CHAPTER 1
Chapter 1: Introduction and Objectives of the Thesis

Oxalic acid (IUPAC name: Ethanedioic Acid; formula: C$_2$H$_2$O$_4$) is a highly oxidized, toxic to living cells, and strong cation (especially sodium and calcium) chelating agent (James, 1972). Oxalic acid has been associated with various pathophysiological conditions in humans such as hyperoxaluria, nephrolithiasis, ureterolithiasis, cardiomyopathy and cardiac conductance disorder (Bhasin et al., 2015; Williams and Smith, 1968). Maintenance of overall human gastrointestinal health is largely dependent on the gut-inhabiting microbiota. Normal balance between gut microbiota and the host has been observed to be disrupted in metabolic disorders like obesity (Turnbaugh et al., 2006), cardiovascular disease (Wang et al., 2011), inflammatory bowel disease (Sartor and Mazmanian, 2012) and chronic kidney disease (Vaziri, 2012). Thus, understanding the extent of perturbation on specific disorder has become an active area of research across the globe. Studies like these are contributing to identifying key players in the disease diagnosis. In-depth understanding of the relationship between oxalic acid metabolism and human health in association with the bacterial metabolic diversity in the gut and molecular mechanism of specific bacterial activities in the human body is essential to design and develop techniques of management of diseases caused by oxalates.

1.1 Oxalic Acid, Environment, and Human Health

Oxalic acid, a major component of the oxalate-carbonate pathway (OCP), is indispensable for the terrestrial ecosystem. The OCP, the major terrestrial carbon cycle wherein autoregulation of atmospheric CO$_2$ in the environment driven only through plants and microorganisms (Cailleau et al., 2011). Especially, biomineralization processes carried through the bacteria that can grow using oxalate as sole carbon and energy sources, and allowing CaCO$_3$ precipitation (Castro et al., 2010; Rowley et al., 2017). The OCP also allows the conversion of atmospheric CO$_2$ into calcite stones (stable geomorphs) (Braissant et al., 2004) apart from CaCO$_3$ precipitation. Importantly, the death and decay material of plants leads to oxalate release into the soil. The Oxalate Metabolising Bacterial Species (OMBS) can degrade...
and transform the oxalate to usually CaCO$_3$ (Marvasi et al., 2010). And thus OCP is only microbiologically mediated process for oxalate sink in the natural environment (Marvasi et al., 2010). Naturally, oxalate in carbon cycle autoregulated in the atmosphere through plants and microorganisms.

Calcium oxalate (CaOx), a bio-mineral form of Ca, is an important ecosystem modulator synthesized by plants (Franceschi and Nakata, 2005), and fungi (Pinna, 1993). Such bio-mineral form of oxalic acid was meant for regulating pH, maintaining the cationic balance of cytoplasm (Franceschi and Nakata, 2005), and decomposition to facilitate nutrient uptake from the outer environment (Kirker et al., 2017; Sayer and Gadd, 2000). CaOx crystals (Ksp $2.57 \times 10^{-9}$) found widely in terrestrial ecosystem and can accumulate in soils; with decomposing leaf litter and fungal mats (Webb, 1999). The significance of CaOx in the ecosystem is not just limited to short- and long-term calcium supply and recycling, wherein insoluble crystals were unavailable for other physiological processes (Franceschi, 1989). On the other hand, CaOx is the causative for adverse effects such as pain, edema to lips, tongue, oral mucosa, conjunctiva, and on the skin in man (Gardner, 1994). Humans acquire oxalate through their diet that includes plants, animal stored food and also via metabolic biosynthesis. Hyperoxaluria is a condition characterized by excessive oxalate production and excretion. Oxalate is also a metabolic byproduct in the systemic fluid of vertebrates, and in hyperoxaluria condition elevated level of this component leads to various detrimental effects.

In hyperoxaluria, elimination of oxalate by the kidneys through glomerular filtration and tubular secretion; can bind with calcium in the kidney leads to urinary CaOx supersaturation, resulting in the formation and putative retention of CaOx crystals in renal tissue (Robertson, 2004; Robijn et al., 2011). This excretory compound tends to crystallize in renal tubules and urine, where it begins cascades of aggregations and blockages (Cochat and Rumsby, 2013). Oxalate is thus, a principal component of crystals found in urinary tract system including kidney (Ivanovski and Drüeke, 2013). The presence of oxalate kidney stones in humans is therefore regarded as a symptomatic phase of oxalosis mainly with hyperoxaluria condition sometimes leading to life-threatening pathophysiological conditions like a chronic kidney
disease (van der Hoeven et al., 2012). Autosomal recessive genetic defect (Robbiano et al., 2010), excessive dietary intake of oxalate (Taylor and Curhan, 2007) and lack of oxalate metabolism ability (Menon and Mahle, 1982) are the possible reasons of hyperoxaluria in humans. Other reasons have been mentioned in Figure 1.1, but these are varied to case-to-case dependent reports.

**Figure 1.1:** Factors responsible for hyperoxaluria condition in humans. These are the most reported reasons mentioned in the literature.

Along with urea and uric acid that oxalate is also being considered as a uremic toxin for the excretory system (Robijn et al., 2011), and can alter the renal cell functions (Jonassen et al., 2003) and will differentially express abnormal proteins in nephritic cells (Koul et al., 2012). Thus, as hypothesized earlier, in hyperoxaluric condition gut lumen acts as a primary excretory system to remove excessive oxalate (Cuvelier et al., 2002). Once in the gut, this oxalate is handled by gut bacteria such as Oxalate Metabolising Bacterial Species (OMBS); because these bacteria possess oxalate utilizing enzymes and hence, can complement the missing oxalate metabolizing ability in the mammalian host (Allison, M.J. and Cook, H.M. 1981). OMBS through
degradation as well as enhanced excretion playing an active role in handling and maintaining homeostasis of oxalate in the gut (Miller and Dearing, 2013) and are important in maintaining reduced levels of oxalate in systemic fluids (Robijn et al., 2011).

Renal calculi, urinary calculi, nephrolithiasis, urolithiasis, ureterolithiasis are the clinical terms used for kidney stone disease. Worlds’ 5 % population is affected by kidney stone disease (Stamatelou et al., 2003). Renal stone is most prevalent in male (12 %) than women (5 %) (Moe et al., 2006) and in children as well (Heilberg and Schor, 2006a). Nevertheless, in certain areas of the world, as in the Middle East, the lifetime risk appears to be even higher (Curhan, 2007; Pak et al., 1985). There has been increased awareness of the renal stone disease which has recurrence rates of 50 % after ten years and 75 % after 20 years (Sutherland et al., 1985; Trinchieri et al., 1999). Calcium oxalate is the major component of about 75 % of all urinary stones (Hesse and Siener, 1997).

CaOx can crystallize into three forms: as the monoclinic monohydrate, tetragonal dihydrate or triclinic trihydrate (Hesse and Siener, 1997). Nevertheless, only the monohydrate form is thermodynamically stable. Thus, in renal stones, only monohydrate and dihydrate forms were found. To obtain the trihydrate form low temperatures are required. On the other hand, the transformation of dihydrate to monohydrate in the solid form has been described and is known as the whewellitization process (Grasesl et al., 1990; Hesse et al., 1976).

1.1.1 Mechanism of CaOx Stone Formation

The biochemical processes involved in CaOx stone formation after super-saturation are nucleation, aggregation, crystal growth, crystal retention, and formation of stone nidus and finally the development of stone (Joshi et al., 2012).

Supersaturation of Urine

For a urinary calculus to form, the urine should have an overabundance of the crystalline material that can create a stone. The concentration at which urine is saturated with the dissolved salts and crystallization commences is known as the
thermodynamic solubility product (Ksp). Ksp is the product of the concentration of the pure chemical components of the solute at the point of saturation. When the concentration of the salt in a solution is less than the solubility product, the solution is supposed to be unsaturated. As the concentration of the salt rises above its solubility product, it will attain a point where the solution turns unstable, and crystallization will spontaneously initiate; this point is designated as the formation product. The region amid the solubility product and the formation product is identified as the metastable region (Asplin et al., 1997). The development of urinary calculi is an outcome of multifarious dynamics of different components, both endorsing and hindering the formation of stone (Sayer et al., 2010; Smith, 1989).

Nucleation

Nucleation is the formation of the minutest unit lattice of a crystal species, the first stage in crystal formation. There are two categories of nucleation: homogeneous and heterogeneous nucleation. The nucleation process is identified as homogeneous when a solution is pure. In human urine, though, the chemical environment is varied, and homogeneous nucleation is not likely to happen; rather, a heterogeneous nucleation process occurs through which crystal nuclei can form on structures such as cellular material, urinary crystals, and urinary casts (Khan, 1997). Most urinary stones comprise of a mixture of crystal types indicating that mode of their formation is primarily heterogeneous nucleation. In general, thermodynamic forces require a higher level of urinary supersaturation for a homogeneous nucleation process than for a heterogeneous nucleation process, favoring the heterogeneous process in the human urinary environment (Crenshaw, 1982; Nancollas, 1982).

Aggregation

Crystal nuclei attach to one another to form larger particles, a process known as aggregation (Chung et al., 2007; Coe et al., 2010). In the urinary environment, chemically or electrically stimulated forces can encourage crystal aggregation; once crystals have amassed with one another, they are kept in place by strong intermolecular forces, and cannot be easily split up. Crystal aggregation is instrumental in stone formation, as a single crystal cannot be retained in the urinary
collecting system owing to its small size (Kok et al., 1990). CaOx urolithiasis concedes that crystal aggregation is possibly engaged in crystal retention in the kidneys as an aggregation of crystals can have a sizable impact on particle size, and aggregated crystals are usually observed in urine and renal stones (Aggarwal et al., 2013).

**Retention**

For a stone to form, crystal retention is necessary; if nucleated and aggregated crystals would be passed out of the renal collecting system with the normal urinary flow, and a clinically evident kidney stone would never form. Therefore, stone formation hinges on the retention of crystal material in the kidney until it achieves a size great enough that it is a clinical renal calculus. There are two mechanisms proposed to account for crystal retention: the free particle hypothesis and the fixed particle hypothesis (Kok and Khan, 1994; Vermeulen and Lyon, 1968). In the free particle scenario, the process of nucleation occurs entirely in the tubular lumen. As crystals move through the renal tubules, nucleation followed by rapid aggregation generates a crystalline structure large enough to be retained at the level of the papillary collecting duct (Finlayson and Reid, 1978). The alternative fixed particle hypothesis relies on the adherence of crystals to a surface point within the renal collecting system, such as renal epithelial cells (Evan et al., 2006). Although normal urothelium is resistant to crystal adhesion, chemically injured urothelium will promote crystal adherence (Gan et al., 2016; Wiessner et al., 2003).

**1.1.2 Inhibitors and Promoters of Stone Formation**

Aggregation or growth of crystals gets reduced, or its adherence to the renal epithelium is inhibited when inhibitors are used. Many inorganic (Citrate, Magnesium, and Pyrophosphate) and organic substances (Tamm-Horsfall protein, Renal lithostathine, Glycosaminoglycans, Osteopontin (Uropontin), Nephrocalcin), high urine volume are known to inhibit crystal growth, aggregation, and adhesion (Aggarwal et al., 2013; Fleisch, 1978). The crystallization processes during stone formation were influenced by regulatory substances present in the urine. Substances which increased crystallization were termed as promoters. Low urine volume, low
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urine pH, calcium, sodium, oxalate, and urate are known to promote stone formation (Basavaraj et al., 2007; Vaitheeswari et al., 2015).

Cell membranes in urine are hypothesized to serve as CaOx kidney stone promoters (Khan et al., 2000, 2002). Membrane degradation products from cell turnover and their phospholipid constituents can be detected in human urine and are thought to act as pre-existing nuclei for secondary nucleation of CaOx crystals (Khan et al., 2002). The RBC is one of the most common cells in urine in many kidney diseases and related disorders (Baggio et al., 1999; Messa et al., 2000). Red blood cell membrane fragments are a promoting factor for CaOx crystal growth and aggregation; they may aggravate CaOx stone formation (Chutipongtanate and Thongboonkerd, 2010).

1.1.3 Role of Bacteria in CaOx Stone Formation

CaOx stones account for 74% of stones (Lieske et al., 2006). Considerable overlap in CaOx urine supersaturation exists between individuals with and without kidney stones; therefore, urine chemistries cannot be the only factor in stone formation (Curhan et al., 2001). Usually, metabolic stones are not induced by bacteria, but recent studies have suggested that calcium oxalate, might originate from infections (Tavichakorntrakool et al., 2012). Emerging evidence indicates an interaction between bacteria and CaOx kidney stones. First, patients with kidney stones are more likely to have UTIs than the general population (Borghi et al., 2012; Holmgren et al., 1989; Hugosson et al., 1989). Second, bacteria have been cultured from 19–32% of CaOx stones, with non-urease-producing Escherichia coli most commonly present (Tavichakorntrakool et al., 2012; Wang et al., 2014).

E. coli was the commonest organism isolated from the urine and stone nidus, and CaOx was the major component of the stone nidus, and urea-splitting bacteria were not the major causative microorganisms (Chutipongtanate et al., 2013; Tavichakorntrakool et al., 2012). E. coli, a major member of the Gram-negative bacterial family Enterobacteriaceae, is a contributor to a wide range of kidney pathologies ranging from pyelonephritis to kidney allograft rejection (Grover et al., 2012; Kaijser et al., 1977).
1.1.4 Bacteria and their Association with Different Urinary Stones

Urolithiasis denotes stones originating anywhere in the urinary tract, including the kidney and bladder. Numerous risk factors responsible for stone formation have identified. In the oxalate synthesis pathway, glycolate oxidase caters oxalate synthesis. Glycolate oxidase converts glycolate to glyoxylate afterward converted into oxalate through oxidation (Mattevi, 2006). Infection stones make up approximately 15% of urinary stone diseases (Bichler et al., 2002). There are majorly five types of renal stones these includes calcium oxalate, calcium phosphate, magnesium ammonium phosphate (struvite), uric acid and cystine stone. The most common type of stones consists of a mixture of calcium oxalate and calcium phosphate, 70-80% of these stones are said to be idiopathic, where there is no known metabolic cause. 10-12% is due to metabolic causes (e.g., hyperparathyroidism, renal tubular acidosis, or hyperoxaluria). Calcium oxalate stones are mainly developed due to hyperoxaluria (Maschio et al., 1981). Struvite stones (infection stones) account for 15-20% of all stones (Stroup and Auge, 2010). Struvite is potentiated by a bacterial infection that hydrolyses urea to ammonium precipitates in alkaline urine forming stones (Kabдашли et al., 2006).

The negative side of some bacteria like in urinary infections can be having the major role in the kidney stone formations. *Proteus* species are the most common bacilli associated with the formation of bacteria-induced bladder and kidney stones (about 70% of all bacteria isolated from such urinary calculi) (Li et al., 2002; Prywer and Torzewaska, 2010). Urease is the essential virulence factor of these bacteria involved in stone formation. Certain bacteria, such as *Proteus mirabilis* and *Ureaplasma urealyticum*, secrete the enzyme urease which hydrolyses urea to carbon dioxide and ammonium ions. This reaction causes the urinary pH to rise (Hedelin, 2002; Hedelin et al., 1985). It has found that, in addition to urease activity, bacterial exopolysaccharides contribute to stone formation. The rapid growth-related embedding of urease-positive bacteria in the crystalline material makes the urinary stone a persistent source of recurrent urinary tract infections (Cicmanec et al., 1980; Meißner et al., 2010). Factors that may predispose one to urinary tract infections increase the likelihood of struvite stone formation (Cohen and Preminger, 1996;
Johnson and Pearle, 2007). Urinary tract obstruction, neurogenic bladder, voiding dysfunction, distal renal tubular acidosis and medullary sponge kidney are considered the main risk factors for developing infection stones (Miano et al., 2007). The role of bacterial infection in stone formation is unclear but may include alteration of urinary components, acting as a nidus for crystallization, or inducing inflammation.

1.2 Human Gut Microbiome and Human Health

The multi-cellular organisms have their microbial flora include bacteria, archaea, viruses, and eukaryotes. The collection of microorganisms that live in peaceful coexistence with their hosts is referred to as the microbiota, microflora, or normal flora. This community is commonly referred to as our hidden metabolic ‘organ’ due to their immense impact on human well-being, including host metabolism, physiology, nutrition and immune function. It is now apparent that our gut microbiome co-evolves with us (Ley et al., 2008), and that changes to this population can have major consequences, both beneficial and harmful, for human health. Indeed, Human gut microbiota is responsible for maintenance of overall human health, influences body physiology, nutritional status, the immune response against foreign pathogens and stress response (Foster and McVey Neufeld, 2013).

Recent advances in DNA sequencing technology has helped to determine the composition of the gut microbiome and their interactions with the host. In most of the studies, the microbial population has been identified based on 16S rRNA gene amplicon sequencing. But nowadays next-generation-sequencing (NGS) helps in determining the whole genome of microbes without culturing them. Bioinformatics tools such as QIIME and mother and databases such as Kyoto Encyclopedia of Genes and Genomes (KEGG) or the Clusters of Orthologous Groups (COG) helps to analyze the data and to understand the important role of microbes and their association with the host. Remarkable information generated through two important projects namely HMP: Human Microbiome Project (Turnbaugh et al., 2006) and MetaHIT: Metagenomics of the Human Intestinal Tract (Qin et al., 2010). These projects are helpful in understanding role of the healthy gut microbiome and their importance in
health and diseases. Microbial population present in the gut have a significant role in nutrient metabolism, immune modulation, xenobiotics-drug metabolism and it provides the antimicrobial protection also (Figure 1.2).

**Figure 1.2:** Positive and negative major impacts of gut microbiome on human health.

### 1.2.1 Positive Front of Microbiome

The gut microbiome obtains their essentials nutrients mainly from dietary carbohydrates. Fermentation of carbohydrates by colonic microbe results in the production of short-chain fatty acids (SCFAs) such as acetate, propionate, and butyrate, which are a rich energy source for the host and maintain the homeostasis also (Corrêa-Oliveira et al., 2016; Vinolo et al., 2011). Also, to this colonic microbes harbor the essentials gene required for the synthesis of vitamins, cofactors and secondary metabolites (Gill et al., 2006). The immune system includes innate and adaptive encounters the number of foreign antigens. The gastrointestinal tract is the main site of interaction of microbes and host immune system. According to various reports gut macrophages responsible for expression receptors such as CD 14 (Smith et al., 2001) and their pro-inflammatory response through production of IL-1, IL-6, IL-12, RANTES, TNF-β, and TNF-α is highly down-regulated (Smythies et al., 2005).
Antigen presenting cells such as dendritic cells and neutrophils also present in lower abundance in the gastrointestinal tract (Haverson et al., 2007; Ohkubo T et al., 1990). Segmented filamentous bacteria found in the small intestine are also known to elicit a pro-inflammatory response by influencing the differentiation of CD4+ T cell to Th17 cells (Wu et al., 2010). There are some gut microbes known to induce specific differentiation of CD4+ T cells in Treg cells. CD4+ T cells are the key component of adaptive immune response and are known to differentiate into IL-10 producing Treg cells in response to gut colonization of clusters IV and XIVa Clostridium species in germ-free mice (Atarashi et al., 2011).

Gut microbes play an essential role in the metabolism of xenobiotics (Maurice et al., 2013). Metabolism of various drugs results in changes in their efficacy and side effects also. Also, gut microbes produce various antimicrobial like peptides such as bacteriocin which helps to inhibit colonizations of the pathogen (Hammami et al., 2013). SCFAs have a significant role in inhibition of enteric pathogens (Spanogiannopoulos et al., 2016). Similarly, gut microbes consume all the essential nutrients which leads to starvation of pathogens which ultimately results in expressions of virulence factors of pathogens which leads to disease condition (Momose et al., 2008). Our gut microbiome evolves with us and has significant interaction with the host. It influences the host metabolism, physiology, and immune system. It has various mechanisms which prevent and survive lance of pathogens (Pacheco et al., 2012). This way gut microbes maintain beneficial interactions with host and maintain homeostasis also.

1.2.2 Negative Front of Microbiome

Nowadays, focus on a study to the investigation of the association of gut microbiome with host and understand alterations in gut microbiome due to metabolic disorders. This alteration occurs due to cause of diseases or consequence of diseases is major area yet to be studied in detail. General attributes to all these studies include reduced diversity with altered compositional and functional properties of gut microbiota in disease subjects. Metabolic perturbations are due to alternations in metabolic pathways which is also linked to the immune system. The reason behind
development metabolic alternations is mainly genetic and environmental factors. Sporadic changes in lifestyle and consumption of high energy food and degradation of complex polysaccharide by gut microbes are linked with the development of metabolic disorders such as cardiovascular diseases, insulin resistance, obesity, and diabetes. In the case of obesity, we can observe major alternations in gut microbiome such increased levels of Firmicutes and decreased the level of Bacteroidetes both in animal models and humans (Ley et al., 2006). Trimethylamine–N-oxide (TMAO), a metabolic by-product of choline metabolism in bacteria has been found to be strongly correlated with cardiovascular disorders (Nell et al., 2010). Gastrointestinal disorders are different forms of Inflammatory Bowel Diseases (IBD) such as Crohn’s disease, and ulcerative colitis is well studied. Reports suggest that gut microbiota responsible for the pathophysiology of the gastrointestinal disorder. Therefore, many therapies in the form of use of pre- and probiotics, synbiotics, antibiotics and fecal transplantation are being conducted to target gut microbiota to reduce the incidences and complications associated with IBD (Serban, 2015). Though IBD showed, there is a significant alteration in the gut microbiome, the exact reason of IBD unknown.

Some chemical compounds produced that are diffused away from GI tract by gut flora. These compounds influence the activity of distal organs; consequently, several axes viz. gut-liver axis, gut-kidney axis, and gut-brain axis have been established. Gut microbiota forms a bidirectional neuro-humoral communication system with the brain and its role in neurological diseases such as stress, anxiety, and multiple sclerosis is now well documented (Foster and McVey Neufeld, 2013). As a result, there is considerable improvement in our understanding of different liver disorders such as non-alcoholic fatty liver disease, alcoholic liver disease and liver cirrhosis (Usami et al., 2015). Similarly, production of toxic end products due to fermentation and translocation of live bacteria from the gut have been linked to chronic kidney disease and end-stage kidney disease (Sabatino et al., 2015).

Thus, understanding the extent of perturbation on specific disorder has become an active area of research across the globe. Studies like these are contributing to identifying key players in the disease diagnosis. Although, whether the extent of gut
microbial perturbation is a cause or consequence of specific disorder is unclear, it has been a prime target for interventions in the prevention and sometimes treatment of many diseases including chronic kidney disease (Ramezani and Raj, 2014; Scott et al., 2015).

1.3 Bacteria, Oxalic Acid Removal, and its Relation to the Human Health

The human GIT is colonized by innumerable different bacterial species (Macfarlane and Macfarlane, 2004), and are largely individual specific. These microbial profiles are steadily conserved but can fluctuate to some degree over time in individuals owing to their diet and numerous factors like ingesting antibiotics and compounds such as oxalate (Iapichino et al., 2008). GIT bacteria participate in various biochemical reactions which impact human health and nutrition and can also influence decomposition of many dietary ingredients that cannot be digested by humans (Hooper et al., 2002). Humans are deficient in enzymes required to metabolize endogenous and dietary oxalate, a toxic compound triggering hyperoxaluria and calcium oxalate urolithiasis (Allison and Cook, 1981). In humans, oxalate is eliminated through various routes such as via urine, as insoluble calcium oxalate salts in the feces and degradation of oxalate by gut microbes (Robbiano et al., 2010; Taylor and Curhan, 2007; Menon and Mahle, 1982). However, microbial oxalate degradation is a negative risk factor for kidney disease, and significant host-microbe metabolic interactions have a promising protective role in stone diseases.

Understanding on the beneficial microbiota has led to the development of probiotics. Some species have been broadly employed as probiotics in food products, and pharmaceuticals due to the indigenous colonization of the gut by these valuable bacteria which may contribute to human health in numerous aspects (Abratt and Reid, 2010). Of late, studies have demonstrated that specified bacterial genera can exploit the oxalate existing in the gut lumen, possibly averting its absorption into the bloodstream and subsequent excretion in the urine (Magwira et al., 2012).

1.3.1 Lactobacillus species as a Probiotics
Lactic acid bacteria (LAB) have been a source of innumerable health benefits to the human kind. Consumption of LAB with food or supplements has found to reduce the level of serum cholesterol (Anderson and Gilliland, 1999). They also exclude pathogenic bacteria from the intestine (Hemarajata and Versalovic, 2013), enhance taste in food substances (Badel et al., 2011), confer immune-modulatory activity with anti-oxidative effects (Liu et al., 2011), and reduce the risk of colon cancer (Jones, 2016; Wollowski et al., 2001). The oxalate-degrading *Lactobacillus* and *Bifidobacterium* sp. are the most probable bacterial candidates with the significant potential for the treatment and management of kidney stone disease (Abratt and Reid, 2010). These genera possess the ‘Generally Regarded as Safe’ (GRAS) status facilitating their commercial use as probiotics. Similar mechanism (their oxalate degradation mechanism) finds similarities to *O. formigenes*, possessing both *oxc*- and *frc*-genes; however, their exact mechanism of oxalate uptake is yet to be elucidated. *Lactobacillus* and *Bifidobacterium* sp. Are identified as “generalist oxalotrophs” due to their inherent potential to grow on a range of alternate energy sources including oxalate (Turroni et al., 2007) allowing them to endure in the gut in low oxalate conditions as well. Most of the genetic research has largely focused on *Lactobacillus acidophilus* and *Lactobacillus gasseri*. In both species, the *frc*- and *oxc*-gene homologs are situated adjacent to one another on the genome, in contrast to *O. formigenes* where the genes are positioned at a distance (Azcarate-Peril et al., 2006; Turroni et al., 2007). Notably, mildly acidic (pH 5.5) growth conditions are essential for the expression of both genes, like those existing in the lower GIT (Azcarate-Peril et al., 2006).

### 1.3.2 In-vitro and Animal Models for Oxalate Utilization by Gut Bacteria

The *in vitro* oxalate degradation capability of the numerous *Lactobacillus* and *Bifidobacterium* sp. is both species and strain specific. It has been established that the best oxalate-degraders (68%) in the *Lactobacillus* group belonged to *L. paracasei*, *L. gasseri* or *L. acidophilus* (Mogna et al., 2014). Turroni et al. (2007) isolated a range of novel *Lactobacillus* sp. (including *L. acidophilus* and *L. gasseri*) that demonstrated more than 50% of the available oxalate degradation. In the *Bifidobacterium* group, Federici et al., (Federici et al., 2004) registered that the *Bifidobacterium lactis* DSM
possessed the highest level of oxalate degradation at 61%, while Bifidobacterium longum MB 282 and Bifidobacterium adolescentis MB 238 exhibited 35% and 57% degradation respectively. In general, all the Bifidobacterium strains tested had recorded reduced degradation activity in comparison to Lactobacilli, probably due to the toxic effects of oxalate on Bifidobacterium. In the search for efficient oxalate degraders, laboratories have also screened and novel isolated from the guts of various animals. Murphy et al. (Murphy et al., 2009) isolated Bifidobacterium and Lactobacillus strains from cats and dogs and demonstrated in-vitro oxalate degradation in 61% of the Lactobacillus isolates. In contrast, the Bifidobacterium sp. showed very little degradation activity for all species tested. Two strains of L. murinus and two of L. animalis were tested in a rat model, but only the L. animalis strains gave a significant reduction in urinary oxalate levels (Campieri et al., 2001). Other workers have screened probiotic strains for oxalate degradation activity and their ability to modulate inflammation in the human GIT. They have found strains of L. plantarum, L. acidophilus, B. breve and B. longum as promising candidates for this purpose in in-vitro experiments (Bhat et al., 2016; Giardina et al., 2014).

1.3.3 In-vivo Oxalate Utilization Studies in Humans by Gut Bacteria

Number clinical trials with human participants have been undertaken to examine the effect of oral administration of lactic acid bacteria probiotics on Dietary hyperoxaluria, plasma oxalate concentration, and urinary oxalate excretion. However, most of these studies are limited in various ways, with low numbers of participants and varying test procedures making an evaluation of their comparative effectiveness difficult (Lieske et al., 2005).

The earliest studies suggested that lactic acid bacteria, such as Lactobacillus and Bifidobacterium reduced urinary oxalate excretion by 40–50%. In a study involving six calcium stone forming participants (Campieri et al., 2001), the reduction continued after the treatment ended. This was comprehended by a study with participants having enteric hyperoxaluria, where the Oxadrop (VSL Pharmaceuticals) probiotic preparation was administered. This formulation contains a mixture of four
lactic acid bacterium species (*L. acidophilus*, *L. brevis*, *S. thermophiles* and *B. infantis*). One packet of Oxadrop per day relegated urinary oxalate excretion by 19%, and this amplified to 24% when two packets per day were administered (Lieske et al., 2005). This study had 20 participants, 10 receiving Oxadrop daily and 10 in the placebo group.

However, recent studies, using randomized, double-blind groups of stone formers with idiopathic hyperoxaluria, did not demonstrate a decrease in urinary oxalate excretion, in spite of using the same Oxadrop probiotic preparation at the same concentration of $4-9 \times 10^{11}$ CFU. The sample size was 20 participants with 10 in each group (10 receiving Oxadrop daily and 10 in the placebo) (Goldfarb et al., 2007). Lieske et al. (Lieske et al., 2010) performed a double-blind study to ascertain the effect of Oxadrop and the symbiotic AKSB preparation comprising fructooligosaccharides, *Enterococcus faecium* and *Saccharomyces cerevisiae* (Agri-King Symbiotic, Fulton, USA) on 40 participants with mild hyperoxaluria receiving an oxalate controlled diet. It was established that limited diet has diminished urinary oxalate while the probiotics and symbiotics did not. Though, the selection of the organisms the AKSB preparation and their impact on oxalate metabolism is uncertain. Recently, Siener et al. (Siener et al., 2013a) placed 20 healthy participants on an oxalate-rich diet for six weeks and administered Oxadrop for five weeks. Urinary oxalate excretion and plasma oxalate concentrations increased with the oxalate-rich diet and did not decrease by the ingestion of probiotics in the treated group.

Liebman and Al-Wahsh (Liebman and Al-Wahsh, 2011), studied the impacts of probiotics on dietary oxalate absorption, recognized that the disparity in the results between laboratories was owed to variances in protocols, dissimilarities in diet and perhaps the choice and management of the specific probiotics. However, there is an indication that probiotics can, in certain cases, cause a decrease in oxalate following a high oxalate diet (Okombo and Liebman, 2010) or in cases of enteric hyperoxaluria (Lieske et al., 2005). Therefore, there is a necessity for more accurate studies on the use of oxalate-degrading lactic acid bacteria for the treatment of patients at risk of
developing kidney stones. Effective bacterial strains and administration protocols are yet to be developed and proven for future therapeutic applications.

It is important to consider the issues around the benefits of chronic administration of the oxalate degrading probiotics as opposed to undertaking permanent re-colonization of the guts of patients suffering from enteric hyperoxaluria. There is currently limited information correlating the lack of an oxalate-degrading bacterial population and the development of kidney stone disease due to the variations in normal baseline microbial numbers (Knight et al., 2013). The factors affecting microbial colonization, including diet and host-microbial interactions, also require further investigation (Peterson et al., 2015; Ursell et al., 2013).

Balaji and Menon (Balaji and Menon, 1997) reported that under normal conditions, the colon is the main site of oxalate absorption, with approximately 3 to 5% of dietary oxalate being absorbed. However, researchers reported that an amplified intestinal absorption of oxalate possibly will lead to hyperoxaluria with a notably higher chance of urinary stone formation (Chadwick et al., 1973; Earnest et al., 1974; Smith et al., 1991). A study performed by Sutton and Walker (Sutton and Walker, 1994) demonstrated that the idiopathic calcium oxalate stone formers with mild hyperoxaluria showed no evidence of any considerable modification in the renal oxalate transport and assumed that an increased dietary load of oxalate with a probable hyper-absorption was the responsible mechanism. The relative amounts of calcium and oxalate in the diet may be an important variable in determining the rate of oxalate absorption and urinary excretion (Smith et al., 1991). There is credible evidence that increased absorption with the consequent hyperoxaluria may be the result of other intestinal disorders, including the syndrome of bacterial overgrowth and ileal resections for inflammatory bowel disease (Chadwick et al., 1973; Earnest et al., 1974; Smith et al., 1991). In fact, since 1968, nephrolithiasis was recognized as a complication of inflammatory bowel disease or intestinal resection (Gelzayd et al., 1968) and Diabetes was associated with an increased risk of incident kidney stone formation (Taylor et al., 2005). Intestinal hyper-absorption of oxalate was reported to make a considerable contribution to urinary oxalate, even in the absence of
gastrointestinal disorders. It was reported that absorption of oxalate was increased in patients with a substantial increase in CaOx stones (Voss et al., 2006).

*Oxalobacter formigenes* was the earliest reported oxalate-degrading gastrointestinal inhabitant. This species, which is common in the mammalian gut, requires oxalate as a carbon and energy source. Previously, a clinical study by Troxel et al. (Troxel et al., 2003) established a relationship amongst low rates of intestine colonization by oxalate-degrading bacteria, precisely *Oxalobacter formigenes*, and a heightened possibility of hyperoxaluria. Moreover, this organism was reported to be a natural inhabitant of the gastrointestinal tract (GIT) of vertebrates, including humans, and it was the best-characterized microorganism of the intestinal microbiota with an oxalate-degrading mechanism (Duncan et al., 2002) involving decarboxylation of oxalate, yielding formic acid and CO$_2$. This reaction was reported to generate a proton gradient that contributes to the generation of one ATP molecule when it is coupled with oxalate/formate transport. Bacteria when utilizing CaOx as a source of carbon and energy are so-called oxalotrophic bacteria (Sahin, 2003), and for studying the oxalotrophic bacterial diversity use of frc-gene as a marker for any the environment (Khammar et al., 2009). Also, other species from the genera *Lactobacillus*, *Bifidobacterium*, *Enterococcus*, and *Eubacterium* can utilize other substrates for growth along with oxalate (Miller and Dearing, 2013). The frc-gene (formyl-CoA transferase) EC: 2.8.3.16, a key enzyme in oxalate metabolism wherein CoA moiety from formyl-CoA transfer to oxalate and well characterized from *Oxalobacter formigenes* (Baetz and Allison, 1990). Exploring the diversity and metabolism of these bacteria, and measuring their oxalate catabolism in-vitro and in-situ has been having a great interest to cross-adulate the oxalate-related complications (Figure 1.3).
Figure 1.3: Illustration of depicted role of gut microbes in oxalate homeostasis for systemic oxalate pool. Oxalate degradation and the gut secretion of oxalate in the gut lumen, these are the major phenotypes involved in maintaining the systemic oxalate in humans. In the intestine, oxalate may coalesce with calcium, sodium, magnesium, potassium, or iron to form non-soluble salts. It is proposed that specific bacteria regulate oxalate homeostasis by degrading free oxalate and thereby decreasing its concentration in urine and plasma, thus preventing its absorption. Intestinal oxalate-degrading bacteria could, therefore, be crucial in sustaining oxalate homeostasis and plummeting the probability of kidney stone development. Another mechanism as the endogenously derived oxalate is secreted into the colon and then reabsorbed, and that the bacterial products reduced the re-absorption either directly or by degrading the secreted oxalate.

1.4 Scope of the Thesis

The only recommended standard therapies for critical kidney stones are surgical interventions suggested by American Urological Association guidelines inclusions of percutaneous nephrolithotomy (PNL), shock-wave lithotripsy (SWL), a combination of PNL and SWL and open surgeries (Preminger et al., 2005). Also, medical dissolution of stones is challenging, and surgical interventions are expensive as well as require extra care during operations. So far oxalate reduction by human gut
bacteria in which from systemic fluid was found possible, and this research orientation configured towards the defining OMBS diversity and their oxalate utilization looking towards intervention therapy.

The colonization of *Oxalobacter formigenes*, a known OMBS in the gut has been critically investigated. And a direct link between lack of colonization of *O. formigenes* as a major risk factor and its inverse association with calcium oxalate kidney stones in humans (Kaufman et al., 2008) and canines (Gnanandarajah et al., 2012a) has been established. In another study, the predominance of other OMBS like *Lactobacillus* spp., *Bifidobacteria* sp. have been shown to be protective in the development of oxalate kidney stone in a specific population (Magwira et al., 2012). Similarly, indigenous gut inhabitants including *Enterococcus faecalis*, *Providencia rettgeri*, *Eubacterium lentum* and *Escherichia coli* have been proposed as therapeutic candidates for the treatment of calcium oxalate disease (Abratt and Reid, 2010). These OMBS including *Oxalobacter formigenes* share common oxalate-degrading enzymes including membrane-associated antiporter (*oxIT*), formyl-CoA transferase (*frc*) and oxalyl-CoA decarboxylase (*oxc*) (Siener et al., 2013b). In all the OMBS studied so far, these genes have been detected, and functional genes such as *frc* gene have been used as a molecular marker to assess the active OMBS diversity from soil niche (Khammar et al., 2009).

In a large portion of the world including India, incidences, and frequency of kidney stone disease is higher; these areas are often called as stone belt areas (López and Hoppe, 2010). Especially in India, the reasons for this high prevalence include but are not limited to: large genetic variations, different dietary habits, and vast geographic distribution. Comprehensive reports on oxalate kidney stone associated dysbiosis in subjects are lacking. There are many questions needs to be answered for an understanding of the gut ecology, the oxalate metabolism through gut inhabitants.

1. Who are there in the gut of hyperoxaluric human subjects and extent of perturbation in bacterial diversity?
2. Is the oxalate handling by the gut-inhabiting OMBS? Who are they? Do they are constitutive microbiome components and do they dwell with the extent of oxaluria concentration, and have dysgenic functional diversity?

3. Since the Archaea, Fungi, and Microeukaryotes along with the Eubacteria are the major components of the microbiome. Do they impact the hyperoxaluria condition?

4. Can we attempt to isolate Oxalate Metabolising Bacterial Species (OMBS) from the human gut and check for its functional attributes towards the remedial approach?

Answers from above question were expected to unravel the black box of ecological understandings in more prevalent disorders like hyperoxaluria in the Indian scenario. The disturbed flora in hyperoxaluria could disrupt the symbiotic relationship that exists under natural environments and leads to the production and absorption of pro-inflammatory and other harmful by-products, and concurrently reduce the positive functions and products contributed by the normal flora. Such episodes can be traceable to contribution to inflammation, uremic toxicity and cardiovascular, nutritional, and further problems at very early stages.

1.5 Objectives of the Thesis

Etiology of hyperoxaluria and the role of colonic bacteria in the oxalate degradation in persons with enteric malfunctions has not been taken into consideration. There is no established therapy employing the use of bacterial-bionts for the reduction of urinary oxalate excretion in calcium oxalate stone patients. This notion is perhaps not quite surprising considering the paucity of the gaps in knowledge of oxalate dynamics in the gut and arithmetical views for actual oxalate metabolizing bacterial diversity. However, dietary supplementation bacterial-bionts with traditionally used probiotics will be a potential strategy for increasing the degradation of dietary oxalate without any side effects. Thus, the study was carried out to explore and correlate oxalate metabolizing bacterial diversity. This study aims to investigate the correlation of oxalotrophic bacteria in human gut associated with the metabolic hyperoxaluria disorders and its occurrence. Present work focused on the assessment of differences between the compositions of the gut microbiota in individuals with
recurrent kidney stones as a symptomatic phase of hyperoxaluria and compared it with healthy individuals. Also, targeted-gene metagenome approach for surveillance of active OMBS in human gut flora employed. This study was started by taking the following objectives to understand the relationship of microbiota and the gut ecology.

1. Understanding the differences in bacterial diversity in the gut of patients suffering from hyperoxaluria as compared to the healthy control individuals.

2. Exploration of the Oxalate Metabolising Bacterial Species (OMBS) diversity from the human gut.

3. Quantitative surveillance of specific bacteria in the human gut.

4. Cultivation and characterization of OMBS from different ecological niches.