CHAPTER 3
Chapter 3: Comparative Exploration of Stool Bacterial Diversity Associated with Recurrent Oxalate Kidney Stone Disease and Control Subjects

3.1 Introduction

Excessive oxalate in the systemic fluids and the excretion is referred to as a hyperoxaluria. Though the oxalate is a by-product of metabolism; impaired metabolism, and ingestion of external oxalate substantially contribute to an excessive oxalate pool generation (Cochat and Rumsby, 2013). Oxalates in the systemic fluid act as the uremic toxin are crystallized in the renal tubule and urine (Ivanovski and Drüeke, 2013). Crystallization in the urinary track is a serial phenomenon that follows salt aggregations and blockage of the urinary tract, is known as kidney stones or the urinary stones. Hyperoxaluria condition is the principle cause of oxalate stones and the recurrent episodes of kidney stones therefore regarded as a symptomatic phase in humans. Sometimes such recurrent episodes and the uncontrolled oxaluria leads to pathophysiological conditions like chronic kidney disease and end-stage renal disease. All these pathologies were mainly due to the impaired oxalate production, digestive absorption and kidney reabsorption in the host body. Progression towards the life threatening is only due to lack of oxalate metabolism ability are some of the possible reasons of hyperoxaluria extremities.

In hyperoxaluria elevated oxalates, being of uremic toxin the gut lumen instead of the urinary system acts as a primary excretory system to remove excessive oxalates (Cuvelier et al., 2002). Once in the gut, oxalates are handled by gut bacteria collectively identified as Oxalate Metabolising Bacterial Species (OMBS) which complement the missing oxalate metabolizing ability in the mammalian host (Allison and Cook, 1981). OMBS, through degradation and enhanced excretion, plays an active role in handling and maintaining homeostasis of oxalate in the gut (Miller and Dearing, 2013) and also maintains reduced levels of oxalates in systemic fluids (Robijn et al., 2011). So far, studies concerning hyperoxaluric-gut-microbiome have
largely focused on *Oxalobacter formigenes*, a known key player in oxalate homeostasis in the gut. A direct link between lack of colonization of *O. formigenes* as a major risk factor and inverse association with calcium oxalate kidney stones in human (Kaufman et al., 2008) and canines (Gnanandarajah et al., 2012a), has been established. The OMBS; including *O. formigenes*, share common oxalate-degrading enzymes, including oxlT, frc, oxcI, genes for membrane-associated antiporter, formyl-CoA transferase and oxalyl-CoA decarboxylase respectively (Siener et al., 2013b). In all OMBS studies 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. We noticed that comprehensive reports on oxalate kidney stone associated dysbiosis in human subjects are lacking.

In search of the impact of hyperoxaluria on the gut microbiota literally, we can find the contribution of gut inhabitants to the health status. In the human gut microbiota and microbiome, Eubacteria population and their gene content shared the largest contribution to the gut ecology and fundamental metabolism. The corroborative contribution of Eubacteria by their presence, diversity and the metabolic capacities to the hyperoxaluria is still elusive. To strive for the comparative Eubacteria, diversity in the hyperoxaluric and healthy control status will give the unique knowledge about the disease associated flora and the extended perturbation in diversity along with the metabolic capacities. So in this chapter, we access the Eubacteria diversity by their presence and their quantitative surveillance in individuals with recurrent kidney stones (KSD), which is a symptomatic phase of hyperoxaluria, and healthy individuals (HLT). *The term “subjects” in this entire thesis refers to all recurrent oxalate kidney stone disease patients (KSD) and healthy enrolled individuals (HLT).* Eubacteria profiling was achieved by 16S rRNA gene sequencing, whereas differences between the compositions of the gut microbiota and their deviations in hyperoxaluria condition were measured.

### 3.2 Materials and Methods

We used the following kits: QIAmp DNA Stool Mini kit (Qiagen, USA), AmpliTaq Gold PCR Master Mix (Life Technologies, USA), Agencourt AMPure XP DNA purification
Bead (Beckman Coulter, USA), Agilent High Sensitivity DNA Analysis Kit (Agilent Technologies, USA), Ion Xpress™ Plus gDNA Fragment Library Preparation kit (Life Technologies, USA), Ion OneTouch™ 200 Template Kit v2 DL (Life Technologies, USA), Ion Sequencing 200 kit (Life Technologies, USA) and SYBR Green PCR Master Mix (Applied Biosystems Inc., USA) were procured from respective vendors.

### 3.2.1 Stool Metagenomic DNA Extraction from Enrolled Subjects

Total 73 stool samples from 73 Indian subjects were collected under ‘case-control study’ and ‘healthy cohort study.’ Fecal samples from the above subjects were collected in a sterile container and stored at -80 °C until they were used for DNA extraction and followed for bacterial diversity and targeted metagenomic analysis. All the procedures were pre-approved and then conducted the experiments for the actual analysis by the National Centre for Cell Science ethical committee and biosafety committee.

To extract total DNA content from stool samples QIAmp DNA Stool Mini kit (Qiagen, USA) was used as per manufacturer’s instructions with little modifications as follow.

In a 15 ml falcon tube approximately 200 mg stool was taken and 1.4 ml ‘Buffer ASL’ was added and vortexed till the stool sample was thoroughly homogenized. Later on for 5 min suspension was kept at 70 °C, vortexed for 15 s and centrifuged at 10,000 rpm for 1 min.

The supernatant was transferred to a new 2 ml microcentrifuge tube, and one tablet of ‘InhibitEX’ provided in the kit was added to it. Samples were vortexed immediately for 1 min to dissolve the tablet completely.

The suspension was kept for 1 min incubation at room temperature to allow inhibitors to adsorb to the InhibitEX matrix.

The suspension was centrifuged at 10,000 rpm for 3 min to pellet inhibitors bound to InhibitEX matrix.

The supernatant was transferred to a new 1.5 ml microcentrifuge tube.
200 μl of supernatant was transferred to a new tube, and 15 μl of proteinase K was added to it.

To this mixture of supernatant and proteinase K, 200 μl ‘Buffer AL’ was added and vortexed for 15 s.

After incubation at 70°C for 10 min, 200 μl of absolute ethanol was added to the lysate and mixed by vortexing.

The whole lysate was then transferred to the QIAamp spin column and centrifuged at 10,000 rpm for 1 min.

A two-time wash of 500 μl ‘Buffer AW1’ and 500 μl ‘Buffer AW2’ was given and QIAamp spin column was transferred to a new 1.5 ml micro-centrifuge tube.

Purified DNA was eluted by adding 100 μl ‘Buffer AE’ and centrifugation at 10,000 rpm for 1 min.

NanoDrop 1000 spectrophotometer (Thermo Scientific, USA) was used to check purity and measure the concentration of extracted DNA.

**3.2.2 16S rRNA gene Amplicon Generation and Sequencing on Ion Torrent PGM**

We used Ion Torrent PGM sequencing technology for 16S rRNA amplicon sequencing (Figure 3.1).

PCR was set up in 50 μl reaction using AmpliTaq Gold PCR Master Mix (Life Technologies, USA) and with 16S rRNA V3 region specific bacterial universal primers: forward primer 341F (5'-CCTACGGGAGGCAGCAG-3') and reverse primer 518R (5'-ATTACCGCGGCTGCTGG-3') (Bartram et al., 2011).

Conditions used for PCR: initial denaturation at 95 °C for 10 min, 20 cycles of 95 °C for 1 min, 56 °C for 30 s, and 72 °C for 30 s with a final extension at 72 °C for 10 min.

Agencourt AMPure XP DNA purification Bead (Beckman Coulter, USA) were used to purify PCR product, end-repaired and ligated with specific barcode adaptor as explained in IonXpress™ Plus gDNA Fragment Library Preparation user guide.
Bioanalyzer 2100 (Agilent Technologies, USA) was used to fragment size distribution and molar concentrations of the amplicon using High Sensitivity DNA Analysis Kit as per manufacturer's instructions.

Ion OneTouch™ 200 Template Kit v2 DL (Life Technologies, USA) was used to carry out emulsion PCR on diluted and pooled amplicon (10 samples).

Finally, sequencing of the amplicon libraries was carried out on 316 chips using the Ion Torrent PGM system and Ion Sequencing 200 kit (Life Technologies, USA).

**Figure 3.1:** Diagrammatic representation of methodology followed for 16S rRNA gene amplicon study by using Ion Torrent PGM. The chart depicts the amplicon
generation from the test subjects DNA to the date interpretation in a stepwise manner.

3.2.3 Raw Sequences deposition

Raw sequences were deposited to NCBI-SRA under accession number SRP066940.

3.2.4 Sequence Processing and Bioinformatics Analysis

Mothur pipeline (Schloss et al., 2009) was used to pre-process all PGM reads with following conditions: minimum 150 bp to maximum 200 bp, maximum homopolymer -5, maximum ambiguity -0, and average quality score -20.

Single FASTA files were generated by pooling all high-quality amplicon reads for further analysis in QIIME: Quantitative Insights Into Microbial Ecology (Caporaso et al., 2010).

Operational Taxonomic Units (OTUs) clusters were obtained by Closed reference based OTU picking approach at 97% sequence similarity using UCLUST algorithm (Edgar, 2010) and a representative sequence from each OTU was selected for further analysis.

All OTUs were assigned to the lowest possible taxonomic rank that is genus by utilizing RDP Classifier 2.2 (Wang et al., 2007) and Greengenes database 13.8 with a confidence score of at 80%.

Alpha diversity was calculated using estimates such as Chao1 and PD whole tree and Simpson and Shannon indices on rarefied sequences. For beta diversity measures to understand the microbial communities in Indian individuals, UniFrac was used. Since ‘UniFrac’ analysis is known to be affected by sequencing depth and evenness, another tool ‘jackknifing’ in which samples are subjected to even subsampling for ‘n’ replicates was used and UniFrac distance matrix was calculated for each replicate (Lozupone and Knight, 2005). We generated 1000 replicates of PCoA coordinates and then applied Procrustes analysis to each PCoA replicate to plot the average position of individuals on PCoA plot. The interquartile range of the distribution of points
among the replicates was represented as an eclipse around the point (Lozupone et al., 2011).

3.2.5 QPCR Based Quantitative Estimation of Bacterial Taxa and Genes

Absolute quantification of 16S rRNA gene copy numbers was estimated using qPCR assay to confirm the increased or decreased abundance of specific bacterial taxa in HLT and KSD subjects. Schematic representation of qPCR assays mentioned in Figure 3.2. Additionally, to confirm the enrichment of OMBS in KSD subjects absolute quantification of \textit{frc}- and \textit{oxc} genes were performed. In Table 3.1 targeted groups of bacteria, primer sequences, and amplicon size have been summarized. Absolute quantification qPCR assays were performed as for each gene, triplicate qPCR reactions tubes were prepared (10 µl each) containing a respective pair of primers, 50 µg of sample DNA and SYBR green master mix (Applied Biosystems Inc. USA). The reactions were performed on 7300 Real-time PCR system (Applied Biosystems Inc. USA).

Reaction was set as follows,

\begin{align*}
\text{Template (50 ng/ µl)} & : 1 \mu l \\
\text{Forward primer (0.1 µM)} & : 0.1 \mu l \\
\text{Reverse primer (0.1 µM)} & : 0.1 \mu l \\
\text{Power SYBR Green PCR Master Mix} & : 5 \mu l \\
\text{Distilled water} & : 3.8 \mu l \\
\text{Total} & : 10 \mu l
\end{align*}

Conditions used for qPCR assay: one cycle of 95 °C 10 min followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min.
Figure 3.2: Diagrammatic representation of methodology followed for the qPCR assays. Assay plate depicts here with the standard gene with known copy number, and the test subjects DNA were tested in triplicate.

A standard curve for each set of primers was generated from 10-fold serial dilutions of a known concentration of PCR products for each group. Additionally, melting curve analysis was performed at the end of qPCR cycles to check the amplification specificity. For enumerations of tested gene copy numbers for each group, average values of the triplicate were used using standard curves generated. For all assays, PCR efficiency was maintained above 90% with a correlation coefficient >0.99. Variations in copy number of targeted bacterial genera and frc- and oxc-genes were assessed using Mann-Whitney U test.
Table 3.1: Table showing the targeted bacterial taxa, bacterial genes and their primers used for qPCR assays. Modified and reprinted (adapted) from Suryavanshi et al. (2016), Hyperoxaluria leads to dysbiosis and drives selective enrichment of oxalate metabolizing bacterial species in recurrent kidney stone endures, Scientific Reports, Copyright © 2016, Nature Publishing Group.

<table>
<thead>
<tr>
<th>Target bacteria and genes</th>
<th>Primers used</th>
<th>Sequence (5’-3’)</th>
<th>Product (bp)</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total bacteria</td>
<td>341F</td>
<td>CCTACGGAGAGCAAGCAG</td>
<td>177</td>
<td>(Bartram et al., 2011)</td>
</tr>
<tr>
<td></td>
<td>518R</td>
<td>ATACCGCGGCTGCTGG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phylum Firmicutes</td>
<td>FirmiF</td>
<td>CTGATGGGACACCCGCGT</td>
<td>429</td>
<td>(Hermann-Bank et al., 2013)</td>
</tr>
<tr>
<td></td>
<td>FirmiR</td>
<td>ACGGTTGACTGCTGTTT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phylum Bacteroidetes</td>
<td>BacterioF</td>
<td>CCGGAWTYATGGTTAAAAAGG</td>
<td>414</td>
<td>(Hermann-Bank et al., 2013)</td>
</tr>
<tr>
<td></td>
<td>BacterioF</td>
<td>GGTAAGGTTCCGCGTA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Genus Lactobacillus group</td>
<td>F_Lacto 05</td>
<td>AGCAGTGGGAATCTTCCA</td>
<td>352</td>
<td>(Furet et al., 2009)</td>
</tr>
<tr>
<td></td>
<td>R_Lacto 04</td>
<td>CGCCACTGGTGTTCTCATATA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Genus Bifidobacterium group</td>
<td>Bif16S3</td>
<td>AGGGTTCAGTTCTGCTCAG</td>
<td>156</td>
<td>(Magwira et al., 2012)</td>
</tr>
<tr>
<td></td>
<td>Bif16S4</td>
<td>CATCCGCATTACCACC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Genus Prevotella group</td>
<td>PrevF</td>
<td>CACCAAGGCAGATCA</td>
<td>283</td>
<td>(Larsen et al., 2010)</td>
</tr>
<tr>
<td></td>
<td>PrevR</td>
<td>GAGTAACGCYGGACCT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Genus Feacalibacterium group</td>
<td>FPF</td>
<td>GGAGGAAAGTTCTTGCG</td>
<td>252</td>
<td>(Bhute et al., 2016)</td>
</tr>
<tr>
<td></td>
<td>FPR</td>
<td>AATTCCGGCATTCTGCTGACT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Genus Megasphaera group</td>
<td>MegaF</td>
<td>CTAGTGCAACGGGTGAGT</td>
<td>179</td>
<td>(Bhute et al., 2016)</td>
</tr>
<tr>
<td></td>
<td>MegaR</td>
<td>CAGACGGCCACTGATCGTC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Species Oxalobacter formigenes (oxc-gene)</td>
<td>F_OFocx</td>
<td>CGACAATGTAAGTTGACTGA</td>
<td>164</td>
<td>(Prokopovich et al., 2007)</td>
</tr>
<tr>
<td></td>
<td>R_OFocx</td>
<td>CGTTTGTGTCGGACGAA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Oxalotrophic bacteria (frc-gene)</td>
<td>frc171_F</td>
<td>CTSTAYTCCASATGCTSAAC</td>
<td>135</td>
<td>(Khammar et al., 2009)</td>
</tr>
<tr>
<td></td>
<td>frc306_R</td>
<td>GDSAAGCCCATVCGRTC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Species Lactobacillus plantarum</td>
<td>Lplant_F</td>
<td>TTACATTGAGTGAGGTGCGGA</td>
<td>62</td>
<td>(Klocke et al., 2006)</td>
</tr>
<tr>
<td></td>
<td>Lplant_R</td>
<td>AGGTGTATTCCCGGGCTTCT</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
3.2.6 Subject grouping Based on Stone Characteristics and Colonization Status of *Oxalobacter formigenes* (*O. formigenes*)

We then divided the study participants based on family history, the frequency of stone formation and colonization status of *O. formigenes*. The rationale of subject segregation especially of KSD subjects was a substantially higher risk of stone formation in male patients having a family history (Curhan et al., 1997). In the extreme case of stone endures (third-time episode) who have a higher number of stone episodes, there are more chances of development of chronic kidney diseases (Rule et al., 2011) and has an inverse relationship of *O. formigenes* with the stone formation (Siener et al., 2013b). Thus, the subjects were grouped into five categories with four subjects in each group viz. KSD with family history (KSD_FH), KSD *O. formigenes* colonizer (KSD_OX_COL), KSD *O. formigenes* non-colonizer (KSD_OX_N_COL), KSD with third episode (KSD_THIRD) and Healthy control (HLT). Please refer the Table 3.2 for the sample characteristics and segregation of samples into the five groups.

**Table 3.2:** Criteria for the grouping the subjects

<table>
<thead>
<tr>
<th>Groups</th>
<th>KSD_FH</th>
<th>KSD_OX_COL</th>
<th>KSD_OX_N_COL</th>
<th>KSD_THIRD</th>
<th>HLT</th>
</tr>
</thead>
<tbody>
<tr>
<td>Characteristics</td>
<td>KSD with family history</td>
<td>KSD with <em>Oxalobacter</em> colonization</td>
<td>KSD without <em>Oxalobacter</em> colonization</td>
<td>KSD with third episode</td>
<td>Healthy control</td>
</tr>
<tr>
<td>Samples Included</td>
<td>KSD3, KSD9, KSD21, KSD25</td>
<td>KSD7, KSD10, KSD22, KSD28</td>
<td>KSD12, KSD20, KSD29, KSD31</td>
<td>KSD1, KSD13, KSD27, KSD32</td>
<td>HLT8, HLT9, HLT10, HLT11</td>
</tr>
</tbody>
</table>

3.2.7 Other Analyses of 16S Amplicon Data

Effect of hyperoxaluric condition on microbial interactions was assessed using network analysis of co-occurrence and co-exclusion as described before (Faust et al., 2012). Also, the metabolic capabilities of the bacterial community were inferred by utilizing a computational approach: PICRUSt (phylogenetic investigation of communities by reconstruction of unobserved states) (Langille et al., 2013). Briefly, reference based OTU picking was performed in QIIME and the OTU table was imported to the online PICRUSt tool at [http://huttenhower.sph.harvard.edu/galaxy/](http://huttenhower.sph.harvard.edu/galaxy/), and functional predictions were made using KEGG Orthology (KO) database. The resulting data was analyzed using STAMP - version
2.0.2 (Parks and Beiko, 2010). Functional gene predictions data was used for identification of oxalate bioconversion pathways present in the gut microbiome.

### 3.3 Results

We concentrated on the Eubacteria population, whereas diversity structure and their perturbations in hyperoxaluria condition had been checked on case-control study population. Some of the major findings have been described below.

#### 3.3.1 Comparative Eubacterial Diversity in HLT and KSD

By using Ion Torrent PGM, we obtained ~1.3 million good quality 16S rRNA gene amplicon reads from all the study participants. Using QIIME and UCLUST algorithm, these sequences were grouped into 19,633 unique OTUs. No significant differences were observed in alpha diversity indices viz. Chao1, observed species, phylogenetic diversity, Shannon and Simpson (Appendix VI) between the HLT and KSD subjects. We were able to recover 11 bacterial phyla from all the subjects. Upon application of Mann-Whitey U test, notable differences were observed in abundance at phylum and class levels (Figure 3.3a). In particular, Firmicutes (p=0.05), Proteobacteria (p=0.0007) and TM7 (p=0.044) were found to be increased, while Bacteroidetes (p=0.007) and Cyanobacteria (p=0.004) were decreased in KSD subjects. Upon closer examination of taxonomic data, we noted that KSD subjects were enriched with class Bacilli (p=0.0005), Gammaproteobacteria (p<0.0001) and TM7-3 (p=0.044) whereas, class Bacteroidia (p=0.007), Betaproteobacteria (p=0.0004), Chloroplast (p=0.013) and Coriobacteriia (p<0.0001) were reduced in KSD subjects. Beta diversity analysis using unweighted (Figure 3.3b) and weighted UniFrac (Figure 3.3c) distance matrices revealed compositional differences in overall microbial communities in HLT and KSD subjects. Both these plots suggest that members of Firmicutes such as Lachnospiraceae, Peptostreptococcaceae, *Streptococcus*, and *Lactobacillus* have a profound effect on segregation of KSD subjects on PCoA plots.
Figure 3.3: 3a. Variations in major bacterial phyla and class in HLT and KSD subjects (*: p=<0.1, **: p=<0.05). PCoAbiplot based on 3b. Unweighted and 3c. Weighted UniFrac distance matrix: Subjects are represented as HLT (red) and KSD (green) whereas taxonomic group influencing sample segregation are shown as a gray sphere whose size demonstrate abundance. Reprinted (adapted) from Suryavanshi et al. (2016), Hyperoxaluria leads to dysbiosis and drives selective enrichment of oxalate metabolizing bacterial species in recurrent kidney stone endures, Scientific Reports, Copyright © 2016, Nature Publishing Group.
Kruskal-Wallis (a non-parametric ANOVA) test revealed 1602 significantly different OTUs ($p<0.01$) in HLT and KSD subjects. Of these, 952 were completely absent, and 341 were augmented in KSD subjects. To identify the strongest taxonomic features responsible for the observed compositional differences in HLT versus KSD subjects, we used Random Forest (RF) a supervised learning model and noticed that there were at least 50 OTUs contributing significantly to the observed differences. Most notably, certain OTUs assigned to *Prevotella* (or *Prevotellacopri*) and *Dialister* were solely present in HLT subjects’ whereas OTUs assigned to order Bacteroidales, family Lachnospiraceae and genus *Bacteroides* were dominating in KSD subjects (Appendix VII). Interestingly, *Faecalibacterium prausnitzii* OTUs, one of the dominant butyrate producers in the gut environments was depleted in KSD subjects.

To detect altered microbial interactions in the gut due to microbial dysbiosis in KSD subjects, we performed co-occurrence and co-exclusion network analysis (Figure 3.4). We used genus-level abundance data containing abundance values of 70 detected genera to understand these mutually exclusive interactions. In the resulting network, we were able to detect a total of 57 interacting nodes representing 29 (45 interactions) and 28 (44 interactions) significantly interacting genera in KSD and HLT subjects’ respectively. We also noted a concomitant decrease in co-occurrence interactions: 23 in HLT versus 18 in KSD and an increase in co-exclusion interactions: 21 in HLT versus 27 in KSD. All these observations indicate decrease as well as compositional enrichment in certain bacterial OTUs in KSD subjects as compared to HLT subjects.
Figure 3.4: Microbial interaction network in HLT (red colored nodes) and KSD (green colored nodes) subjects represented at the genus level. The size of the node is indicative of abundance and color of connecting edge indicate interaction type; co-presence: white and co-exclusion: yellow while its thickness represents the weight of interactions. Reprinted (adapted) from Suryavanshi et al. (2016), Hyperoxaluria leads to dysbiosis and drives selective enrichment of oxalate metabolizing bacterial species in recurrent kidney stone endures, Scientific Reports, Copyright © 2016, Nature Publishing Group.

3.3.2 Correlation of Eubacterial Population with the Clinical Parameters

Simple linear regression analysis revealed that nine bacterial genera negatively correlated while three positively correlated with the oxalate content (Figure 3.5).
**Figure 3.5:** Spearman correlation indicating positive and negative responses of various microbial genera with oxalate concentration in 24-hr urine. Linear regression plot displaying best fit blue line with 95% confidence bands and size of sphere corresponds to oxalate concentration. Reprinted (adapted) from Suryavanshi et al. (2016), Hyperoxaluria leads to dysbiosis and drives selective enrichment of oxalate metabolizing bacterial species in recurrent kidney stone endures, Scientific Reports, Copyright © 2016, Nature Publishing Group.

Most notably, *Prevotella* and *Roseburia*, members of Bacteroidetes phyla and *Faecalibacterium* negatively correlated while *Veillonella*, *Clostridium* and *Weisella* members of Firmicutes positively correlated with urine oxalate content. Further, we looked at whether the microbial composition was related to the clinical parameters by performing co-inertia analysis (COIA). COIA (with RV coefficient=0.307, Monte-
Carlo test for 1000 replication p=0.06) revealed a modest relationship between genus level abundance data and clinical parameters (Figure 3.6).

Figure 3.6: Co-inertia analysis of bacterial genera and subject characteristics. Upper score plot indicates the best matching of 39 subjects with the origin of arrows indicating bacterial genera and arrowhead indicating where spots would move about subject characteristics. Lower plots show the contribution of two groups of the variable to the canonical space. Reprinted (adapted) from Suryavanshi et al. (2016), Hyperoxaluria leads to dysbiosis and drives selective enrichment of oxalate...

### 3.3.3 Metagenomic Imputation for Metabolic Profiling of Gut Bacteria

Having observed the microbial dysbiosis in KSD subjects, we were curious to know whether the dysbiosis was associated with specific metabolic enrichments involved in oxalate utilization in these subjects. Hence, metagenomic contributions of gut microbiota were assessed on their ability to utilize oxalate and associated functions using PICRUSt tool. Several gene families were down-regulated or up-regulated in KSD subjects at weighted NSTI (Nearest Sequenced Taxon Index) values of below 0.1. Protein families, downregulated in KSD subjects, were involved in energy metabolism, glycan synthesis, and metabolism of cofactors and vitamins. Whereas, protein families up-regulated in KSD subjects were linked to lipid metabolism, carbohydrate metabolism and xenobiotic degradation and metabolism (Figure 3.7a).
Figure 3.7: Graphical presentation of imputed metagenome in HLT (red) and KSD (green) subjects. 3.7a. A cladogram is showing a differential abundance of microbial originating metabolic functions. 3.7b. Variation in abundance of known KOs involved in oxalate metabolism. Reprinted (adapted) from Suryavanshi et al. (2016), Hyperoxaluria leads to dysbiosis and drives selective enrichment of oxalate metabolizing bacterial species in recurrent kidney stone endures, Scientific Reports, Copyright © 2016, Nature Publishing Group.
Oxalate metabolism of bacterial origin has been studied and well annotated at KEGG server (Figure 3.8). From KOs, we observed that the enzymes which are involved in oxalate degradation were enriched in KSD subjects, these include formate dehydrogenase (K08349), oxalate/formate antiporter (K08177), formyl-CoA transferase (K07749), oxalyl-CoA decarboxylase (K01577) and oxalate decarboxylase (K01569) (Figure 3.7b).

Figure 3.8: Putative oxalate degradation pathways derived from the enzymes obtained from KEGG-Orthology Maps. Reprinted (adapted) from Suryavanshi et al. (2016), Eubacterial Diversity and Oxalate Metabolizing Bacterial Species (OMBS) Reflect Oxalate Metabolism Potential in Odontotermes Gut, Journal of Pure and Applied Microbiology, Copyright © 2016.

3.3.4 Colonization Status of *O. formigenes* in tested Subjects

We tested the colonization of *O. formigenes* using PCR for all subjects included in the ‘case-control study’ and ‘healthy-cohort study’ collectively. Interestingly, we found that in the HLT population, 15 from ‘case-control study’ and 34 from ‘healthy-cohort study’ found to be colonized. We found that only 4 subjects were positive for colonization in the KSD subjects (n=24). In all, only about 17 percent KSD subjects and another side 100 percent HLT subjects were positive for the *O. formigenes*.
Figure 3.9: Detection *Oxalobacter formigenes* colonization using PCR: genus-specific 16S rRNA gene amplification (416bp) (upper row) and oxc-gene amplification (318bp) (lower row) in a representative subpopulation of HLT (annotated as H) and KSD (annotated as K) subjects. Reprinted (adapted) from Suryavanshi et al. (2016), Hyperoxaluria leads to dysbiosis and drives selective enrichment of oxalate metabolizing bacterial species in recurrent kidney stone endures, Scientific Reports, Copyright © 2016, Nature Publishing Group.
The insignificant difference in abundance of *O. formigenes* but the concomitant increase in oxalate metabolizing enzymes KOs in KSD subjects prompted us to find out whether the oxalate metabolism ability is only conferred to well-characterized OMBS in human gut or if there are many other gut residents possessing the ability to utilize oxalate. We, therefore, segregated the subjects into five groups based on the colonization pattern of *O. formigenes*, kidney stone episodes and family history of stone. Colonization of *O. formigenes* was confirmed by PCR using specific sets of primers (Figure 3.9). Accordingly, all HLT group subjects and only 4 KSD subjects were found colonized with *O. formigenes*. Hence five groups consisting of four subjects each were made (Table 3.2). We performed the remaining analysis of these groups which included identification of shared OTUs among the groups. After an analysis of shared phylotypes among the KSD groups and HLT subjects, we found that all members of the individual KSD groups shared maximum phylotypes, while there was gradation in the shared phylotype pattern among the different KSD groups. All the members of all KSD groups (except KSD 22 sample) shared least phylotypes with the members of HLT group which in turn, shared maximum of phylotypes among themselves (Figure 3.10).

We found the colonization of *Oxalobacter* in the KSD subjects retains the 118 out of 785 OTUs found (Kruskal-Wallis test, \( p<0.001 \) & FDR=0.05), reflects the 20.08% of HLT group flora. These observations indicate that the *O. formigenes* colonization could achieve the retention of HLT microbiome.
Figure 3.10: Heatmap is representing pairwise inter-individual sharing of phylotypes amongst five groups. Gradation of yellow color indicates sharing of phylotypes within KSD groups, and blue color indicates sharing of phylotypes between HLT and KSD groups. Reprinted (adapted) from Suryavanshi et al. (2016), Hyperoxaluria leads to dysbiosis and drives selective enrichment of oxalate metabolizing bacterial species in recurrent kidney stone endures, Scientific Reports, Copyright © 2016, Nature Publishing Group.

3.3.5 Eubacterial Family and their Metabolic Contributions in Health Status

We found that certain microbial families are characteristically present in diseases such as in the KSD subjects and other conditions. Certain bacterial families like Bacteroidaceae, Enterobacteriaceae, and Porphyromonadaceae were found enriched, and Mogibacteriaceae, Paraprevotellaceae, Alcaligenaceae, Pasterallaceae, Spirochaetaceae, and Streptococcaceae were depleted in KSD (Welch's t-test, p=<0.001, FDR=0.05) group (Figure 3.11).
**Figure 3.11:** Heatmap representing Eubacterial families detected through 16S rRNA gene amplicon libraries in HLT and KSD and respective five groups. Families who are significantly reported in group wise study were shown.

Using the hypergeometric test function found that such differential bacterial families present and have their role in KSD; not by chance alone. The metagenomic imputation demonstrated the differential abundance of genes involved in another metabolism in KSD (Wilcoxon sum rank test, p=<0.005) (Figure 3.12).
Figure 3.12: Diagrammatic representation of Eubacterial metabolic potentials depicted through PICRUSt, and found in HLT and KSD subjects. Respective KEGG Orthology for the enzymes involved in metabolic phenotype.

3.3.6 Quantitative Estimation of Diversity Players

By using qPCR, we were able to detect and support the amplicon based results from the quantitative measurements of selected bacteria. Substantial enrichment of *Lactobacillus* group (p=<0.0001) and *frc*-gene (p=<0.0001); whereas depletion in Bacteriodetes phylum (p=<0.0001), *Bifidobacterium* (p=0.0009), *Fecalibacterium* (p=0.0028), *O. formigenes* (p=<0.0001) and *Lactobacillus plantarum* (p=<0.0001) microbial taxa were detected in KSD subjects (Figure 3.13).
**Figure 3.13:** Dot plots are representing the counts of selected bacterial taxa and genes by qPCR assay in HLT and KSD subjects. Minimum and maximum count values are displayed for each plot in all tested subjects. Modified and reprinted (adapted) from Suryavanshi et al. (2016), Hyperoxaluria leads to dysbiosis and drives selective enrichment of oxalate metabolizing bacterial species in recurrent kidney stone endures, Scientific Reports, Copyright © 2016, Nature Publishing Group.
Quantitative PCR results revealed that known OMBS *O. formigenes* varied from $3.2 \times 10^{05}$ to $8.9 \times 10^{06}$ counts per gram of fecal sample in HLT subjects and that it was significantly higher. The *frc*-gene is referred to as a molecular marker for oxalate metabolizing bacteria, and its higher copies can be considered as an indication for the enrichment of range of OMBS bacteria in the human gut. The *frc*-gene copies in HLT subjects ranged from $7.8 \times 10^{05}$ to $7.7 \times 10^{07}$ counts per gram which were significantly lower than its copies in KSD subjects $7.4 \times 10^{7}$ to $1.7 \times 10^{11}$. Further, we obtained the overall abundance of OMBS bacteria by taking the ratio of *frc*-gene copy number to 16S rRNA gene copy number and observed that the ratio was much higher for KSD subjects (range: 0.03 to 20.1%) than in HLT subjects (range: 0.00018 to 0.01%). This confirmed the fact that in diseased condition active OMBS are highly enriched. We next checked the contribution of *O. formigenes* to total OMBS population, with the help of ratio of *Oxalobacter* copy number to *frc*-gene copy number. A higher ratio of *O. formigenes* in HLT subjects (range: 1.2 to 88.13%) indicates that it is an important contributor for oxalate homeostasis in the gut of HLT subjects. However, the significantly low ratio in KSD subjects (range: 0.004 to 0.22%) indicated its inhibition and associated enrichment of other OMBS in KSD subjects.

### 3.4 Discussion

A kidney stone is believed to be a multifactorial disease influenced by lifestyle as well as food habits. In general population, calcium oxalate kidney stones are the most common type of kidney stones and are predominantly found in males than in females (Daudon et al., 2004). Although a kidney stone is perceived as an acute disease, evidence suggests the fact that for many individuals, hyperoxaluria is a chronic condition and often leads to recurrent stone episodes (Cochat and Rumsby, 2013). In the last two decades, major efforts have been taken in diagnosis and treatment of recurrent kidney stones, however; satisfactory regimes are still awaited. There are a few reasons for unsuccessful treatments including insufficient information on oxalate content in food, the relation between dietary-oxalate precursors and oxalate excretion, and the factors involved in intestinal oxalate handling (Asplin, 2002). Uremic Toxin including oxalate may alter the gut biochemical milieu, which may consequently affect structure and composition of gut
microbial communities (Robijn et al., 2011). Previous observations confirm altered microflora in chronic kidney disease (Ramezani and Raj, 2014; Vaziri et al., 2013), but specific reports on alteration in gut microbiome associated with oxalate stones in human subjects are scanty (Stern et al., 2016). In the present study, we revealed differences in bacterial community structure in 24 male subjects suffering from idiopathic hyperoxaluria and showed enrichment of oxalate metabolizing microbes in their gut.

Our results, both at broader and refined levels of microbial taxonomy indicate dysbiosis in major gut microbial communities in these subjects and are by the previous study in canines (Gnanandarajah et al., 2012b). Members of two bacterial phyla Firmicutes and Bacteroidetes dominate endogenous gut microflora of many mammals including humans, forming complex interactions among themselves and with the host to ensure the stability in this ecosystem.

3.4.1 Eubacteria-structural Diversity is Changed in Hyperoxaluria Condition and Deflected with the Urine Oxalate Concentration

Increased abundance of Firmicutes has been linked with metabolic disorders such as obesity (Turnbaugh et al., 2006) and diabetes (Qin et al., 2012). Thus augmentation of Firmicutes in hyperoxaluria can be easily perceived. Bacteroidetes are often involved in the metabolism of complex polysaccharides mainly derived from the food (Filippo et al., 2010). Recently, it has been shown that this rather copious phylum possess contact-dependent inter-bacterial antagonism which is essential to maintain the stability of gut microbiota in healthy subjects and also presents a barrier to most of the pathogens like members of Proteobacteria (Russell et al., 2014). Indeed, a significant decrease in Bacteroidetes and a concomitant increase in Proteobacteria in KSD subjects indicates disruption of Bacteroidetes driven inter-bacterial antagonism. Using beta diversity analysis based on unweighted and weighted UniFrac distance matrix, we further confirmed the dominance of Firmicutes and deprivation of Bacteroidetes in KSD subjects leading to their distinct segregation on PCoA plots from HLT subjects. Furthermore, dispersal of KSD subjects on PCoA plots indicates
enhanced beta diversity associated with enrichment of specific microbial communities in them compared to the HLT subjects.

Random Forest machine learning approach is an effective way to identify discriminating taxa between different physiological states or disease conditions (Knights et al., 2011). Our observation of a higher number of *Prevotella* and *Lachnospiraceae* OTUs in HLT and KSD subjects respectively further supports the fact KSD subjects were depleted with Bacteroidetes. Our findings of decreased abundance of *Prevotella* and *Eubacterium* in KSD subjects are in congruence with a recent study reporting variations between gut microbial communities of stone formers and non-stone formers (Stern et al., 2016). The same study reported an increase in abundance of Bacteroidetes, which was not observed in our study. The observed differences could be attributed to recruitment of more female subjects, stone formers with mixed types of stones and stone formers with other disease-associated complications (Stern et al., 2016).

Competitive and cooperative interactions are very common microbial interactions prevalent in various ecosystems. To obtain a complete overview of microbial interactions in the human gut is a task primarily due to the complex nature of this ecosystem and partly because many members are yet to be cultured. Hence, few indirect methods have been developed to model these interactions using microbial abundance data (Faust et al., 2012). Accordingly, increased competitive interactions in KSD subjects could be related to dysbiosis of healthy microflora in them. Such competitive interactions have been linked with the evolution of cooperation in yeast communities (Celiker and Gore, 2012).

We also observed that some of the prominent gut residents to be negatively correlated with the urinary oxalate, even though they have the ability to metabolize oxalate. This indicates that above a certain concentration, oxalate could be toxic to these common gut inhabitants; similar results have also been observed on *O. formigenes* (Siener et al., 2013b). The co-inertia analysis used to examine global similarity between clinical parameters and genus abundance profile also revealed segregation of *Prevotella, Sutterella, Roseburia* (negatively correlated genera) from
Veillonella and Weissella (positively correlated genera) and we believe that this could be influenced by oxalate concentration, stone number, and stone episodes.

3.4.2 Hyperoxaluria Effectuate Accountable Change in Diversity Players and their Functional Capacities inside the Gut Environment

Although PICRUSt infers metabolism of given microbiome based on 16S rRNA amplicon sequences, lower NSTI scores are often indicative of good metagenomic predictions. Using KEGG server (Kanehisa and Goto, 2000), we identified five metabolic pathways involved in oxalate degradation in microbes. Enrichment of some of these KOs in KSD subjects further strengthened the notion that microbiota not only alters structurally but also leads to enrichment of oxalate metabolic function due to hyperoxaluria drove selective pressure. While our PICRUSt analysis revealed several other important differences in the inferred metagenomic data about calcium oxalate stone formation on a broader scale (Figure 3.12); their augmentation or diminution in KSD subjects needs experimental validation.

Bacterial communities are often checked for their ability to metabolize oxalate by molecular characterization of frc-gene. Such studies have highlighted the fact that soils enriched with oxalate, bear the highest load of microbes involved in oxalate degradation (Khammar et al., 2009). In another study, presence and distribution of oxalate utilizing bacterial consortia have been demonstrated throughout the gut ecosystem of herbivorous mammals (Miller et al., 2014). For the first time, report the frc-gene in human gut microbial communities in the context of their abilities to metabolize oxalate present constitutively. Most significant finding from this study is that in addition to O. formigenes, several gut inhabitants possess the frc-gene and hence the ability to utilize oxalate. This becomes especially important because humans do not have the ability to metabolize oxalates, and are dependent on a gut microbial reservoir for oxalate clearance from the gut environment. Furthermore, our results show an increased abundance of frc-gene in KSD subject indicating enrichment of particular microbial communities involved in the utilization of oxalates; this could be a consequence of hyperoxaluria which was not observed in healthy subjects. Since gut-inhabiting E. coli uses homolog of frc- and oxc-gene viz.
YfdW and YfdU for acid tolerance (Fontenot et al., 2013), we further hypothesize that this increased diversity of frc-gene could be linked with the acquisition of oxalate-induced acid tolerance phenotype, and it may also be due to the interspecies horizontal gene transfer. Earlier studies, the presence of frc-gene is limited to Actinobacteria, Firmicutes and Proteobacteria phyla (Hervé et al., 2016). We were able to detect its presence in Bacteroidetes as well as Spirochaetes phyla suggesting that catalog of frc-gene is incomplete and we speculate that it could be extended in many other bacterial phyla.

Absolute quantification of specific bacterial groups in stool samples has been precisely determined by using real-time PCR (qPCR) (Matsuki et al., 2004; Yang et al., 2015). QPCR has also been used as a method to validate the findings of next-generation sequencing and micro-RNA data (Git et al., 2010). In our opinion, this is the first study to report the frc-gene frequency associated to the human gut and its strong relation to the oxalate stones. Further, high abundance of O. formigenes in HLT subjects, low abundance in KSD subjects and its minimal contribution to oxalate metabolism in KSD subjects suggests that in the hyperoxaluria condition, the gut milieu may become unfavorable for its growth. It should also be noted that the frc is just one route involved in oxalate metabolism. As per our PICRUSt analysis using KEGG server, there are at least five different oxalate degrading pathways. Hence, the possibility of the existence of alternative pathways of oxalate metabolism should not be neglected (Miller et al., 2014; Miller and Dearing, 2013; Samuel et al., 2007). However, to prove the existence of such alternative pathway, there is a need for large-scale metagenomic studies.

Some strong aspects of our study are the inclusion of subjects with matching age, who followed a vegetarian diet and were from similar socioeconomic class. Exclusion of female subjects and the cross-sectional nature of the study could be the limitations of our study. Further, our findings are largely based on culture-independent studies, considering the huge number of positively associated microbial taxa with oxalate content, there is the great scope of culture-dependent studies to understand the contribution of individual microbial taxa in oxalate homeostasis in the human gut. Moreover, since the diet is known to alter the gut microbiota (Filippo
et al., 2010), it is essential to evaluate the effect of specific diet (e.g. oxalate rich food) and associated microbiota towards the development of stones.

### 3.4.3 *O. formigenes* and *Lactobacillus plantarum* Colonization Retains much Healthy Gut Flora in Hyperoxaluria

Association of the inverse relationship between *O. formigenes* with an incidence of kidney stones is well established (Gnanandarajah et al., 2012a; Kaufman et al., 2008). Several studies have described *O. formigenes* as potential probiotics in the treatment of hyperoxaluria (Hoppe et al., 2006; Sidhu et al., 2001) and several commercial probiotics products containing *O. formigenes* are already available in the market (Hoppe et al., 2011). One striking observation in our study was that the taxonomic assignments to the *O. formigenes* OTUs could maximally be observed up to family level and that this does not differ significantly between HLT and KSD subjects. This could partly be attributed to a low abundance of this bacterium in the gut environment as well as its detection sensitivity using V1-V3 region of 16S rRNA gene amplicon sequencing (Barnett et al., 2016). This was further confirmed by using *Oxalobacter* specific 16S rRNA gene primers which demonstrated its presence in 100% (49 out of 49) HLT subjects as against ~17% (4 out of 24) KSD subjects. Furthermore, our observation of minimum sharing of phylotypes between different KSD groups and HLT subjects, and their gradation pattern in KSD subject signifies the role of hyperoxaluria in dysbiosis and may be attributed to disease state, family history, stone episodes and the presence of *O. formigenes*. Alongside, *Lactobacillus plantarum* has a negative correlation to the disease condition, and quantitative measures were signified their colonization status in Indian population study. *Lactobacillus* genus was already correlated with the population-specific urolithiasis prevalence status (Magwira et al., 2012). In the context of the Indian population, it has been observed that the presence of *O. formigenes* and *Lactobacillus plantarum* led to a maintenance of healthy gut flora. Metabolic abilities like oxalate degradation and tolerance capacity, colonization of *O. formigenes* and *Lactobacillus plantarum* were speculated to anchor the healthy gut microbiome and normal gut physiology.
In conclusion, our study using the high throughput DNA sequencing reports the dysbiosis of gut microbial communities in recurrent oxalate kidney stone sufferers. Also, it highlights dysbiosis of the structural and functional diversity of Eubacteria in hyperoxaluria condition (Figure 3.13). Considering the incidence rate (Romero et al., 2010) and recommended therapy for removal of kidney stones (Preminger et al., 2005), our study provides important avenues of gut microbiota as a potential target for treatment and controlling the recurrent episodes of oxalate-associated stones.

**Figure 3.13:** Diagrammatic illustration of actual observations in the tested subjects. Eubacteria species diversity and functional attributes were analyzed by amplicon sequencing and qPCR counts.