity in each sample was evaluated as OTUs at 97% similarity and 1,223 OTU were identified amongst the two groups. Table 2 summarizes the number of the OTUs obtained in each sample. Rarefaction curves illustrate the comparative species richness among the samples (Figure 1).

Table 2. Summary of number of sequences obtained from healthy (H1-5) and CaOx dogs (C1-5)

ID	Total Sequences	Qualified Sequences	OTU
H1_PO7M	2269	1313	180
H2_MS10F	2593	1609	264
H3_JT11M	3384	1914	237
H4_ST6F	2747	1707	232
H5_YT6M	10029	6128	494
C1_BI7M	7595	4532	353
C2_WT11M	7090	4747	325
C3_MS11M	4367	2710	298
C4_JT11M	4967	3215	244
C5_CH10M	2490	1364	132
Healthy Pooled	21,022	12,671	843
CaOX Pooled	26,509	16,568	843

Figure 1. Rarefaction analysis of sequences

Rarefaction analysis based on analyzed sequences from each sample. Operational Taxonomic units were generated at 97% similarity. Each line represents a single sample. Black and gray line represents healthy and CaOx dogs respectively.



Diversity indices were evaluated to compare the richness, evenness and proportion of the bacterial species among samples. Estimated Chao and ACE diversity indices are summarized in Table 3. The Shannon and Simpson evenness indices for all samples are shown in Table 3. Diversity and evenness indices were comparable between the two groups of dogs.

According to the pooled sample analysis, 38% (463) of the OTUs were shared by both healthy and CaOx stone dogs and 380 OTUs were unique to each group. Based on individual samples only 2% (24 OTUs) and 0.6% (8 OTUs) of OTUs were shared (core) among dogs in the healthy and CaOx stone groups, respectively.

Based on differential relative abundance (metastatistic) analysis of individual samples, relative abundance of 152 OTUs was significantly ($p < 0.05$ and $q < 0.05$) different between two groups while 51 and 28 OTUs were unique to healthy and CaOx stone dogs respectively. Relative abundance of 39 OTUs was comparatively higher in healthy dogs than in CaOx stone dogs whereas 34 OTUs were comparatively higher in CaOx stone dogs than in healthy dogs.

Distribution of the phylotypes

OTUs were phylotyped using an RDP classifier at 50% bootstrap confidence threshold. Relative abundance of the phyla in pooled samples and individual samples are shown in Figure 2. Firmicutes were the predominant bacterial phylum in dogs regardless of health status. The relative abundance of bacterial species in the phylum Firmicutes ranged from 40 to 86%. Relative abundance of the phylum Bacteroidetes in the samples ranged from 0.1 to 21%. OTUs with similarity to the bacteria in the phylum Fusobacteria

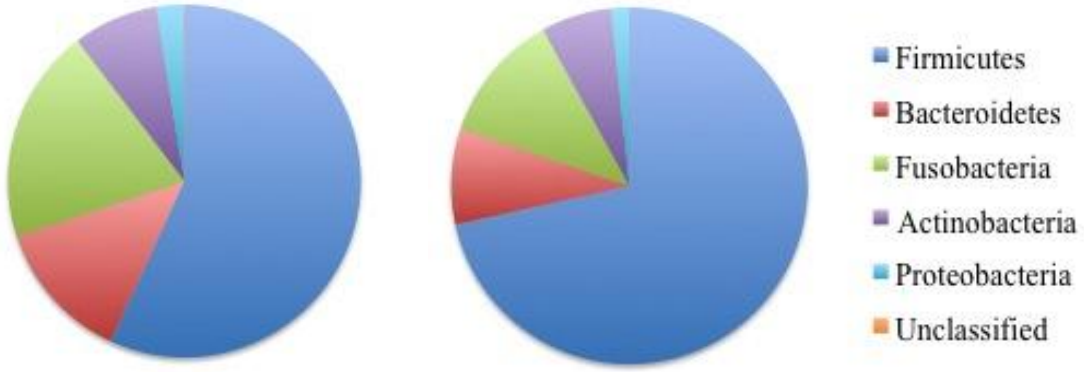
Table 3. Diversity indices of sequences obtained from healthy (H1-5) and CaOx dogs (C1-5)

ID	Shannon	Simpson	Ace	Chao
H1_PO7M	3.7 (3.6 - 3.8)	0.94	408 (350 – 486)	295 (246 - 380)
H2_MS10F	4.3 (4.2 - 4.4)	0.96	636 (560 - 732)	464 (389 - 584)
H3_JT11M	4.0 (3.9 - 4.1)	0.95	443 (391 - 511)	363 (312 – 447)
H4_ST6F	4.0 (3.9 - 4.1)	0.95	360 (317 – 427)	364 (311 – 454)
H5_YT6M	4.6 (4.6 - 4.7)	0.98	861 (792 – 945)	779 (688 – 913)
C1_BI7M	3.9 (3.8 - 3.9)	0.93	512 (464 – 580)	488 (440 – 563)
C2_WT11M	3.9 (3.9 - 4.0)	0.95	579 (522 – 652)	477 (422 – 565)
C3_MS11M	3.9 (3.8 - 4.0)	0.93	767 (676 – 879)	608 (494 – 791)
C4_JT11M	3.8 (3.7 - 3.8)	0.93	346 (310 – 401)	350 (305 – 427)
C5_CH10M	3.2 (3.1 - 3.3)	0.92	325 (272 – 400)	230 (183 – 318)
Healthy Pooled	5.06 (5.03- 5.09)	0.98	1194(1119–1289)	1252 (1144-1397)
CaOX Pooled	4.8 (4.77 - 4.86)	0.97	1184 (1111-1277)	1218 (1118-1352)

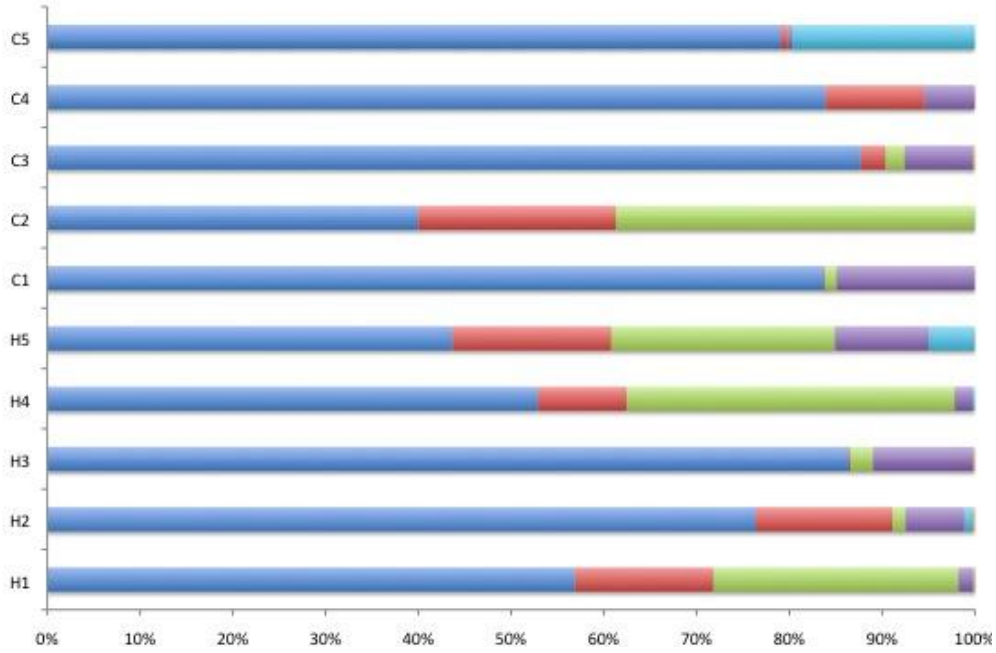
Figure 2. Distribution of phyla identified based on V3 region of 16S rDNA phylotyping

The comparative relative abundance of phyla in A) pooled healthy, B) pooled CaOx samples and C) individual samples (H1-H5 healthy dogs and C1-C5 CaOx dogs).

A) B)



C)



were detected only in 80% (8 out of 10) of the dogs and the relative abundance ranged from 1.3% to 39%. Phylum Actinobacteria accounted for 0.6 to 37% of the fecal bacterial community. Relative abundance of phylum Proteobacteria was very low and ranged from 0 to 16.8%. Additionally, five OTUs were identified as bacteria but were not classified.

Relative abundances of Bacteroidetes, Fusobacteria and Actinobacteria were significantly higher in healthy dogs compared to dogs with CaOx stones ($p < 0.05$). Relative abundance of Firmicutes was significantly higher in CaOx stone dogs compared to healthy dogs ($p < 0.05$).

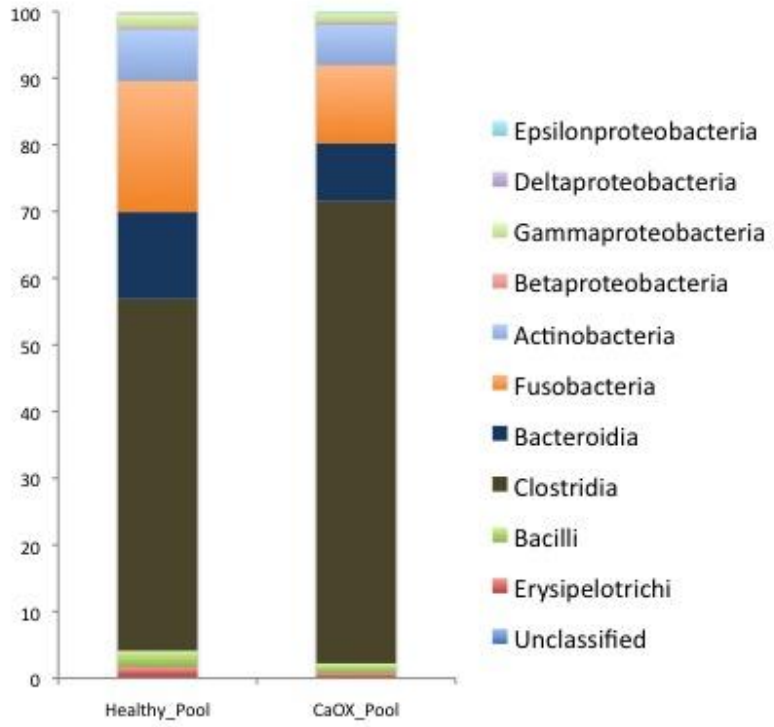
The distribution of bacterial classes is shown in Figure 3. Regardless of health status, fecal microbiota of dogs was dominated by the Class Clostridia. Relative abundance of classes Betaproteobacteria, Fusobacteria, Bacteroidia, Actinobacteria, and Deltaproteobacteria were significantly higher in healthy dogs compared to dogs with CaOx stones. Relative abundance of classes Clostridia and Bacilli are significantly higher in dogs with CaOx stones compared to healthy dogs.

Only 30% (370) of OTUs were classified to the genus level at 50% confidence by the RDP taxon classifier. Therefore during the genus level analysis, sequences were assigned to their closest-matched genus with a less stringent threshold (< 50%) to phylotype all the identified OTUs. OTUs were assigned to 82 phylotypes at the genus level. Identified genera are listed in Table 4. The most abundant genus was *Blautia* (~20%) followed by *Sporacetigenium* (~14%) and *Fusobacterium* (~11%). Relative abundance of 36 phylotypes were found to be significantly different between healthy and CaOx stone dogs (Table 4).

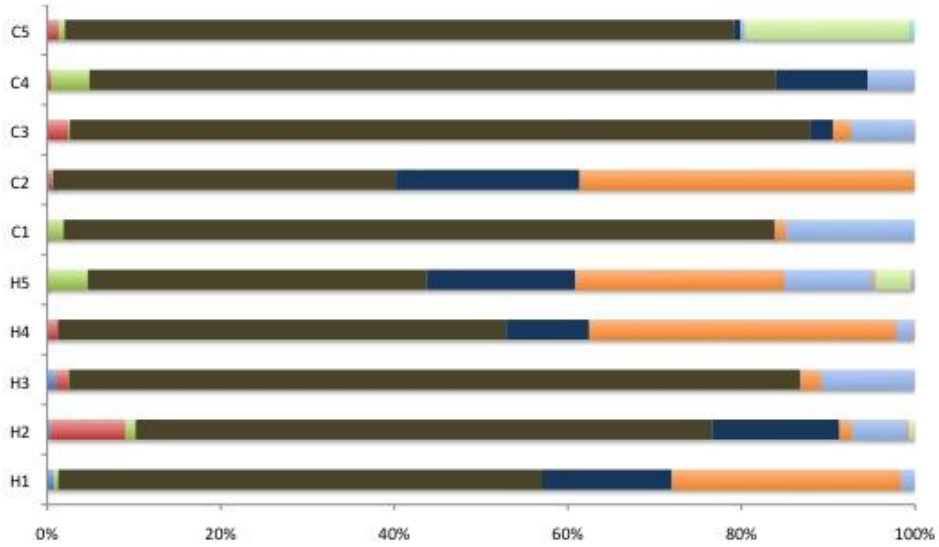
Figure 3. Relative abundance of bacterial classes in healthy and CaOx dogs

Relative abundance of bacterial classes identified. Comparative relative abundance of bacterial class in A) pooled and B) individual samples.

A)



B)



Genera *Megasphaera, Parabacteroides, Peptococcus, Solobacterium, Desulfovibrio, Catonella* and *Sporobacterium* were only found in healthy dogs. *Turicibacter and Lactobacillus* were found only in dogs with CaOx stone (Table 4). The relative abundance of 14 genera was significantly higher in healthy dogs compared to dogs with CaOx stones. The relative abundance of 13 genera was significantly higher in dogs with CaOx stones compared to healthy dogs (Table 4).

Clustering patterns of the individual samples were analyzed by UniFrac, based on the fraction of the branch length that is shared by the individual samples on a common phylogenetic tree constructed from all 16S rRNA sequences. Principal coordinate analysis (PCoA), based on UniFrac distance matrices, revealed that healthy dogs were distinctly clustered together, with the first and third principal coordinates (PC1 and PC3) accounting for 19.0% and 16.3 % inter-sample variances, respectively (Fig 4). In the PCoA analysis, dogs with CaOx stones were dispersed with no distinct pattern.

Discussion

This is the first study to our knowledge that undertook a culture-independent approach to understand gut microbial community diversity in healthy and CaOx dogs and to identify alternative oxalate-metabolizing bacteria. In this study, sequences of V3 regions of 16S rDNA with 97% similarity were binned into 1,223 OTUs, allowing us to analyze in detail the fecal microbiome differences between healthy versus CaOx stone-producing dogs. Rarefaction curves indicate that sampling completeness was only modest, but still provided enough data to effectively analyze higher-abundance sequences. Chao and ACE revealed that the estimated diversity indices overlap between

Table 4. Relative abundances of genera found in healthy and CaOx stone formed dog

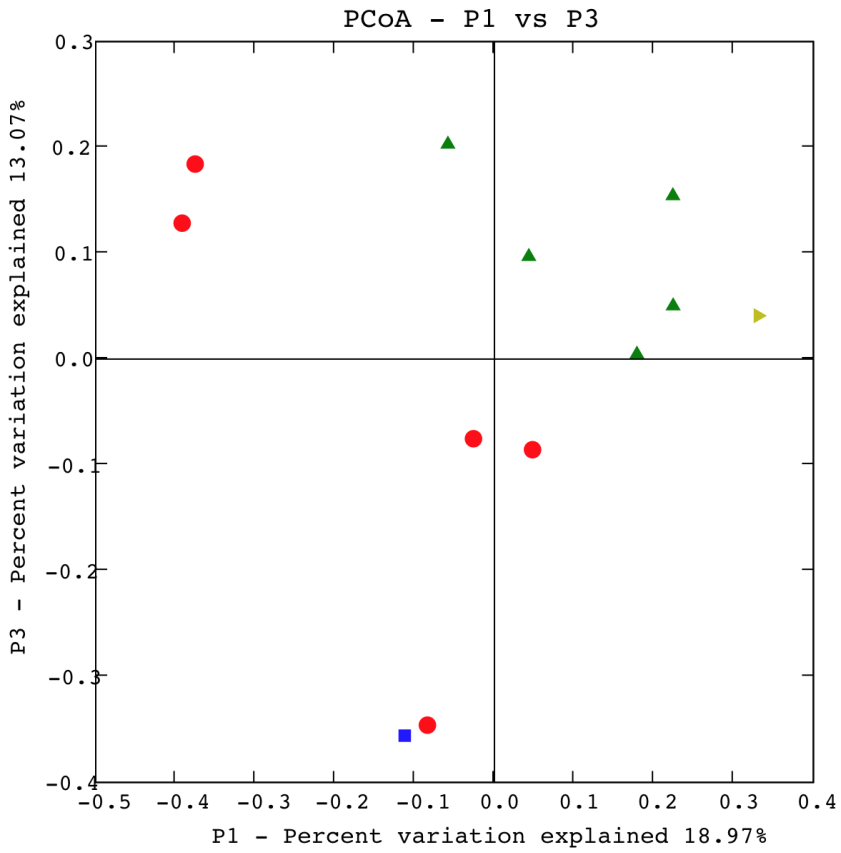
Name	Mean Relative Abundance %		p value	q value
Genus	**Healthy**	**CaOx**		
Megasphaera**	0.337	0.000	**8.20E-14**	**6.19E-13**
Parabacteroides**	0.325	0.000	**5.98E-35**	**1.03E-33**
Peptococcus**	0.249	0.000	**5.41E-08**	**2.72E-07**
Solobacterium**	0.199	0.000	**1.54E-06**	**6.89E-06**
Desulfovibrio**	0.107	0.000	**1.25E-11**	**8.39E-11**
Catonella**	0.076	0.000	**1.24E-03**	**4.55E-03**
Sporobacterium**	0.023	0.000	**2.87E-03**	**9.63E-03**
Fusobacterium*#	15.226	8.287	**4.32E-14**	**3.73E-13**
Bacteroides*#	7.606	6.439	**2.93E-11**	**1.77E-10**
Collinsella*#	5.918	4.222	**1.63E-18**	**1.65E-17**
Megamonas*#	3.595	1.943	**1.39E-08**	**7.66E-08**
Psychrilyobacter*#	2.558	0.080	**8.44E-205**	**1.02E-202**
Prevotella*#	2.111	0.205	**3.12E-35**	**6.29E-34**
Catenibacterium*#	1.504	0.376	**1.06E-12**	**7.57E-12**
Streptococcus*#	1.235	0.054	**1.20E-99**	**7.27E-98**
Hallella*#	0.743	0.007	**1.01E-21**	**1.22E-20**
Eubacterium*#	0.571	0.161	**2.03E-03**	**7.00E-03**
Coprobacillus*#	0.549	0.213	**1.17E-03**	**4.41E-03**
Subdoligranulum*#	0.465	0.088	**1.01E-05**	**4.36E-05**
Tannerella*#	0.379	0.360	**3.21E-05**	**1.34E-04**
Sutterella*#	0.190	0.007	**2.00E-11**	**1.27E-10**
Turicibacter*$	0.000	0.264	**1.68E-07**	**8.15E-07**
Lactobacillus*$	0.000	0.092	**1.82E-03**	**6.49E-03**
Blautia*Δ	20.945	24.753	**1.66E-59**	**5.02E-58**
Coprococcus*Δ	8.094	9.770	**4.72E-18**	**4.39E-17**
Clostridium*Δ	2.577	6.731	**5.87E-14**	**4.73E-13**
Hespellia*Δ	3.049	4.066	**1.95E-09**	**1.12E-08**
Dorea*Δ	2.222	3.842	**2.72E-31**	**4.12E-30**
Anaerobacter*Δ	0.015	3.522	**1.07E-60**	**4.33E-59**
Bifidobacterium*Δ	0.078	1.243	**2.82E-39**	**6.83E-38**
Enterococcus*Δ	0.010	0.837	**9.73E-31**	**1.31E-29**
Lactococcus*Δ	0.003	0.352	**1.24E-18**	**1.36E-17**
Fastidiosipila*Δ	0.012	0.234	**2.91E-08**	**1.53E-07**
Acetitomaculum*Δ	0.012	0.217	**5.98E-07**	**2.78E-06**
Alkaliphilus*Δ	0.003	0.142	**6.30E-04**	**2.46E-03**
Anaerostipes*Δ	0.010	0.137	**5.87E-05**	**2.37E-04**
Escherichia/Shigella	0.899	3.783	5.19E-03	1.70E-02
Reichenbachiella	0.071	0.000	6.62E-03	2.11E-02
Cetobacterium	0.154	0.025	8.24E-03	2.55E-02
Anaerotruncus	0.016	0.000	1.53E-02	4.62E-02
Allobaculum	0.015	0.080	1.68E-02	4.96E-02
Campylobacter	0.000	0.103	2.16E-02	6.22E-02
Syntrophococcus	0.111	0.058	2.37E-02	6.67E-02

Oribacterium	0.130	0.233	3.60E-02	9.89E-02
Propionigenium	0.029	0.013	3.84E-02	1.03E-01
Roseburia	0.706	0.723	6.86E-02	1.80E-01
Acetanaerobacterium	0.020	0.015	8.44E-02	2.17E-01
Oscillibacter	0.057	0.025	8.98E-02	2.26E-01
Weissella	0.025	0.099	9.46E-02	2.33E-01
Sarcina	0.035	0.087	1.12E-01	2.72E-01
Butyricicoccus	0.102	0.051	1.75E-01	4.15E-01
Asaccharobacter	0.025	0.000	1.88E-01	4.20E-01
Dialister	0.025	0.000	1.88E-01	4.20E-01
Succinivibrio	0.025	0.000	1.88E-01	4.20E-01
Helicobacter	0.000	0.026	2.63E-01	5.79E-01
Paraprevotella	0.111	0.071	4.11E-01	8.32E-01
Allisonella	0.012	0.000	4.33E-01	8.32E-01
Klebsiella	0.003	0.000	4.33E-01	8.32E-01
Lawsonia	0.003	0.000	4.33E-01	8.32E-01
Melissococcus	0.003	0.000	4.33E-01	8.32E-01
Nubsella	0.012	0.000	4.33E-01	8.32E-01
Phascolarctobacterium	0.012	0.000	4.33E-01	8.32E-01
Proteiniphilum	0.012	0.000	4.33E-01	8.32E-01
Anaerofustis	0.054	0.161	4.51E-01	8.52E-01
Howardella	0.000	0.015	5.09E-01	9.32E-01
Sedimentibacter	0.000	0.009	5.09E-01	9.32E-01
Centipeda	0.025	0.007	5.83E-01	1.00E+00
Faecalibacterium	1.156	1.244	6.35E-01	1.00E+00
Parasporobacterium	0.050	0.027	7.34E-01	1.00E+00
Sporacetigenium	14.927	14.178	8.02E-01	1.00E+00
Slackia	0.122	0.148	1.00E+00	1.00E+00
Acinetobacter	0.007	0.026	1.00E+00	1.00E+00
Turicella	0.000	0.015	1.00E+00	1.00E+00
Finegoldia	0.000	0.015	1.00E+00	1.00E+00
Jonquetella	0.007	0.022	1.00E+00	1.00E+00
Lactonifactor	0.000	0.015	1.00E+00	1.00E+00
Leuconostoc	0.010	0.004	1.00E+00	1.00E+00
Peptoniphilus	0.000	0.015	1.00E+00	1.00E+00
Proteocatella	0.030	0.037	1.00E+00	1.00E+00
Pseudobutyrivibrio	0.000	0.004	1.00E+00	1.00E+00
Raoultella	0.000	0.015	1.00E+00	1.00E+00
Robinsoniella	0.000	0.007	1.00E+00	1.00E+00

*Relative abundances are significantly different between two groups (q <0.01)
Among the significantly different genera:
** Present only in Healthy dogs.
*$ Present only in CaOx dogs.
*# Relative abundance is high in healthy dogs compared to CaOx stone formed dogs.
*Δ Relative abundance is high in dogs with CaOx stone formed dogs compared to healthy dogs.

Figure 4. Principal coordinate analysis on un-weighted UniFrac distance metric

Principal coordinate analysis on un-weighted UniFrac distance metric based on presence and absence of unique V3 region in samples obtained from healthy (green triangles) and CaOx dogs (red dots). Healthy samples were clustered together with their group mates along with PC1 at 19.0% and PC3 13.1%. Dogs with CaOx stones were dispersed. We also included the pooled samples (healthy pooled –yellow triangle, CaOx pooled -blue square) to observe the clustering trend.



the healthy and CaOx groups. The evenness of species was comparable between the two groups. Relative abundances of the 152 OTUs (candidate OTUs) were significantly different between the groups. Significant differences were observed in relative abundance at phylum and class level identification of the bacterial species. Nevertheless, it is essential to identify the bacteria at the species level to further explore potentially oxalate-metabolizing bacteria. Identified OTUs, when grouped at a similarity comparable to the genus level, resulted in 36 genera that were significantly different between the two dog groups.

In this study we expected less diversity in microbiota among individuals within healthy or CaOx groups. While 38% of the OTUs were shared (core microbiota) among the pooled samples representing each group, only ~2% of OTUs were shared (core microbiota) by all the individuals within each group. Despite the low level of core microbiota, all the healthy samples were found clustered together in PCoA analysis but dogs with CaOx stones were dispersed without a distinct pattern.

Apart from breed variation, we also expect that diet and environmental factors may have contributed to the significant diversity in microbiota among dogs (124). Recent studies on the canine gut microbiome revealed that fiber-based diet significantly altered the microbial diversity (150). The dogs used in this study were client-owned and therefore the factors like diet and environment were highly variable. Dietary oxalate likely is the major exogenous source of oxalate that could lead to hyperoxaluria in dogs.

Past studies in humans have established that the oxalate-metabolizing bacteria play a vital role in controlling the enteric oxalate absorption by limiting the availability of

the oxalate ion in the gut (7, 89, 90, 198, 203). Oxalate decarboxylase (OXDC), oxalyl CoA decarboxylase (OXC) and oxalate oxidoreductase (OOR) are the three oxalate-metabolizing enzymes that have been identified in bacteria (174, 178, 224). Several culture-dependent and independent methods have been used to identify specific oxalate-metabolizing bacteria such as *Oxalobacter formigenes*, *Bifidobacterium spp*, *Lactobacillus spp, Enterococcus faecalis, Eubacterium lentum, Providencia rettgeri*, *Bacillus subtilis, Moorella thermoacetica* and *E. coli* in the gastro-intestinal tract of mammals (2, 13, 45, 58, 84, 85, 174, 224, 232, 242, 245). Currently available genome sequences indicate that a wide variety of bacterial species harbor *oxdC* and *oxc* genes. We intended to determine whether the observed differences in the microbial diversity could provide insights into oxalate-metabolizing bacteria in healthy and CaOx dogs. We found the following potential oxalate-metabolizing bacteria; *Bifidobacterium, Leuconostoc, Lactobacillus, Lactococcus, Enterococcus, E. coli, Shigella, Clostridium, Streptococcus* and *Desulfovibrio.*

In healthy dogs the relative abundance of OTUs with similarity to *Desulfovibrio, Eubacterium and Streptococcus* was significantly higher compared to dogs with CaOx stones. Interestingly, relative abundance of OTUs with similarity to *Bifidobacterium, Lactobacillus, Enterococcus, E. coli, Shigella and Clostridium* was higher in dogs with CaOx stones compared to healthy dogs. The above observation needs to be interpreted cautiously as all the species or strains in certain genera cannot metabolize oxalate (154, 245). Therefore, systematic metagenomic studies are essential to understand the potential oxalate-metabolic activity of the microbial community in the gut. It is worth mentioning

that a high number of sequences from *Bifidobacterium spp*, and *Lactobacillus spp* were observed from one dog in the CaOx group. Although dogs that were on a probiotic diet were excluded from our study, the aforementioned observation leads us to suspect that this particular dog was likely on a probiotic diet or comparable diet.

It is also interesting to note that *O. formigenes*, the bacterium known to be an efficient oxalate metabolizer, was not identified in this study. But, it has already been reported that *O. formigenes* is not abundantly present in dogs, thus likely too rare to be sequenced in our dataset (242).

Another salient finding is that sequences of phylum Fusobacteria were found more frequently in healthy dogs. Bacteria in this group were not evaluated for lower rank diversity due to lack of taxon data within phylum. Fusobacteria were less abundant in dogs with CaOx stones except in one dog . The most extensively studied Fusobacterium species are pathogenic strains. Studies in the past indicate that Fusobacteria are not abundant in other animal species (219).

In a previous study, Middlebos *et al* found that the relative abundances of phylum Fusobacterium and phylum Firmicutes were reduced to 14.5% from 23.38% and increased to 28% from 15%, respectively, when the dogs were switched to a beet pulp-based diet from control diet (150). Beet pulp is known to contain high levels of oxalate and it is plausible that the gut microbial composition of dogs on the beet pulp diet adapted to metabolize oxalate. Their result resembles the phylum patterns of dogs with CaOx stones in our study. Based on this information, it is possible that dogs with CaOx

stones in our study might have consumed a diet with oxalate, which could have altered the enteric microbiota.

Although the present study shed light on the global changes in gut microbial community in CaOx dogs, there are some limitations that restrain complete interpretation of the findings. The major limitation is that the samples were not collected from animals that were matched for age, gender or breed, all factors that could have a significant impact on gut microbial flora. Since the study subjects were client-owned dogs, the oxalate content of their diet was not available. Samples were collected from the CaOx stone-formed dogs but oxalate concentration in the urine was not known to confirm the hyperoxaluric phenotype. Due to a preference for non-invasive sample collection, a limitation was that we studied microbial communities in fecal samples as representative of the gut microbial flora, although significant difference between gut mucosal, luminal intestinal and fecal microbial communities has been observed in dogs and humans (149, 221).

Apart from oxalate-metabolizing bacteria, on the overall distribution of phylotypes provides more insight to the enteric microbiota of the small breed dogs in non-research housing. We found that Firmicutes were the predominant phylum in the dog intestinal tract. Middlebos *et al* reported the co-dominance of phyla Firmicutes, Bacteroides and Fusobacteria in the dog fecal microbiota (150). Bacterial metagenomic analysis on the same set of samples used by Middlebos *et al* revealed that Bacteroides and Firmicutes were the dominant phyla (150). These different results obtained in the same study indicate that the methods employed also contribute to variability in the

outcome (223). It is worth noting that the primers used to amplify the V3 region of 16S rDNA in our study and by Middlebos *et al* were the same (150). Breed differences may have significantly contributed to the observed differences in phyla abundance in these two studies. Middlebos *et al* analyzed fecal samples obtained from 1.7 year-old hound x female litter-mate dogs that were housed under controlled environments, including diet (223), whereas our samples were collected from client-owned, small breeds with variable diets. As expected, we found variation in abundance at different levels of taxonomy among breeds and between individuals. In contrast to previous studies (27-34%), Bacteroidetes were not a highly abundant phylum (<21%) in small breeds (150). A large-scale comparative microbiota study also reported that Bacteroidetes were less abundant in carnivores (124). Abundance of Proteobacteria was very low in small breeds, compared to large breeds (54). Previous studies reported that the relative abundance of Proteobacteria in dogs was 47% in jejunal samples (220). Abundance of Proteobacteria was reported to be low in fecal microbiota (representative of the hindgut microbiota) of dogs and humans. Strict anaerobic environment in the hindgut is not favourable for many of the facultative anaerobic bacteria in the phylum Proteobacteria.

Prior studies in human gut microbiota have clearly shown that intestinal microbiota is highly diverse and variable within individuals of same species (54). Interestingly, human microbiome studies observed that bacterial genes ("core microbiome") are shared significantly among individuals with diversified microbiota (115). Observations of a stable microbiome, which might reflect the function of the gut environment, lead us to speculate that a core microbiome is identifiable within dog breeds

despite the absence of a shared microbial community between breeds. It is likely that low-abundance bacterial species also significantly contribute to gut metabolism. Oxalate metabolism in *O. formigenes* is one good example in which ~20% of the expressed proteins are enzymes involved in oxalate metabolism. We hypothesize that bacterial genes that encode enzymes involved in oxalate metabolism are differentially expressed in dogs affected with CaOx stones. Comparative analysis of fecal transcripts ("Meta-transcriptome") would enable us to test this hypothesis (69). Information on metabolic activity of the intestinal microbial species would enable us to rationalise the co-existence of the bacterial species, responses to environmental changes and host-microbe interaction. It is a well-known fact that multiple risk factors contribute to CaOx stone formation in dogs. Therefore exploring the microbial diversity in breeds that are not at risk will provide more insights on the role of oxalate metabolizing bacteria in CaOx urolithiasis.

CHAPTER IV

Oxalate degrading activity by an engineered probiotic *E. coli* Nissle 1917

In humans, enteric oxalate-metabolizing bacteria minimize the absorption of oxalate. Thus, oxalate-metabolizing probiotics have been used to prevent CaOx stone formation. However, in the absence of oxalate, stable enteric colonization of these probiotic bacteria was not successful. Limitations of the existing oxalate-metabolizing probiotics warranted studies aimed towards colonizable and efficient oxalate metabolizing probiotic bacteria. Here, we hypothesized that the probiotic strain *E. coli* Nissle 1917 (EcN) expressing heterologous oxalate-metabolizing enzymes will degrade oxalate. The bacterium was engineered to metabolize oxalate by ectopic plasmid expression of the *oxc*, *frc* and *OxlT* genes of *O. formigenes* and *oxdC* gene of *B. subtilis*. Engineered EcN strains that expressed OXDC of *B. subtilis* metabolized oxalate *in vitro*. In the absence of the expression of OxlT of *O. formigenes*, engineered EcN failed to metabolize oxalate. This result demonstrates the feasibility of a promising oxalate metabolizing probiotic candidate.

Introduction

Calcium oxalate (CaOx) urinary stones are one of the most common types of urinary stones causing clinical manifestations in dogs. Reducing the urinary concentrations of stone components is essential to avoid reformation of CaOx stones in affected dogs. Enteric oxalate-metabolizing commensal bacteria limit absorption of dietary oxalate (198). For example, presence of specific oxalate-metabolizing bacteria in the gastro-intestinal tract reduced the risk of CaOx stone formation by 70% in humans (101). Also, oxalate metabolizing probiotic therapy significantly reduced the dietary oxalate absorption in humans and rats (27, 96, 198). In this context, *Oxalobacter formigenes*, a commensal bacterium that solely relies on oxalate for its energy generation, has been evaluated most often. Stable colonization of viable probiotic *O. formigenes* is difficult to achieve in the absence of oxalate within the intestinal tract (42, 67, 96). Oxalate-metabolizing lactic acid bacterial probiotic has also been used in humans and mice although consistent reduction in urinary oxalate levels was not observed among different studies (27, 68, 125, 126). Under *in vitro* culture conditions, expression of the oxalate-metabolizing enzymes in *Lactobacillus acidophilus, L. gasseri* and *Bifidobacterium animalis* is induced by acidic pH (13, 123, 231). Further, successful induction of *oxc* and *frc* genes was observed only in strains that were pre-adapted in oxalate-supplement medium (13, 231). Therefore, oxalate-metabolizing activity of the lactic acid probiotics *in vivo* is uncertain. Limitations in the available oxalate-metabolizing probiotic strains prompted us to develop an oxalate-metabolizing probiotic bacterium to express essential enzymes in an oxalate-independent mode. In this study, we

hypothesized that the probiotic strain *E. coli* Nissle 1917 (EcN) expressing heterologous oxalate-metabolizing enzymes would degrade oxalate.

OXC, FRC and OxlT are the essential proteins involved in oxalate metabolism of *O. formigenes*. In this study, we evaluated co-expression of OXC, FRC and OxlT in EcN. The *oxc*, *frc* and *OxlT* genes of *O. formigenes* are not part of a polycistronic operon. Therefore all the above genes were constructed as part of an artificial polycistronic operon within an expression plasmid to co-express OXC, FRC and OxIT.

By contrast, oxalate decarboxylase (OXDC) of *B. subtilis* by itself catalyzes oxalate to formate and CO_2 using manganese as a co-factor in the presence of O_2. OXDC is expressed in the cytosol and is present in the spore coat of *B. subtilis.* It is inactive under strict anaerobic conditions (224, 225). The gene *oxdC* (*yvrK)* encodes OXDC and is induced in acidic environments (pH 5) (225). We evaluated expression of OXDC as a fused protein with maltose binding protein and its signal peptide for secretion as a secretory enzyme in EcN.

E. coli Nissle 1917 (EcN) has been mainly used in Europe as a probiotic in humans and animals with inflammatory diseases of GIT (111, 112, 188, 193, 236). Efficient iron-uptake system, production of microcin and lack of expression of virulence factors are the salient phenotypic characters of probiotic EcN (72, 234). Genetically modified EcN has also been used as a delivery vehicle to express therapeutic molecules within the intestine (179, 246, 261). Oxalate-metabolic activity by *E. coli* was not observed during *in vitro* culture. In this study, we characterized oxalate metabolism in

EcN, engineered to express oxalate degrading enzymes by transformation with commercially available *E. coli* expression vectors.

Materials and Methods

Construction of recombinant expression plasmid.

a) Expression plasmid with *oxc, frc* and *OxlT* protein of *O. formigenes*

Open reading frame (ORF) sequences of the *frc, oxc* and *OxIT* genes were amplified by PCR using genomic DNA from *O. formigenes* as template. The primers were designed based on the sequences of *O. formigenes* (GenBank Accession No.U82167.1, M77128.1 and U40075.1) (Table 1). Amplification was performed in a GeneAmp PCR system 2400 (Perkin Elmer) with activation at 95° C for 10 min, followed by 35 cycles of denaturation at 95° C for 30 seconds, annealing at 57° C for 30 seconds and extension at 72° C for 90 seconds and final elongation at 72° C for 10 minutes. The amplicons were resolved by agarose gel electrophoresis and visualized by ethidium bromide staining. The resolved DNA bands with appropriate size in agarose gel were extracted and purified using Qiagen PCR clean up kit (Qiagen, Valencia, CA). Purified fragments were digested with appropriate restriction enzymes (Table 1). All the restriction enzymes, Antarctic phosphatase and Quick ligation kit were purchased from New England BioLabs (New England Bio Lab, Ipswich, MA). The pTrc-*frc-oxc-OxlT* was constructed by serial restriction digestion and ligation. Briefly, pTrcHis-*OxlT* was generated by insertion of a 1277 bp fragment of *OxlT* ORF with PstI-Kpn1 into pTrcHis A vector (Invitrogen, Carlsbad, CA) by restriction digestion and ligation. The pTrcHis-*oxc-OxIT* was generated by insertion of 1725 bp fragment of *Oxc* ORF with BamHI-BglII into pTrcHis-*OxlT*

vector by restriction digestion and ligation. Finally, recombinant pTrc-*frc*-*oxc*-*OxIT* was generated by insertion of 1306 bp fragment of *frc* Nco I-BamH into pTrcHis-*Oxc*-*OxIT*. Recombinant expression plasmids with RBS in front of the *Oxc* and *OxIT* ORF were also generated as described above. Forward primers were designed to include the following sequences of *OxlT* ORF;

CCAATCTGTGTGGGCACTCGACCGGAATTATCGATTAACTTTA

TTATTAAAAATTAAAGAGGTATATATTA, that include RBS at the 5'of the ORF of oxc and *OxIT* (Table 1). In each round of subcloning, plasmids were transformed into *E. coli* XL10 gold cells (Agilent Technologies Inc., Santa Clara, CA) and transformants were screened on Luria-Bertani (LB) agar plates containing 100 µg/ml ampicillin. Positive clones were screened again by colony PCR. Plasmids were extracted from positive clones and DNA sequences were confirmed by Sanger sequencing (Advanced Genetic Analysis Center, University of Minnesota).

b) Expression plasmid with *oxdC* of *B. subtilis*

Open reading frame (ORF) sequences of the *oxdC* gene were amplified by PCR using genomic DNA from *B. subtilis*, and primers designed based on Genbank sequence NP_301204.1) (Table 1). The DNA fragment was purified as described above and digested with Nde1 and BamHI. Expression vector pMAL-p5X (New England Bio Lab, Ipswich, MA) was also digested with Nde1 and BamHI. The *oxdC* ORF sequences were ligated at the 3' end of the maltose binding protein sequence. Recombinant expression plasmid pMAL-*oxdC* was transformed into *E. coli* XL10 gold cells and positive clones

Table 1. List of PCR primers used to amplify target sequences.

Restriction enzyme sites are underlined and italicized sequence segments contain the ribosome binding site.

Primer name ID	Sequences
NcoI-*frc* F'	CATGCCATGGATGACTAAACCATTAGATGGAATT
frc-BamHI R'	CGCGGATCCTCAAACTACCTGTTTTGCATG
BamHI-*oxc* F'	CGCGGATCCATGAGTAACGACGACAATGTAGAG
oxc-BglII R'	GGAAGATCTTTATTTCTTGCCAACTTTACTTACAA
Pst I-*OxIT* F'	AACTGCAGATGAATAATCCACAAACAGGACAA
OxlT- KpnI R'	CGGGGTACCTTAATGTACAGCTTTTTCTTCTGGA
BamH I-RBS-*oxc* F'	CGCGGATCC*AATCTGTGTGGGCACTCGACCGGAATTATCGATTAA CTTTATTATTAAAAATTAAAGAGGTATATATTA*ATGAGTAACGACGA CAATGTAGAG
Pst I-RBS-*OxIT* F'	AACTGCAG*AATCTGTGTGGGCACTCGACCGGAATTATCGATTAAC TTTATTATTAAAAATTAAAGAGGTATATATTA*ATGAATAATCCACAA ACAGGACAA
NdeI -*oxdC* F'	GGAATTCCATATGATGAAAAAACAAAATGACATTCCGCAGC
oxdC-BamHI R'	CGCGGATCCTTATTTACTTTCTTTTTCACTACTGG
Frc- F'	GGTTCCGGAGATATGACTCG
Frc-R'	TCCCAAGTAAAGCCCATACG
Oxc –F'	GTGTTGTCGGCATTCCTATC
Oxc – R'	GGGAAGCAGTTGGTGGTT
OxlT –F'	GCCGTTACAAAACCATGTCC
OxlT –R'	ACCGAAAATATCGCTGTTGG

were screened as described above. DNA sequences were confirmed by Sanger sequencing (Advanced Genetic Analysis Center, University of Minnesota).

Transformation of pTrc-*frc-Oxc-OxlT* and pMAL-oxdC into EcN strain

EcN cells were processed to obtain electrocompetetent cells. A single colony of EcN strain was inoculated into 10 ml 2XYT medium and incubated for 14 hours at 37°C. One ml of the broth culture was inoculated into 100 ml of 2XYT medium and cultured with shaking at 37°C until the OD reaches 0.6. Cells were harvested by centrifugation at 4000 rpm for 25 min at 4°C, washed twice with 100 ml of ice-cold H_2O and harvested by centrifugation. The bacterial cell pellet was gently re-suspended in 10 ml of ice-cold 10% glycerol and harvested by centrifugation at 4000 rpm for 10 min in 4°C. Then cells were resuspended in 500 µl of ice-cold 10% glycerol and 80 µl aliquots were prepared. Expression plasmids, pTrc-*Frc-Oxc-OxlT* and pMAL-*oxdC* were transformed into electrocompetent EcN by electroporation using MicroPulser^{TM} (Bio-Rad, Hercules, CA) with EC1 program (25 µFD, 200Ω and 1.8 kV). Transformants were screened on Luria-Bertani (LB) agar plates containing ampicillin (100 µg/ml).

A single colony harbouring the plasmid pTrc-*frc-Oxc-OxlT* was inoculated into 5 ml LB broth with ampicillin (100 µg/ml) and incubated at 37^{o} C for 16 hours. 100 µl of the above culture was inoculated into 10 ml of SOB media (pH 6.8) supplemented with 10 mM sodium oxalate and incubated at 37^{o} C until the OD reached 0.3. Then, 0.5 mM IPTG was added to induce the expression of the genes.

A single colony harbouring the pMAL-*oxdC* construct was also induced as described above with the following modifications to the protocol: cells were inoculated

into SOB media (pH 5) supplemented with 5 mM sodium oxalate and incubated at 37^{o} C until the OD reached 0.3. During induction, 5mM $MnCl_2$ was added along with 0.5 mM IPTG.

Expression of OXC, FRC, OxlT and OXDC in engineered EcN

a) Expression of mRNA

One ml of the culture was taken at 0 (before induction), 2, 4, 8, 16 and 32 minutes after induction with IPTG and stabilized immediately by adding 2 ml of RNAprotect Bacteria Reagent (Qiagen, Valencia, CA). Total RNA was extracted using RNeasy Mini kit following protocol 1 (Enzymatic lysis) and protocol 7 (Qiagen, Valencia, CA). Extracted RNA was quantified by Nanodrop spectrophotometry (Thermoscientific, Wilmington, DE). Genomic DNA was removed by treating the RNA extract with TURBO DNase (Ambion-Applied Biosystems, Carlsbad, CA) and cDNA was synthesized using QuantiTect Reverse Transcription Kit (Qiagen, Valencia, CA). To confirm gene expression (of *frc*, *Oxc*, and *OxlT*), qRT-PCR was carried out in ABI 7500 Real time PCR system (Applied Biosystems, Carlsbad, CA) using Quanta Biosciences SYBR green kit (Quanta Biosciences, Gaithersburg, MD). *GapA* was used as the housekeeping gene control for normalization. Primers used for qRT-PCR are listed in Table 2.

b) Protein expression

Four hours after induction with IPTG, 100 µl of culture was collected to analyse the protein expression. Cells were harvested by centrifugation at 8,000 rpm for 5 min. Cell lysates were resolved in SDS-PAGE and visualized by Coommassie Blue staining.

Resolved bands with expected sizes were subjected to in-gel trypsin digestion (196). The digested peptide mixtures were analyzed using LTQ-LC/MS/MS **(**ThermoFinnigan, San Jose, CA). Peptides were first fractionated by liquid chromatography (LC) in an acetonitrile gradient of 5 % - 95 %. Then each fraction was analyzed by tandem mass spectrometry. Tandem mass spectra were extracted by Sequest version **2.2** (ThermoFinnigan, San Jose, CA**).** Mass spectra were searched against protein databases of *E. coli*, *O. formigenes* and *B. subtilis.*

EcN-1 and EcN-2 cell lysate were resolved in SDS-PAGE and transferred to a PVDF membrane (Immobilon-P; Millipore). The membrane was probed with rabbit anti-OxIT N-terminal peptide antibody (1:100 dilution) for 1 h, followed by incubation with a 1:2000 dilution of horseradish peroxidase-labelled anti-rabbit IgG (Bethyl Laboratories Montgomery, TX) (1). Enhanced chemiluminescence western blotting detection reagents (Thermo Fisher Scientific, Rockford, IL) were used according to the manufacturer's instructions to visualize the bands. Recombinant OxIT was used as positive control (1).

Oxalate metabolic activity

Oxalate degrading activity of the engineered EcN clones was tested by reduction in the oxalate concentration of bacterial cultures. Culture supernatant of EcN with pTrc-*frc-oxc-OxlT* was collected before the induction at OD 0.3, and 12 and 48 hours after induction. Culture supernatant of EcN with pMAL-*oxcD* was collected before the induction at OD 0.3, and 2, 4, 8, and 12 hours after induction. Oxalate concentrations of the supernatant were analysed by ion-chromatography. Samples were diluted 10-fold with de-ionized water and analyzed on a Dionex ICS-2000 ion chromatography system consisting of an

AS19 analytical column, ASRS 300 suppressor, AS40 autosampler, and integrated dual piston pump and conductivity detector. The eluent was generated by Reagent Free eluent generator system (Dionex, Sunnyvale, CA), which produced a variable concentration KOH eluent, regulated by Chromeleon control software. The control program used a comprehensive anion elution scheme.

Results

Expression of the FRC, OXC and OxlT in EcN

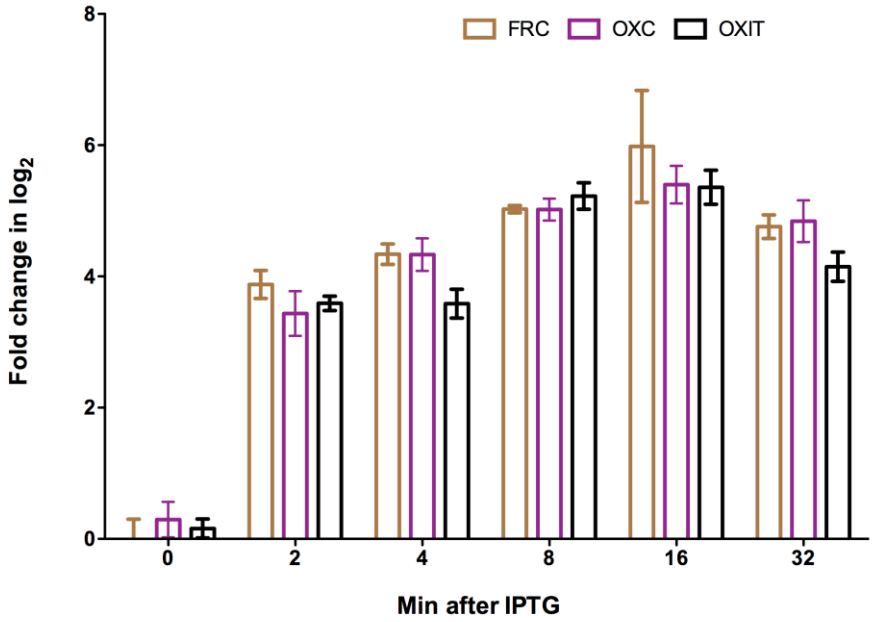
The ORF sequences of *frc, oxc and OxlT* were amplified and ligated into an expression vector, driven by the *trc* promoter, which contains a -35 region of the *trp* promoter together with -10 region of the *lac* promoter. Expression plasmids, pTrc-*frc*-*Oxc*-*OxlT* with and without multiple RBS, were successfully transformed into EcN. Here onwards, recombinant EcN with expression plasmid containing single and multiple RBS site will be symbolized as rEcN-1 and rEcN-2, respectively while the wild type EcN will be denoted as EcN-W. As the expression plasmid contains a copy of the $lacI^q$ gene that encodes Lac repressor, we expected to observe efficient repression before induction and robust induction upon addition of IPTG.

Within 30 minutes of induction, the fold increase of *oxc*, *frc* and *OxlT* mRNA expression was 16 – 32 in rEcN-1 while there was a 2- 4 fold increase in these mRNAs in rEcN-2 (Fig 1a and 1b) compared to un-induced rEcN-1 and rEcN-2 respectively. We also noted that even in the absence of IPTG, mRNA were expressed at a satisfactory level (Ct= 20). This indicates that the expression plasmid has a "leaky" promoter, despite the presence of the $lacI^q$ gene. Amplification was not observed in the samples that were not

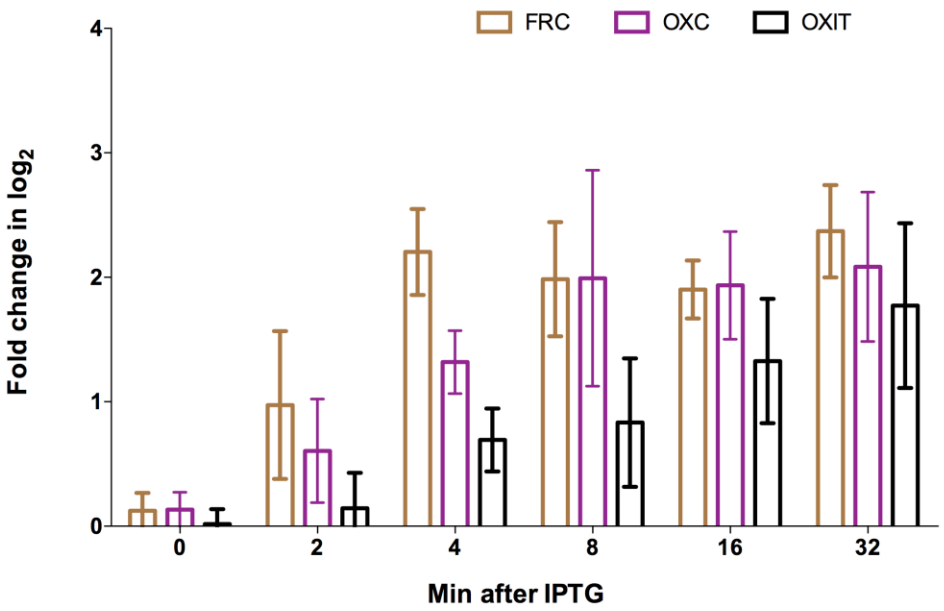
Figure 1. Transcription of *oxc, frc* and *OxlT* in rEcN.

Relative fold-change of *Frc, Oxc* and *OxlT* mRNA in a) rEcN-1 and b) rEcN-2 compared to uninduced controls and normalized against housekeeping gene *GapA.*

a)



b)



treated with reverse transcriptase, indicating that the templates were free of contaminating DNA.

The expected molecular weights of FRC, OXC, and OxlT proteins are 47 kD, 60 kD and 44 kD, respectively. In rEcN-2, a differentially expressed protein at 60 kD was observed compared to EcN-W and rEcN-1 (Fig 2, white arrow). It was identified as *O. formigenes* OXC protein by tandem MS (Fig 3a). Further, the protein band at 47 kD was identified as *O. formigenes* FRC protein in both rEcN1 and rEcN2 (Fig 2, orange arrow, and Fig 3b). Antibody against rOxlT N-terminal sequence did not detect the OxlT in western blot, indicating OxlT protein was not expressed. Further, oxalate concentrations of the rEcN-1 or rEcN-2 culture media were not altered.

Expression of the OXDC

The ORF sequences of *oxdC* were amplified and ligated at the 3' end of the maltose binding (MAL) protein into an expression vector, driven by *tac* promoter. Expression plasmids, pMAL-*oxdC* or pMAL was successfully transformed into the EcN. Here onwards, the recombinant EcN with pMAL and pMAL-*oxdC* will be symbolized as rEcN-3 and rEcN-4.

Expected molecular weights of the MAL protein, OXDC and fusion proteins (MAL-OXDC) are 43 kD, 43.4 kD and 86 kD, respectively. Differential protein expression was observed between un-induced and induced rEcN-3 and rEcN-4 cell lysates in SDS-PAGE (Fig. 4). The 43 kD in EcN-3 lysate (Fig 4, white arrow) was identified as *E. coli* MAL protein by tandem MS (Fig 5b). The 86 kD in EcN-4 lysate (Fig 4, red arrow) was identified as the OXDC - MAL fusion protein, and the 43 kD band

Figure 2. Expression of OXC, FRC and OxlT in rEcN-1 and rEcN-2.

Cellular proteins of EcN-W, rEcN-1and rEcN-2 from SOB-OX induced cultures were resolved in a 10-20% Tris-Glycine PAGE gel and stained with coomassie blue. Lane 1, molecular weight markers; Lane 2, EcN-W; Lane 3, rEcN-1; and Lane 4, rEcN-2. OXC and FRC proteins are shown by the white and orange arrows, respectively.

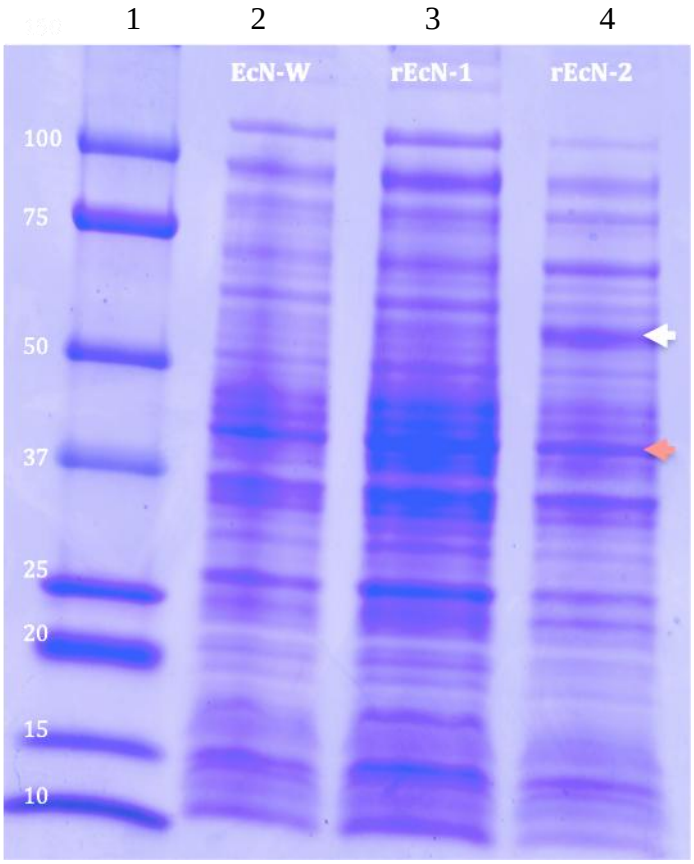
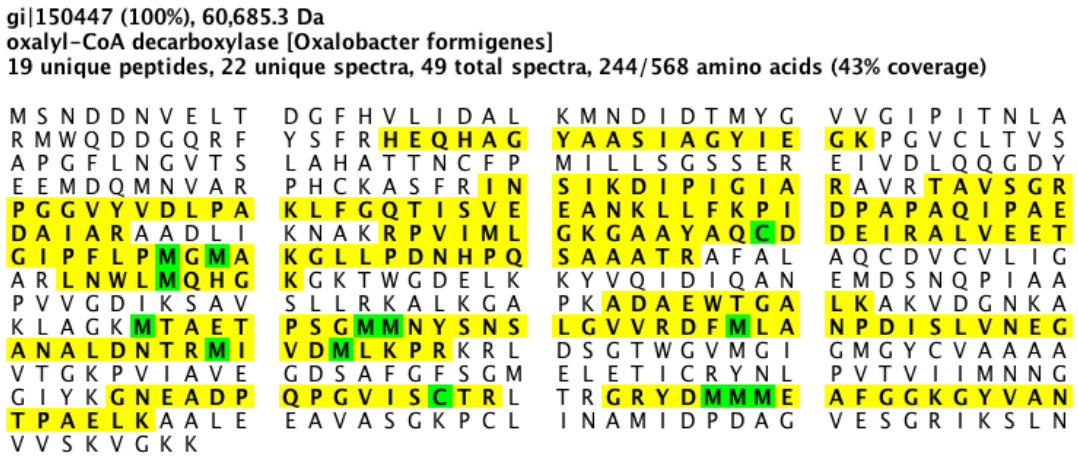


Figure 3. Identification of OXC and FRC peptides by MS.

Unique peptide sequences of (a) OXC and (b) FRC of *O. formigenes* were identified by tandem MS from the SDS-PAGE resolved protein bands. Unique identified peptide sequences are highlighted in yellow. Green highlight indicates the modifications made to the amino acids during database search.

(a)



(b)

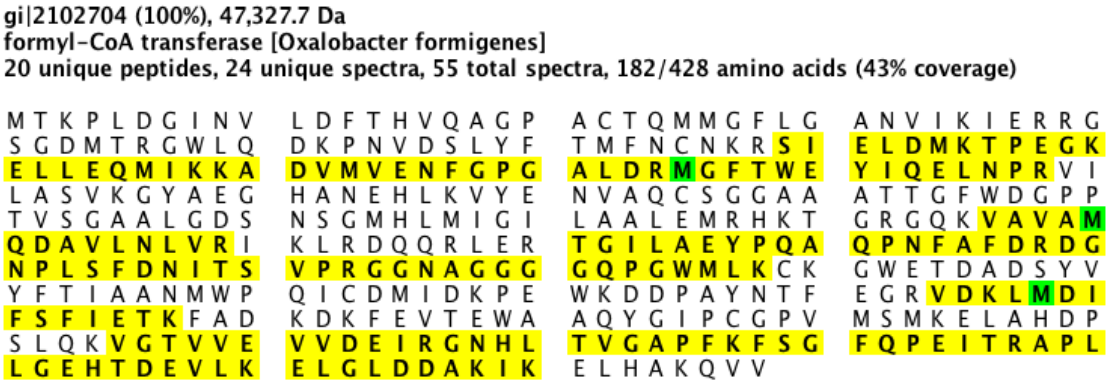


Figure 4. Differential protein expression in rEcN-3 and rEcN-4.

Cellular proteins of rEcN-3 and rEcN-4 from SOB-OX induced or uninduced culture were resolved in a 10-20 % Tris-Glycine PAGE and stained with coomassie blue. Lane 1, rEcN-3 un-induced ; Lane 2, rEcN-4 un-induced ; Lane 3, rEcN-3 induced ; Lane 4, rEcN-4 induced; Lane 5, molecular weight markers (kD). Arrows in white, green, and red point to the bands that were identified by MS as *E. coli* MAL, *B. subtilis* OXDC and MAL-OXDC fusion protein, respectively.

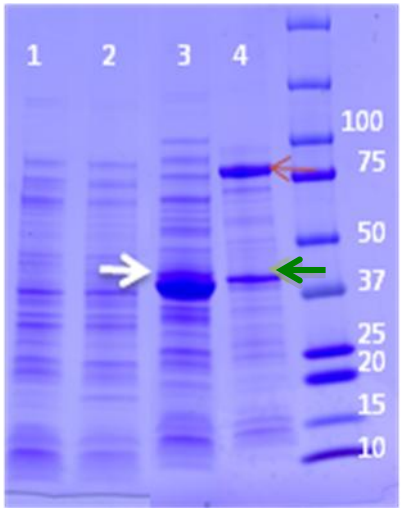
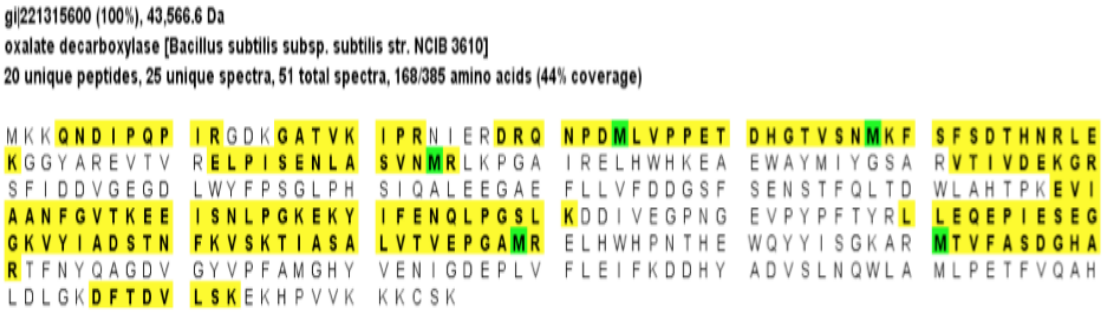


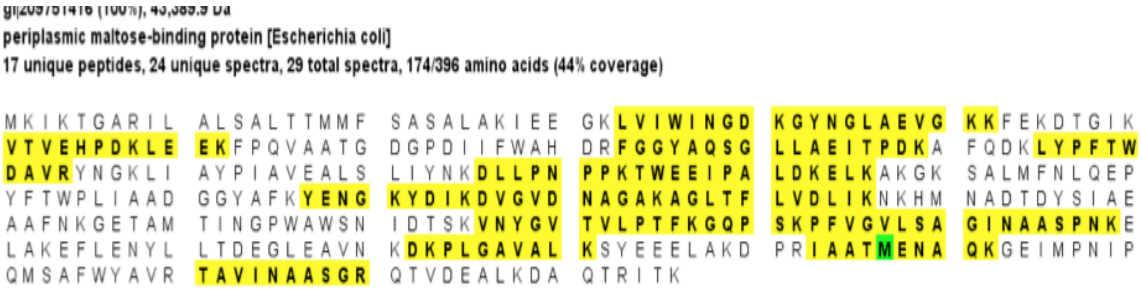
Figure 5. Peptides of MAL and OXDC identified by MS.

Unique peptide sequences of (a) OXDC of *B. subtilis* and (b) MAL protein of *E. coli* that were identified from the SDS-PAGE resolved protein by MS. The unique sequences are highlighted in yellow. Green highlight indicates the modifications made to the amino acids during database search.

a)



b)



(Fig 4, green arrow) was identified as *B. subtilis* OXDC (Fig 5a). Interestingly, oxalate concentration of the rEcN-4 culture medium was reduced by 60% after 12 h induction (Fig 6). The oxalate-metabolizing activity of rEcN-4 was time-dependent, with approximately 50% of total reduction occurring within 4 hr of induction, whereas rEcN-3 did not show time-dependent oxalate metabolism after induction with IPTG and $MnCl_2$ (Fig. 7). Interestingly, growth of rEcN-4, but not rEcN-3, was inhibited following induction (Fig. 8). This unexpected effect was not due to change in pH, which also occurred after induction, since rEcN-3 culture also showed the same pH change, but cell growth was not affected (Fig 9).

Discussion

In this study, *E. coli* Nissle 1917 was engineered to express and secrete OXDC of *B. subtilis* and oxalate-metabolizing activity by the recombinant EcN was observed *in vitro.* In a previous study, optimum activity of OXDC was observed between pH 4 - 5 (222). In our investigation, we observed a decrease in the rate of oxalate depletion in the medium after four hours of induction and that corresponded to an increase in the pH of the medium. Further, the growth of rEcN-4 cells was also affected upon induction. It is likely that the high levels of recombinantly expressed heterologous proteins are toxic to the bacterium. This possibility can be tested by reducing the level of OXDC expression, by decreasing the concentration of IPTG (less than 0.5 mM). Further, since OXDC enzymes were engineered to secrete into the periplasm, it is possible that OXDC in the periplasm of dying cells also contributed to the observed oxalate depletion. Presence of OXDC in the culture supernatant was not detected by SDS-PAGE. It is plausible that the

Figure 6. Oxalate metabolizing activity of rEcN-4

Comparative oxalate depletion in the SOB oxalate and $MnCl_2$ supplemented culture medium by the rEcN-3 (vector control) and rEcN-4 (pMALOXDC) after 14 hours of induction.

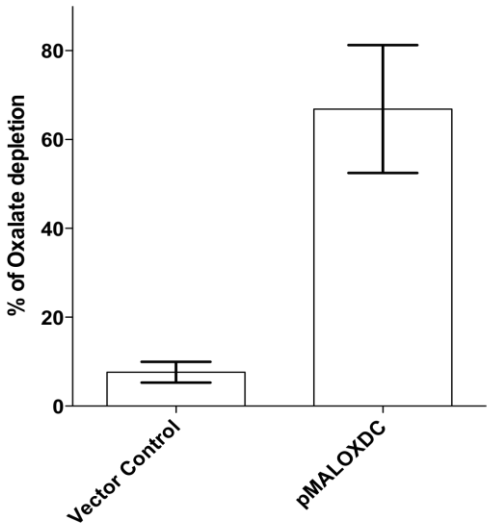


Figure 7. **Comparative oxalate metabolizing activity of rEcN-3 and rEcN-4.** Comparative and time-dependent oxalate depletion in the SOB-oxalate supplemented culture medium by rEcN-3 (vector control) and rEcN-4 (pMALOXDC). Arrow indicates the time at which 5 mM $MnCl_2$ and 0.5 mM IPTG were added.

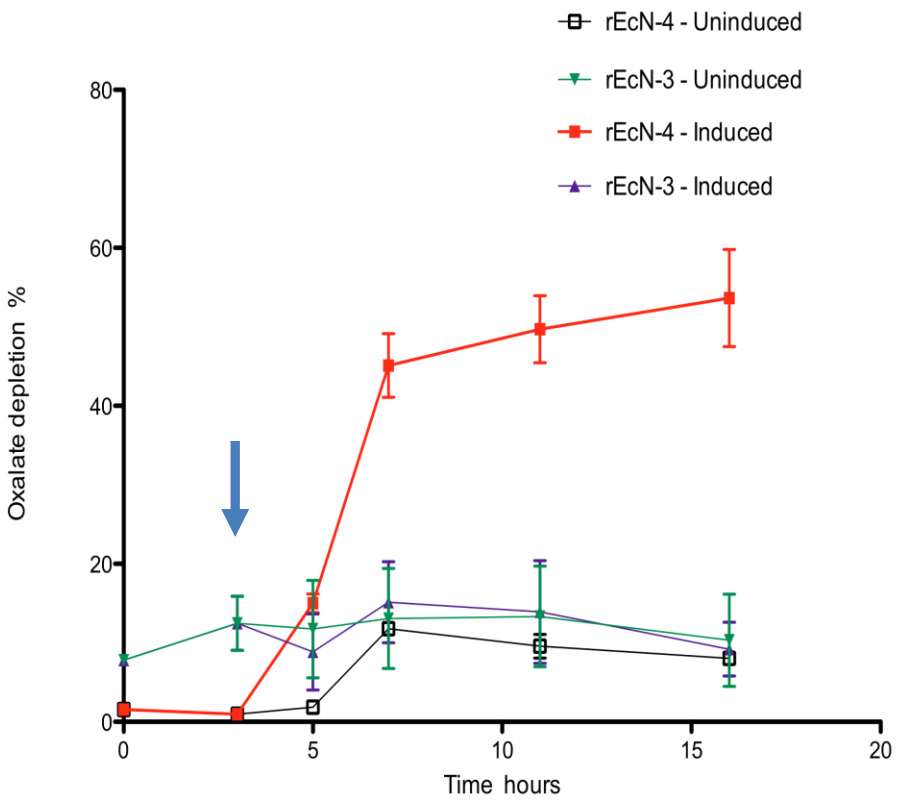


Figure 8. **Comparative growth curves of the engineered EcN.**

Comparative growth curve of induced EcN-3, and uninduced and induced rEcN-4 in SOB-oxalate supplemented culture medium. Arrow indicates the time at which 5 mM $MnCl_2$ and 0.5 mM IPTG were added.

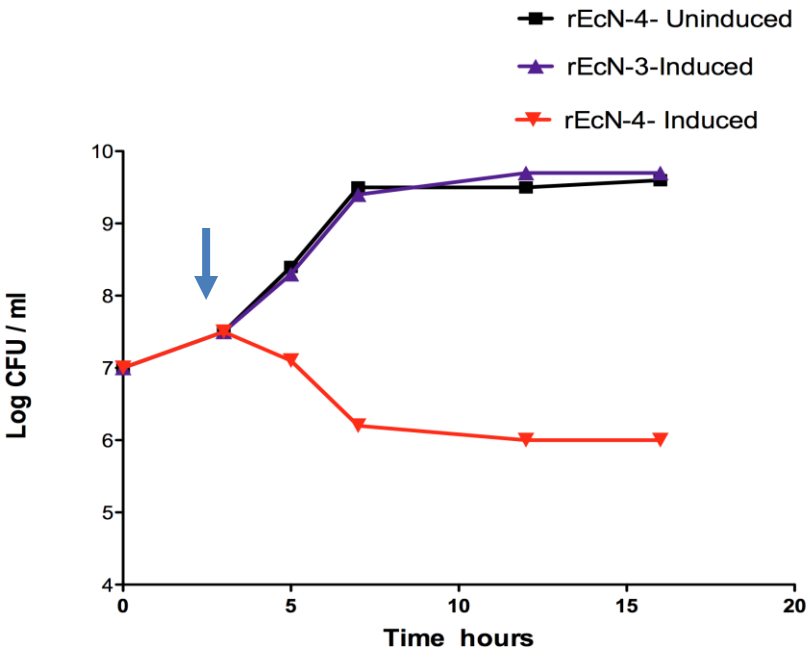
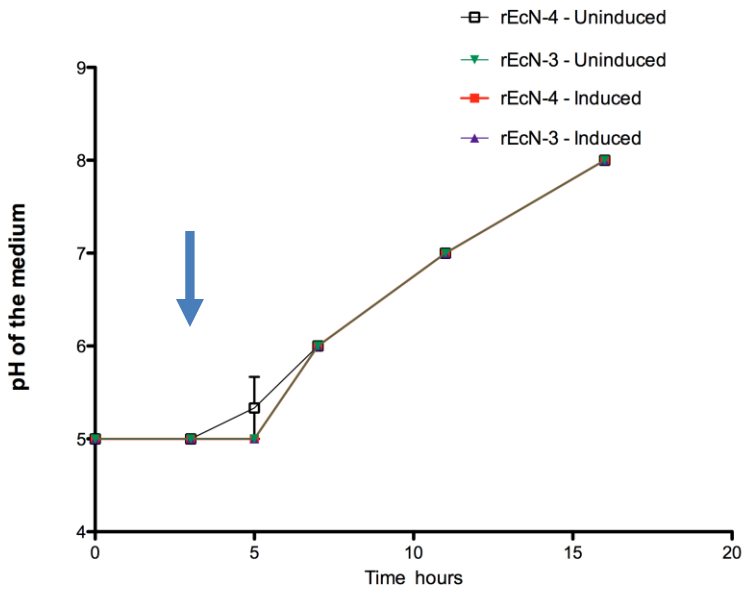


Figure 9. Medium pH changes in rEcN-3 and rEcN-4 cultures

Changes in pH of the SOB-oxalate supplemented culture medium upon inoculation of rEcN-3 and rEcN-4. Arrow indicates the time at which 5 mM $MnCl_2$ and 0.5 mM IPTG were added.



lower concentrations of the enzymes were much below the detection limit of the Coomassie blue staining. Follow up experiments need to focus on determining the subcellular localization of the expressed OXDC enzyme. Alternatively, nutrient levels necessary for rapid growth may have been insufficient under conditions of intense heterologous proteins expression.

Even though we observed oxalate metabolic activity in culture media, no attempt was made to purify the OXDC enzyme to study the kinetics of the heterologously expressed OXDC enzymes.

Tanner *et al* generated an active form of recombinant OXDC enzyme by heat shocking the host cells (*E. coli* DE3) prior to induction (225). Some metalloproteins require metallo-chaperones to assist the metal binding and proper folding. However, no direct evidence was available to confirm the requirement of chaperone proteins for the folding of OXDC or Mn^{2+} binding (222). Interestingly, in this study rEcN-4 was not heat-treated but still the oxalate metabolic activity was observed. Another novel aspect in our study is that, unlike the previous studies where the heterologous expression was localized in the cytoplasm, MAL-OXDC fusion proteins were directed to the periplasm where protein folding is more feasible. According to previous cyrstallographic studies, the OXDC enzyme crystallizes as a hexamer (225). In this study, we did not purify the enzyme therefore we were unable to confirm the formation of the hexamerized proteins. Prior studies on recombinant OXDC expression observed that His tags on C or N terminal of the OXDC altered the enzyme activity of the OXDC (9, 99, 225). Even though OXDC was expressed as a fusion protein in our study, presence of maltose

binding protein did not appear to affect the oxalate metabolic activity of OXDC, but its effect on efficacy of the OXDC activity was not addressed in our study. As described in the results and shown in figure 6, the protein band corresponding to the molecular weight of 43 kD in the rEcN-4 lysate was identified as OXDC by MS. The Factor Xa cleavage site is also present between MAL and OXDC proteins. However, the possibility of the bacterial enzymes cleaving the Factor Xa sequence site is not known.

Studies showed that urinary oxalate concentration was significantly reduced in mice treated with commercially available OXDC ("oxazyme") (38). If the viability of rEcN-4 expressing OXDC is confirmed, *E. coli* Nissle 1917 can be genetically modified to constitutively express and secrete OXDC in the gut environment. Since, OXDC activity is dioxygen, pH and Mn^{2+} -dependent, the metabolic activity of rEcN-4 cells *in vitro* environments reflecting different gut segments need to be assessed prior to such genetic modification (224, 225).

Inherently efficient oxalate-metabolizing activity of *O. formigenes* prompted us to select the genes of the bacterium for heterologous expression. The genes *frc, oxc and OxlT* are critical for oxalate metabolism in *O. formigenes* and they are not part of a single polycistronic operon. Therefore reproducing the oxalate-metabolizing system of *O. formigenes* in *E. coli* is complicated. We decided to clone two expression vectors, one with a single RBS followed by *frc, oxc and OxlT* ORF and the second vector with the RBS in front of each gene. We anticipated that the cloned expression plasmid with multiple RBS would express all three proteins to comparable levels. All three genes were transcribed efficiently in both rEcN-1 and rEcN-2 and the expression increased

significantly upon IPTG induction. Therefore both engineered rEcN 1 and rEcN 2 were evaluated for the expression of the protein.

The rEcN-2 containing plasmid with multiple RBS successfully expressed FRC and OXC but failed to express the OxlT. In addition, if OxIT is not expressed and/or localized in cell membrane, recombinant *E. coli* cannot actively take up the oxalate ions, resulting in lack of intracellular enzymatic activity. This observation explains the failure to demonstrate the oxalate degrading activity by the EcN clones carrying the genes of *O. formigenes*. It is not clear why the OxIT was not expressed by the expression system used in this study. It is possible that OxIT transcript was not translated in the heterologous expression system. OxIT, a membrane transporter protein, is composed of 12 transmembrane domains. It is also possible that OxIT was translated but improper folding resulted in premature degradation of the protein.

In summary, I engineered *E. coli* Nissle 1917 to mimic the *O. formigenes* oxalate degradation, but it failed to metabolize oxalate due to the absence of OxlT expression. By contrast, engineered *E. coli* Nissle 1917 expressing OXDC of *B. subtilis* degraded oxalate *in vitro.* This engineered strain demonstrates the feasibility of a promising oxalate metabolizing probiotic candidate.

CHAPTER V

General Discussion

An alarming increase in the incidence of CaOx stones in humans, dogs and cats has been observed over the last two decades and thus warranted the exploration of novel prophylactic and preventive measures. Increased relative supersaturation of CaOx in urine is the driving force for CaOx crystallization. Therefore preventive measures need to focus on halting the increase in Ca or oxalate levels or both in the urine. Oxalate is a toxic metabolic end product and is excreted in urine. Since dogs are not affected by primary hyperoxaluria, only a few studies have been carried out to explore the risk factors that contribute to the urine oxalate levels in dogs.

One possible reason for the increased incidence of CaOx uroliths in dogs and cats is changes in their diet. Some of the dietary changes are aimed at controlling struvite urinary stones. Acidifiers were used to reduce the urine pH in order to control formation of struvite uroliths. However, acidic urine reduces the solubility of CaOx. Another potential change in commercial pet foods is the use of plant-based nutrient sources. Plant-based raw materials are good dietary sources of oxalate that can readily contribute to increased urinary oxalate. However, complete removal of oxalate-containing plant-based feed materials is impossible to achieve when formulating a nutritious and affordable pet food. Thus, it is essential to explore novel and unconventional approaches to control urinary oxalate levels.

Oxalate-metabolizing enteric commensals express enzymes to metabolize oxalate within the GIT of mammals and thus act to minimize the enteric absorption of oxalate. Oxalate metabolizing commensals are present in the GIT of the dog, but their biological

importance in CaOx urolithiasis causation or prevention is not known. In this context, through this thesis research, we observed an association between the presence of oxalate metabolizing bacteria and absence of CaOx uroliths in dogs. Presence of *O. formigenes, B. animalis* and *L. acidophilus* were more frequently observed in healthy dogs compared to dogs affected with CaOx uroliths. More than 75% of healthy dogs were colonized with at least one of the above bacteria while only 25% of the CaOx urolith dogs harbored any one of these bacteria, indicating that absence of the enteric oxalate metabolizing bacteria increases the likelihood of CaOx stone formation in dogs. Elevated fecal prevalence of *O. formigenes*, *B. animalis* and *L. acidophilus* in healthy stone forming and non-stone forming dogs supports a potential preventive role by these oxalate metabolizing bacteria in enteric oxalate absorption.

Although the present study shed light on the prevalence of oxalate metabolizing bacteria in dogs affected with CaOx uroliths, there are some limitations in the study that restrain the direct pathophysiological interpretation of the findings. Colonization of oxalate metabolizing bacteria was indirectly detected by the presence of *oxc* genes in the feces. *Oxc* is also present in a wide range of oxalate degrading bacteria, but sequence variations among bacterial genera allowed amplification specifically from *O. formigenes*, *B. animalis* and *L. acidophilus.* However, isolation of viable organisms would provide additional, direct evidence of colonization. Urinary oxalate concentrations were not measured, therefore urine oxalate excretion levels were not compared between dogs that were colonized with and without oxalate metabolizing bacteria. Such a comparison would better demonstrate the association between the colonization and urinary oxalate excretion

The frequency of *O. formigenes* occurrence between CaOx urolith dogs and their breed-matched controls was not significantly different. It is possible that the limitations in the current study "masked" the differences between the healthy and CaOx urolith groups, or that other oxalate-reducing bacteria carried out this function. Oxalate content in the feed or fecal samples of the respective dogs was not analyzed therefore it was not possible to assess whether these two groups were equal in their oxalate intake. Stable colonization of *O. formigenes* is observed only in an oxalate-rich environment as the bacterium solely relies on the oxalate. Studies on human subjects observed that no oxalate hyperabsorbing individuals were colonized with *O. formigenes*. It is not known if oxalate hyperabsorbers exist in dogs or, if they do exist, if they were among the convenient sample of animals we recruited for the study. In this study, dogs had no antibiotics two months prior to the sample collection. Recent epidemiological studies on humans claim that the *O. formigenes* prevalence is high in individuals who never had antibiotics, suggesting the 2 month "withdrawal period" may not be sufficient for re-colonization (102). Since there is substantial evidence that antibiotic usage affects *O. formigenes* colonization, future studies evaluating risk factors should include the history of antibiotic therapy.

Further, varying degrees of oxalate metabolizing activity were observed in fecal cultures and thus indicated that other oxalate metabolizing bacteria are present in the GIT flora. In light of this observation, we expanded the scope of our study by adopting a microbiome-based approach to characterize commensal populations in healthy and CaOx urolith dogs. Bacterial diversity in the feces of stone formers and non-stone formers was explored by analyzing the hypervariable region-V3 16S rRNA library. Principal

coordinates analysis revealed that based on the composition of the fecal microbiota, healthy dogs were clustered together whereas the CaOx urolith dogs were dispersed in a random manner. This observation confirms that the microbiota of the healthy gut is distinct from that of dogs affected with CaOx uroliths. This observation suggests that enteric microbial flora of CaOx stone formers is altered possibly due to the diet or use of antibiotics. Microbiome-based data has opened up avenues to use CaOx urolithiasis as a metabolic disease model in which microbial flora play an important role. Further, fecal microbiota were explored for the presence of known bacterial genera that harbor oxalate-metabolizing genes. Relative abundance of these genera between stone formed dogs and healthy dogs were comparable. Alternatively, presence of the genes encoding the oxalate metabolizing enzymes in the genome of these bacteria did not necessarily mean that the oxalate metabolizing enzymes were expressed or functional in the GIT. For instance, *in vitro* studies failed to demonstrate oxalate metabolic activity by *E. coli* or lactic acid bacterial species even though the genes were present. Studies have also shown that the low-abundant bacterial species significantly contribute to gut metabolism (11). In this context, the oxalate metabolizing enzymes of *O. formigenes* are good example in which only ~20% of the expressed proteins are enzymes involved in oxalate metabolism, yet the bacterium impacts the gut oxalate metabolism significantly. Some other bacterial species express oxalate-metabolizing enzymes only under specific environmental conditions, such as acidic pH.

In the microbiome study, is that the samples were not collected from animals that were matched for age, gender or breed. These factors could have a significant impact on

gut microbial flora. We studied microbial communities in fecal samples as representative of the gut microbial flora, although significant differences between gut mucosal, luminal intestinal and fecal microbial communities have been observed in dogs and humans (221)

Since multiple risk factors are associated with CaOx urolithiasis, addressing and controlling all those factors in a single study is an impossible task. However, future prevalence studies on oxalate metabolizing bacteria could be improved by taking into account the oxalate levels in the diet, feces and urine. Comparative analysis of fecal transcripts ("Meta-transcriptome") in healthy and CaOx urolith dogs would also enable us to quantify the differences in oxalate metabolizing gene pools between CaOx stone formers and healthy dogs. Further, finding a correlation between dietary oxalate, fecal oxalate, urinary oxalate and microbiome would provide an insight on aetiopathogenesis of CaOx urolithiasis in dogs, cats and humans.

The observed high prevalence of the oxalate metabolizing bacteria in healthy dogs compared to CaOx stone formed dogs led to a hypothesis that administration of oxalate metabolizing probiotics minimizes the urinary oxalate excretion in dogs. The efficient oxalate metabolizer *O. formigenes* is an ideal probiotic candidate. However, the stable colonization of *O. formigenes* is challenging if oxalate level is limited in the gut environment. Few strains among the commercially available lactic acid bacterial pool also metabolize oxalate *in vitro*. But the induction of an oxalate metabolizing gene of the lactic acid bacteria is pH-dependent. Therefore, probiotics that express the oxalate metabolizing genes constitutively and independent of environmental pH, may be ideal prophylactic candidates to minimize enteric oxalate absorption. In this study, I

engineered the *E. coli* Nissle 1917 to express the oxalate metabolizing genes of the *O. formigenes* and *B. subtilis* and explored the feasibility of oxalate degrading activity *in vitro*.

Expression of three genes, *oxc*, *frc* and *OxIT*, are essential to carry out the oxalate metabolism in *O. formigenes* and successful heterologous expression of such a complicated system is challenging. Engineered *E. coli* Nissle 1917 successfully expressed *O. formigenes* OXC and FRC enzymes. The *OxIT* gene was successfully transcribed but protein was not expressed. Since OxIT is a membrane protein with 12 transmembrane domains it is possible that the expressed protein mis-folded and was degraded. As OxIT is critical for oxalate import, in spite of expressing OXC and FRC, engineered *E. coli* Nissle 1917 failed to metabolize oxalate. Another potential oxalate-metabolizing enzyme, OXDC, was evaluated. Thus, *E. coli* Nissle 1917 was engineered to express *B. subtilis* OXDC into the periplasm as a secretory protein. *E. coli* Nissle 1917 expressing OXDC metabolized oxalate *in vitro* upon induction by IPTG in the presence of $MnCl_2$. Here OXDC was expressed as fusion protein at the C-terminus of maltose binding protein. We also observed a reduction in viable cell population in the induced culture compared to the un-induced controls indicating that high-level expression of heterologous protein may affect the viability of the expressing cells. Future experiments need to be carried out to observe the viability of the cells when OXDC is expressed in lower levels. In our study, *E coli* Nissle 1917 was engineered to metabolize oxalate *in vitro* to evaluate the feasibility of our genetic manipulation. Future experiments need to focus on engineering the *E. coli* Nissle 1917 to metabolize oxalate *in vivo*. One potential approach will be to clone *oxdC*

into a food grade expression vector that is driven by a constitutively expressed promoter (eg. 16S rRNA). To facilitate the secretion into the periplasm, *oxdC* need to carry a signal sequence, 5'UTR region of *fliC*, at its N terminal (141). Another feasible approach is to integrate the *oxdC* gene into the chromosome of the *E. coli* Nissle 1917 by displacing the *fliC* gene (49).

In conclusion, this is the first study that has identified an association between the presence of oxalate-metabolizing bacteria, specifically *O. formigenes, L. acidophilus* and *B. animalis*, and absence of CaOx urolithiasis in dogs. This observation indicates that oxalate metabolizing commensal bacterial species are critical to minimize the enteric oxalate absorption and absence of the commensal organisms increase the likelihood of CaOx stone formation in dogs. Interestingly, we observed that fecal microbiota of healthy dogs is distinct from that of CaOx stone formed dogs and thus indicates that the microbial flora is altered in CaOx stone formers. Microbiome-based study also confirms that potential oxalate metabolizing bacteria are present in the GIT of the dog. Engineered *E. coli* Nissle 1917 expressing oxalate decarboxylase of *B. subtilis* metabolize oxalate and thus proves the technical feasibility of engineering probiotic to metabolize oxalate. Engineered *E. coli* Nissle 1917 that expressed oxalate metabolizing genes of *O. formigenes* did not metabolize oxalate due to the failure in translation of the critical oxalate membrane transporter protein OxIT. Further, in this study *L. reuteri* and *L. acidophilus* were also identified as efficient oxalate metabolizing commensals from the healthy dogs. Along with these strains, engineered oxalate metabolizing *E. coli Nissle 1917* can be used as potential probiotic candidates in clinical trials. This thesis research

has identified that oxalate metabolizing bacteria are critical to minimize the risk CaOx stone formation in dogs and has opened up avenues for the use of oxalate metabolizing probiotic therapy as a prophylactic measure in dogs, cats and humans.

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Appendix 1

Isolation of *Bifidobacterium spp* and *Lactobacillus spp*

Bifidobacterium spp and *Lactobacillus spp* were isolated from the fecal cultures that demonstrated a high level of oxalate depletion (Chapter 1-Figure 6). *Bifidobacterium spp* and *Lactobacillus spp* were selected by plating the aforementioned cultures in BIM-25 and MRS agar plates, respectively. Each colony (53 colonies) was re-inoculated into MRS-Ox medium and incubated anaerobically. Oxalate concentration of the supernatant was measured after 48 hours. Fifteen isolates, metabolized more than 50% of oxalate in the medium and were identified as *L. reuteri* (1), *L. acidophilus* (3) , *B. animalis* (3) *and B. pseudolongum* (8) by sequencing the 16S rDNA amplicons using genus-specific primers (Biomedical Genomics Center, University of Minnesota). Further, the selected isolates were compared for their oxalate metabolizing activity per CFU. *L. acidophilus* and *L. reuteri* metabolized oxalate efficiently than the *B. animalis and B. pseudolongum.*