Received: 1 April 2020 | Revised: 20 May 2020 | Accepted: 16 June 2020

DOI: 10.1096/fj.202000786R

RESEARCH ARTICLE

THE FASEB JOURNAL

The relationship between gut microbiota and short chain fatty acids in the renal calcium oxalate stones disease

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Funding information
National Natural Science Foundation of China, Grant/Award Number: 81770703 and 81970602; Foundation of Science & Technology Department of Sichuan Province, Grant/Award Number: 2018SZ0118; Sichuan University, Grant/Award Number: ZYGD18011, ZYJC18015, Z and Y2016104

Abstract

The relationship of gut microbiota and calcium oxalate stone has been limited investigated, especially with no study of gut microbiota and short chain fatty acids (SCFAs) in nephrolithiasis. We provided Sprague Dawley rats of renal calcium oxalate stones with antibiotics and examined the renal crystals deposition. We also performed a case-control study by analyzing 16S rRNA microbial profiling, shotgun metagenomics and SCFAs in 153 fecal samples from non-kidney stone (NS) controls, patients with occasional renal calcium oxalate stones (OS) and patients with recurrent stones (RS). Antibiotics reduced bacterial load in feces and could promote the formation of renal calcium crystals in model rats. In addition, both OS and RS patients exhibited higher fecal microbial diversity than NS controls. Several SCFAs-producing gut bacteria, as well as metabolic pathways associated with SCFAs production, were considerably lower in the gut microbiota among the kidney stone patients compared with the NS controls. Representation of genes involved in oxalate degradation showed no significance difference among groups. However, fecal acetic acid concentration was the highest in RS patients with high level of urinary oxalate, which was positively correlated with genes involvement in oxalate synthesis. Administration of SCFAs reduced renal crystals. These results shed new light on bacteria and SCFAs, which may promote the development of treatment strategy in nephrolithiasis.

Abbreviations: ANOVA, analysis of variance; EG, ethylene glycol; HE, Hematoxylin-Eosin; IQR, interquartile range; KS, kidney stone; LEfSe, linear discriminant analysis Effect Size; LPS, lipopolysaccharide; NAFLD, nonalcoholic fatty liver disease; NS, non-kidney stone; OS, occasional stone; OTUs, operational taxonomic units; PCoA, principal coordinates analysis; PCR, polymerase chain reaction; PSM, propensity score match; RS, recurrent stone; SCFAs, short chain fatty acids; VK, Von Kossa.

Yu Liu and Xi Jin are co-first authors.

KEYWORDS

16s Rrna, renal calcium oxalate stones, sgut microbiota, short chain fatty acids, shotgun metagenomics

1 | INTRODUCTION

Nephrolithiasis, with a worldwide prevalence rate of 5%-10%, is one of the most common urologic diseases.[1,2] Kidney stone (KS) returns frequently with a recurrence rate of 6%-17% within one year, 21%-53% within three to five years, and a lifetime recurrence rate of 60%-80%.[2] Nephrolithiasis has increasingly strained the health care system with high prevalence, severe pain to patients, and burdensome medical costs.

Calcium oxalate stone is the most common type of KS, accounting for 60%-90% of the cases.[2] Limited knowledge of the mechanisms underlying stone formation has been available. For example, it is known that KS formation may result from poor fluid intake, high temperature, or excessive consumption of high oxalate foods, which could increase urinary excretion of oxalate.[2] The oxalate in the body was directly absorbed from diet, and produced by the liver as the final metabolite product of many metabolites, such as glyoxalate, glycine, hydroxyproline, and ascorbic acid.[3] Recently, Tasian et al reported that antibiotic exposure was associated with an increasing nephrolithiasis prevalence, which indicated that the intestinal microbiota may affected the formation of renal calcium oxalate stone.[4] In addition, some studies have found that intestinal microbiota was related to calcium oxalate stone with the discovery of *Oxalobacter formigenes*, which could degrade oxalate.[5-7] Thus, the underlying mechanisms of the formation of renal calcium oxalate stones remain obscure, and need more in-depth exploration.

Short chain fatty acids (SCFAs), as a major product from the microbial fermentative activity in the gut, have been involved in energy metabolism, hormone secretion, immune inflammation, and cancer.[8,9] While growing evidence has suggested that SCFAs play an important role in kidney disease through the gut-kidney axis by regulating inflammation, oxidative stress, and fibrosis,[10,11] SCFAs in nephrolithiasis are rarely explored.

Recently, the emerging techniques in 16S rRNA amplicon sequencing and shotgun metagenomics have enabled us to better understand the roles of gut microbiota in the formation of renal calcium oxalate stones. This study, to our knowledge, registers the first attempt to disclose the relationship between gut microbiota and short chain fatty acids in renal calcium oxalate stones and non-kidney stones.

2 | MATERIALS AND METHODS

2.1 | Renal calcium oxalate stone models and treatment regimes

We purchased Sprague Dawley rats (6-week-old, male) (Dossy Experimental Animals Co., Ltd, Chengdu, Sichuan, China). The animal experiment was performed in accordance with the policies of the West China Hospital of Sichuan University Medical Research Ethics Committee (2017063A). Twenty rats were randomly divided into four groups. Rats were first acclimatized for 1 week before experiment in the specific pathogen free animal facility with free access to standard food and water at Animal Experiment Center of West China Hospital, Sichuan University. Rats in control group had free access to drinking water. Antibiotics group rats received an extra cocktail of antibiotics resolved into drinking water, including 0.5 mg/mL of ampicillin, 0.5 mg/mL of metronidazole, 0.5 mg/mL of neomycin, and 0.25 mg/mL of vancomycin. Ethylene glycol (EG) group rats received drinking water containing 1% (v/v) EG. EG + antibiotics group rats had 1% (v/v) EG and antibiotics at the same time. Another 25 rats were also randomly divided into five groups, including control, EG, EG + Acetate, EG + Propionate, and EG + Butyrate groups. The last three groups received drinking water with EG plus sodium acetate, sodium propionate, and sodium butyrate, respectively. The amount of drinking water was recorded every three days, and the body weight of rats was recorded every week. After 4 weeks, we injected chloral hydrate (4% (w/v), 0.8 ml/100g) (JUHUI CHEMICAL, Chengdu, Sichuan, China) intraperitoneally to euthanize rats and collected kidneys and feces in colon. Kidneys were stored in 10% of formaldehyde and fixed in paraffin. Feces were stored at −80°C.

We extracted microbial DNA from fecal samples using Qiamp Fast DNA Stool extraction kit (Qiagen, Hilden, Nordrhein-Westfalen, Germany) according to manufacturer's protocols. Then, we amplified DNA with primers 27F (5′-AGAGTTTGATCMTGGCTCAG-3′) and 1492R (5′-CGGTTACCTTGTTACGACTT-3′).

Kidneys were stained with Hematoxylin & Eosin (HE) and Von Kossa (VK) using HE staining kit (Thermo Fisher Scientific, Waltham, Massachusetts, USA) and VK staining kit (Solarbio, Beijing, China) according to the manufacturer's protocols. HE staining scoring system reported by Xiang

et al was used to examine the formation of crystals.[12] The level of calcium oxalate crystals in VK staining was evaluated by image pro plus6 software.

2.2 | Human study designs, subjects, and sampling

A case-control study was performed at the West China Hospital in China from June 2018 to August 2019. Patients were diagnosed as renal stone by using the X-ray of kidney-ureter-bladder, ultrasound of the urinary system, or abdominal computed tomography. All patients received percutaneous nephrolithotomy or flexible ureteroscopy. Patients with at least two episodes of renal stones and those who had renal stones within one year after surgery by follow-up were regarded as recurrent stones (RS) patients. All the other KS patients were considered as occasional stones (OS) patients. To eliminate the bias caused by age, we mainly recruited non-kidney stone (NS) controls without renal stones between 40 and 60. They were confirmed by the absence of the history of renal colic and of renal stones using abdominal ultrasonic examination in Health Promotion Center of West China hospital. This study was approved by the West China Hospital of Sichuan University Medical Research Ethics Committee (2018182), and informed consents were obtained from each participant.

The following types of KS patients were excluded: non-calcium oxalate stones, infectious stones, abnormality of the urinary system, hyperthyroidism, hyperparathyroidism, long-term use of drugs that may cause KS. The component of renal stones was confirmed by infrared spectroscopy. Participants were also excluded if they used antibiotics or immunosuppressants within three months before fecal sampling, had inflammatory bowel disease, irritable bowel syndrome, digestive tract infection, tumors of the digestive system, intestinal surgery, or diarrhea or constipation within one month before fecal sampling.

A questionnaire was designed to query participants' demographic information, dietary habits, sitting, and sleeping time, history of smoking and alcohol, family history of stone and history of metabolic diseases and surgery. Body mass index was calculated by dividing weight in kilograms by the square of height in meters. Fecal and urinary samples from each participant were collected during the visit of the clinic of Urology of West China Hospital, prior to any treatment, such as antibiotics. Samples were immediately frozen in liquid nitrogen, and subsequently stored under −80°C until analysis.

2.3 | 16S rRNA microbial profiling analyses

Microbial DNA was extracted from the fecal samples by using QIAampFastDNAStoolMiniKit,Cat#51604(Qiagen,Germany) according to the manufacturer's protocol. The V3-V4 hypervariable regions of the bacteria 16S rRNA gene were amplified with primers 338F (5′-ACTCCTACGGGAGGCAGCAG-3′) and 806R (5′-GGACTACHVGGGTWTCTAAT-3′) by PCR system (GeneAmp 9700, ABI, USA). The resulting PCR products were extracted from a 2% of agarose gel and further purified by using the AxyPrep DNA Gel Extraction Kit, Cat# AP-GX-50 (Axygen Biosciences, USA) and quantified by using QuantiFluor-ST (Promega, USA) according to the manufacturer's protocol. Then, purified amplicons were pooled in equimolar and paired-end sequenced (2 × 300) on an Illumina MiSeq platform (Illumma, San Diego, USA) according to the standard protocol.

Raw fastq files were quality-filtered by Trimmomatic and merged by FLASH. All the sequences were clustered into different operational taxonomic units (OTUs) at 97% similarity using UPARSE (version7.1 http://drive5.com/uparse/), and chimeric sequences were identified and removed by using UCHINIE. The taxonomy of each 16S rRNA amplicon sequence was analyzed by the RDP Classifier algorithm (http://rdp.cme.msu.edu/) against the Silva (SSU132) 16S rRNA database with the confidence threshold of 70%. Each sample was rarefied to the same amount of sequences (10 000).

We compared alpha diversity indices, such as Chao, ACE, Shannon, and Simpson across the OS, RS, and NS groups. Coverage index was calculated to determine if the sequencing depth covered the whole bacterial diversity. Inter-individual variability (beta diversity) among these three groups was evaluated by the principal coordinates analysis (PCoA) and the Adonis test. Linear discriminant analysis Effect Size (LEfSe) analysis was used to identify differentially abundant bacteria among the three groups with a cutoff of 2.0. The Kyoto Encyclopedia of Genes and Genomes metabolic pathways were predicted by the PICRUSt package.

2.4 | Shotgun metagenomics analysis of fecal samples

DNAs extracted from 15 fecal samples (five NS controls, five OS patients, and five RS patients), representing the average composition of controls and KS patients, were used for deep shotgun metagenomics sequencing. Bacterial DNAs were extracted from stool samples. The metagenome sequencing was performed and analyzed using Illumina Hiseq system. The raw sequences were decoded, denoised, trimmed, and assembled for gene prediction by using MetaGene (http://metagene.cb.k.u-tokyo.ac.jp/). The predicted genes were clustered (with identity of 95% and coverage of 95%) by CD-HIT (http://www.bioinformatics.org/cd-hit/) into a nonredundant gene catalog. KEGG (Kyoto Encyclopedia of Genes and Genomes, http://www.genome.jp/kegg/) databases were used to predict gene functions.

2.5 | Fecal short chain fatty acids quantification and detection of urinary oxalate

Fecal SCFAs were measured using gas chromatography-mass spectrometry. Each SCFA was calculated from a calibration curve produced for serially diluted SCFA standards. The SCFA calibration curve was linear ($R^2 \geq 0.999$). Urinary oxalate was measured and analyzed by liquid chromatography-mass spectrometry. The oxalate calibration curve was linear ($R^2 = 0.999$). The mean recovery of oxalic acid of known concentration added to urine samples was 92.91%-99.25%.

2.6 | Statistical analysis

Continuous variables were summarized with mean and standard deviation if they were symmetrically distributed. Otherwise, they were reported with median and interquartile range (IQR). Analysis of variance (ANOVA), Kruskal-Wallis test, and Mann-Whitney test were used for testing quantitative variables, and Chi-square test was used for categorical variables. All statistical analyses were performed with R v.3.6.0 (R Project for Statistical Computing, www.r-project.org).

3 | RESULTS

3.1 | Effect of antibiotics on gut microbiota and renal calcium oxalate crystals

To verify the role of gut microbiota in renal calcium oxalate stone, we directly depleted bacterial load in rat gut with antibiotics. All rats were alive at the end of experiment after four weeks. The change of body weight of rats was shown in Figure S1A). The amount of drinking water of rats was not different between EG and EG + Antibiotics groups (Figure S1B). Rat fecal bacterial load decreased sharply by 150 times after administration of antibiotics, when compared to the control group (Figure 1A,B). Then, we observed the formation of renal crystals in rat kidney with or without administration of antibiotics. The result showed that EG successfully induced the formation of renal crystals in rat kidney (Figure 1C,D,E). Furthermore, VK staining showed that antibiotics significantly promoted the formation of renal crystals (Figure 1C,E).

3.2 | General characteristics of non-kidney stone controls, occasional, and recurrent stone patients

To identify what specific bacteria were associated with renal calcium oxalate stone, we recruited 76 renal stone patients. Seven of them were excluded because their renal stones were infectious stones or not calcium oxalate. Finally, 69 KS patients (26 RS patients and 43 OS patients) and 84 NS controls were enrolled (Figure S1C). Age ($P = .460$) and BMI ($P = .198$) were not significantly different among RS, OS, and NS groups. However, the distribution of gender was significantly different among the RS, OS, and NS groups (76.9% male in RS, 65.1% male in OS, and 40.5% male in NS, $P = .001$). Consumption of fat or red meat, intake of milk and fruit, sitting and sleeping time, active smoking, family history of KS, hypertension, and nonalcoholic fatty liver disease (NAFLD) were significantly different among NS, OS, and RS groups (Table 1). The results of a multiple logistic regression model, after controlling for these ten variables, indicated that less fruit intake, sitting longer, hypertension, and NAFLD were associated with occurrence of renal calcium oxalate stones (Table 2).

3.3 | Richness and diversity of the gut microbiota in KS patients and NS controls

The fecal samples from 153 participants were applied to 16S rRNA sequence analysis. A total of 8,026,841 high-quality sequences were obtained from these 153 samples with an average length of 436. All the sequences were clustered into 1108 OTUs, which belonged to 442 genera and 23 phyla. The coverage indices of the NS, OS, and RS groups were 98.34%, 97.97%, and 97.98%, respectively. In addition, nearly all the rarefaction curves reached the saturation plateau (Figure S1D).

The Wilcoxon rank-sum test showed that alpha diversity indices (Ace and Chao), representing the richness of gut microbiota, were higher in KS patients than in controls (Figure 2A, Table S1). There were no statistically significant differences in Shannon and Simpson indices across the NS, OS, and RS groups (Figure 2A, Table S1). PCoA at the OTU level showed that the overall microbiota composition of the NS, OS, and RS groups were different, which was confirmed by the Adonis test ($R^2 = 0.031$, $P = .002$) (Figure 2B).

3.4 | Taxonomic analysis of microbiota composition between KS patients and NS controls

Taxonomic assignment of the OTUs revealed the composition of the bacterial population down to the phylum and genus level. At the phylum level, Firmicutes (61.4%) was the most common bacteria in gut microbiota, followed by Bacteroidetes (18.9%) and Proteobacteria (11.7%). The ratio of Firmicutes/Bacteroidetes was higher in KS patients than that in NS controls (3.70 vs 2.72). At the genus level, the common bacteria of 153 samples were *Bacteroides* (9.8%), *Fecalibacterium* (9.5%), *Blautia* (4.5%), *Escherichia-Shigella* (4.4%), *Megamonas* (4.2%), *Lactobacillus* (4.1%), and [*Eubacterium*]-rectale-group (4.0%) (Figure 2C).

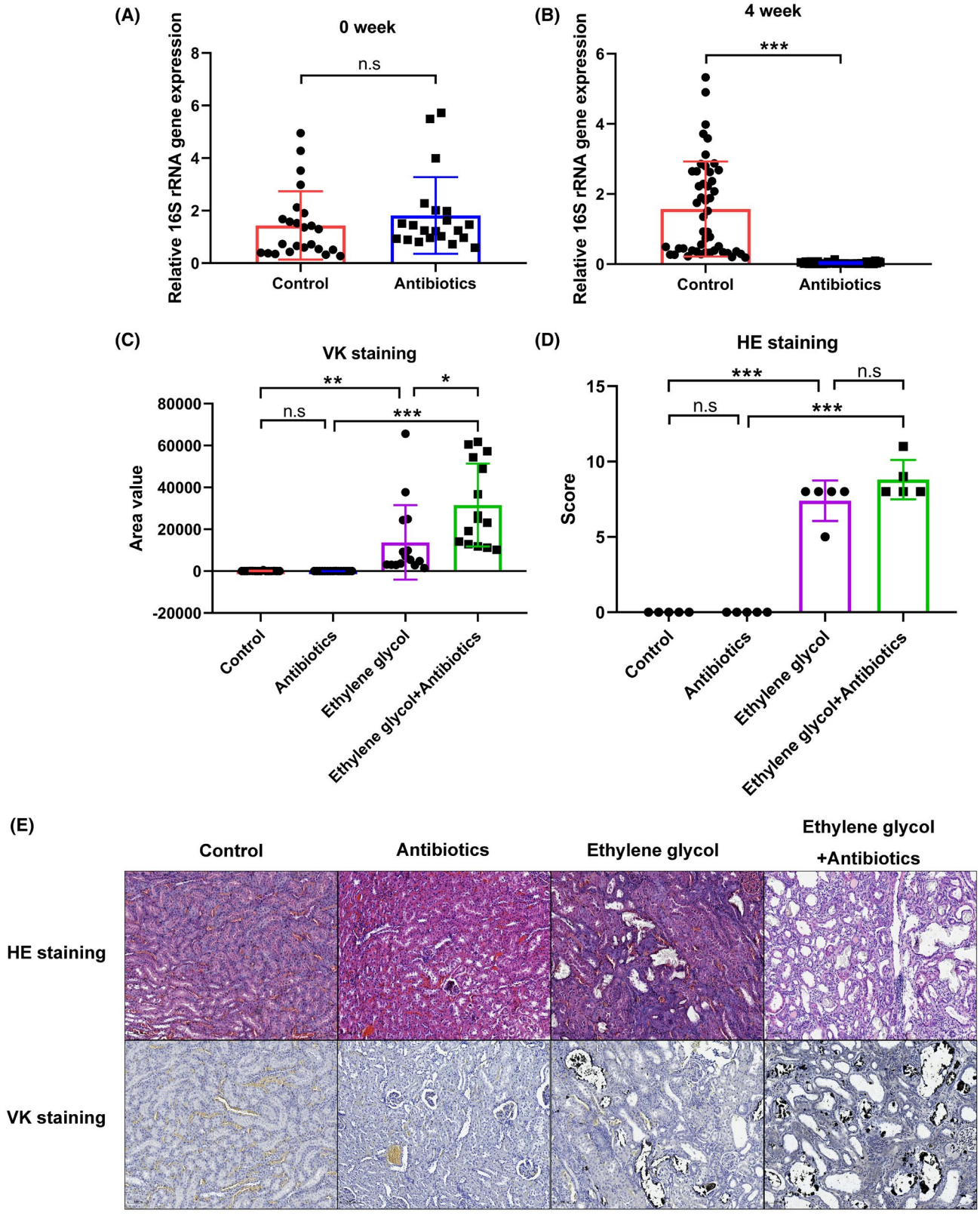

FIGURE 1 Effect of antibiotics on gut microbiota and renal calcium oxalate crystals in rats (n = 5/group). Rats received an extra cocktail of antibiotics resolved into drinking water, including 0.5 mg/mL of ampicillin, 0.5 mg/mL of metronidazole, 0.5 mg/mL of neomycin, and 0.25 mg/mL of vancomycin with or without ethylene glycol. Rat microbial DNA from fecal samples were detected before (A) and after (B) antibiotics administration. The level of calcium oxalate stones was evaluated by image pro plus6 software using Von Kossa (VK) staining. Three different sections of each Von Kossa staining were selected, which resulted in 15 data in Figure 1C (C and E). Kidneys were also stained with Hematoxylin-Eosin (HE), and HE staining scoring system was used to examine the formation of crystals (D and E)

TABLE 1 Comparison of general characteristics between NS controls, OS and RS patients

	NS controls	OS patients	RS patients	*P* value
Age	50.4 (7.0)	50.6 (12.0)	53.0 (11.1)	.460[a]
BMI	24.4 (2.5)	23.6 (2.9)	24.7 (3.5)	.198[a]
Gender				
Female	50 (59.5%)	15 (34.9%)	6 (23.1%)	.001[b]
Male	34 (40.5%)	28 (65.1%)	20 (76.9%)	
Water				
<1000 (mL/day)	30 (35.7%)	14 (32.6%)	7 (26.9%)	.604[b]
1000-2000 (mL/day)	31 (36.9%)	14 (32.6%)	13 (50.0%)	
>2000 (mL/day)	23 (27.4%)	15 (34.9%)	6 (23.1%)	
Salt				
Less-salt	27 (32.1%)	11 (25.6%)	3 (11.5%)	.345[b]
Medium-salt	43 (51.2%)	23 (53.5%)	17 (65.4%)	
More-salt	14 (16.7%)	9 (20.9%)	6 (23.1%)	
Fat				
Less-fat	31 (36.9%)	3 (7.0%)	2 (7.7%)	.001[b]
Medium-fat	35 (41.7%)	25 (58.1%)	17 (65.4%)	
More-fat	18 (21.4%)	15 (34.9%)	7 (26.9%)	
Spicy				
Non-spicy	11 (13.1%)	11 (25.6%)	7 (26.9%)	.094[b]
Medium-spicy	53 (63.1%)	17 (39.5%)	12 (46.2%)	
Super-spicy	20 (23.8%)	15 (34.9%)	7 (26.9%)	
Pickled vegetables				
<10 (times/month)	58 (69.0%)	30 (69.8%)	19 (73.1%)	.420[b]
10-20 (times/month)	14 (16.7%)	3 (7.0%)	4 (15.4%)	
>20 (times/month)	12 (14.3%)	10 (23.3%)	3 (11.5%)	
Tea or coffee				
<10 (times/month)	67 (79.8%)	27 (62.8%)	15 (60.0%)	.172[b]
10-20 (times/month)	2 (2.4%)	1 (2.3%)	1 (4.0%)	
>20 (times/month)	15 (17.9%)	15 (34.9%)	9 (36.0%)	
Red meat				
<100 (g/day)	67 (79.8%)	28 (65.1%)	18 (69.2%)	.004[b]
100-200 (g/day)	15 (17.9%)	6 (14.0%)	7 (26.9%)	
>200 (g/day)	2 (2.4%)	9 (20.9%)	1 (3.8%)	
Milk				
<250 (mL/day)	54 (64.3%)	37 (86.0%)	22 (84.6%)	.012[b]
>250 (mL/day)	30 (35.7%)	6 (14.0%)	4 (15.4%)	
Fruit				
<200 (g/day)	42 (50.0%)	31 (72.1%)	20 (76.9%)	.010[b]
>200 (g/day)	42 (50.0%)	12 (27.9%)	6 (23.1%)	
Sitting time				
<4 (h/day)	67 (79.8%)	12 (27.9%)	6 (23.1%)	<.001[b]
4-7 (h/day)	17 (20.2%)	19 (44.2%)	9 (34.6%)	
>7 (h/day)	0 (0.00%)	12 (27.9%)	11 (42.3%)	
Sleeping time				
<6 (h/day)	5 (6.0%)	10 (23.3%)	7 (26.9%)	.022[b]
6-9 (h/day)	77 (91.7%)	32 (74.4%)	19 (73.1%)	
>9 (h/day)	2 (2.4%)	1 (2.3%)	0 (0.00%)	
Smoking				
No	67 (79.8%)	22 (51.2%)	20 (76.9%)	.003[b]
Yes	17 (20.2%)	21 (48.8%)	6 (23.1%)	
Alcohol				
No	60 (71.4%)	28 (65.1%)	20 (76.9%)	.562[b]
Yes	24 (28.6%)	15 (34.9%)	6 (23.1%)	
Family history of stone				
No	73 (86.9%)	26 (60.5%)	15 (57.7%)	<.001[b]
Yes	11 (13.1%)	17 (39.5%)	11 (42.3%)	
Diabetes mellitus				
No	80 (95.2%)	39 (90.7%)	21 (80.8%)	.067[b]
Yes	4 (4.8%)	4 (9.3%)	5 (19.2%)	
Hypertension				
No	80 (95.2%)	30 (69.8%)	15 (57.7%)	<.001[b]
Yes	4 (4.8%)	13 (30.2%)	11 (42.3%)	
NAFLD				
No	80 (95.2%)	36 (83.7%)	19 (73.1%)	.005[b]
Yes	4 (4.8%)	7 (16.3%)	7 (26.9%)	
Surgery of gastrointestinal tract				
No	80 (95.2%)	37 (86.0%)	21 (80.8%)	.053[b]
Yes	4 (4.8%)	6 (14.0%)	5 (19.2%)	
Gastritis or enteritis				
No	74 (88.1%)	37 (86.0%)	19 (73.1%)	.169[b]
Yes	10 (11.9%)	6 (14.0%)	7 (26.9%)	

Note: [a]Analysis of variance (ANOVA). [b]Chi-square test. NS, non-kidney stone. OS, occasional stones. RS, recurrent stones. BMI, body mass index. NAFLD, nonalcoholic fatty liver disease.

To identify the association between calcium oxalate stones and gut bacteria, LEfSe analysis was used to compare bacteria among NS controls, OS patients, and RS patients. At

TABLE 2 Multivariate logistic regression analysis for risk and protective factors of kidney stone patients

Factors	*P* value	Coefficients	95% CIs	
			Lower bound	Upper bound
Fat				
Less-fat	0.018	−1.569	−2.866	−0.272
Medium-fat	0.267	−0.529	−1.463	0.405
More-fat		0		
Red meat				
<100 (g/day)	0.743	0.245	−1.220	1.710
100-200 (g/day)	0.771	−0.243	−1.879	1.394
>200 (g/day)		0		
Milk				
<250 (ml/day)	0.473	0.364	−0.631	1.360
>250 (ml/day)		0		
Fruit				
<200 (g/day)	0.003	1.335	0.455	2.215
>200 (g/day)		0		
Sitting time				
<4 (h/day)	<0.001	−3.326	−4.629	−2.024
4-7 (h/day)	<0.001	−2.402	−3.631	−1.173
>7 (h/day)		0		
Sleeping time				
<6 (h/day)	0.677	0.626	−2.318	3.570
6-9 (h/day)	0.428	−1.145	−3.974	1.684
>9 (h/day)		0		
Smoking				
No	0.744	0.142	−0.710	0.995
Yes		0		
Family history of stone				
No	0.132	−0.687	−1.581	0.207
Yes		0		
Hypertension				
No	0.003	−1.485	−2.478	−0.492
Yes		0		
NAFLD				
No	0.043	−1.244	−2.447	−0.040
Yes		0		

Abbreviations: CIs, confidence intervals; NAFLD, nonalcoholic fatty liver disease.

the genus level, 10, 17, and 21 significantly different bacteria exhibited in NS controls, OS and RS patients, respectively ($P < .05$) (Table S2).

In addition, when comparing NS controls vs KS patients, 15 and 40 significantly different bacteria genera were abundant in the gut microbiota of NS and KS patients ($P < .05$) (Table S3). When comparing the gut microbiota between OS and RS patients, only three (*Eubacterium*, *Lautropia*, and *Ruminococcaceae*_UCG_005) and four bacteria (*Blautia*, *Eubacterium*_hallii_group, *Fusicatenibacter*, and *Lachnospiraceae*_ND3007_group) were higher in OS and RS patients, respectively ($P < .05$).

3.5 | Metabolic function of gut microbiota

Based on the classification of bacteria by using 16S data, differentially abundant metabolic pathways were detected among OS, RS patients and NS controls. LEfSe analysis revealed that the abundance of porphyrin and chlorophyll metabolism (ko00860) was higher in NS controls, and that of citrate cycle (ko00020) was higher in KS patients (Figure 2D). When comparing between NS controls and KS patients (OS/RS), more abundant metabolic pathways related to inflammation and oxidative stress, such as lipopolysaccharide (LPS) biosynthesis (ko00540), were identified among KS patients. On the contrary, the abundance of pathways in methane metabolism (ko00680), arginine and proline metabolism (ko00330) were higher in NS Controls ($P < .05$) (Figure 2D).

3.6 | Taxonomic analysis of gut microbiota between NS controls and KS patients selected by propensity score match (PSM)

To eliminate the possible confounding effects of the above-mentioned factors (such as gender, hypertension, and NAFLD), we selected 24 NS controls and 24 KS patients by using PSM, which was a statistical tool to balance the possible confounders across comparison groups tool.[13] These factors were not statistically different between these two groups after this operation ($P > .05$) (Table S4). Using LEfSe, we found 12 and 27 significantly different bacteria genera in the gut microbiota of NS and KS patients (Table S5) ($P < .05$). The results were similar before and after PSM.

3.7 | Functional analysis of fecal microbiota by metagenomics

To investigate the functional profile of the gut microbiome in KS patients, we also performed metagenomics analysis of the microbial DNA extracted from fecal samples of OS, RS patients and NS controls. A total of 678,658,648 filtered reads and 4,037,497 ORFs were used for functional annotation in the KEGG databases.

LEfSe analysis of metagenomics data was used to compare bacteria among NS controls, OS and RS patients at the species level, and detected nine significantly different bacteria exhibited between NS controls and KS patients (Figure 3A). When comparing between OS and RS patients, only three significantly different bacteria (*Thermorudis_peleae*,

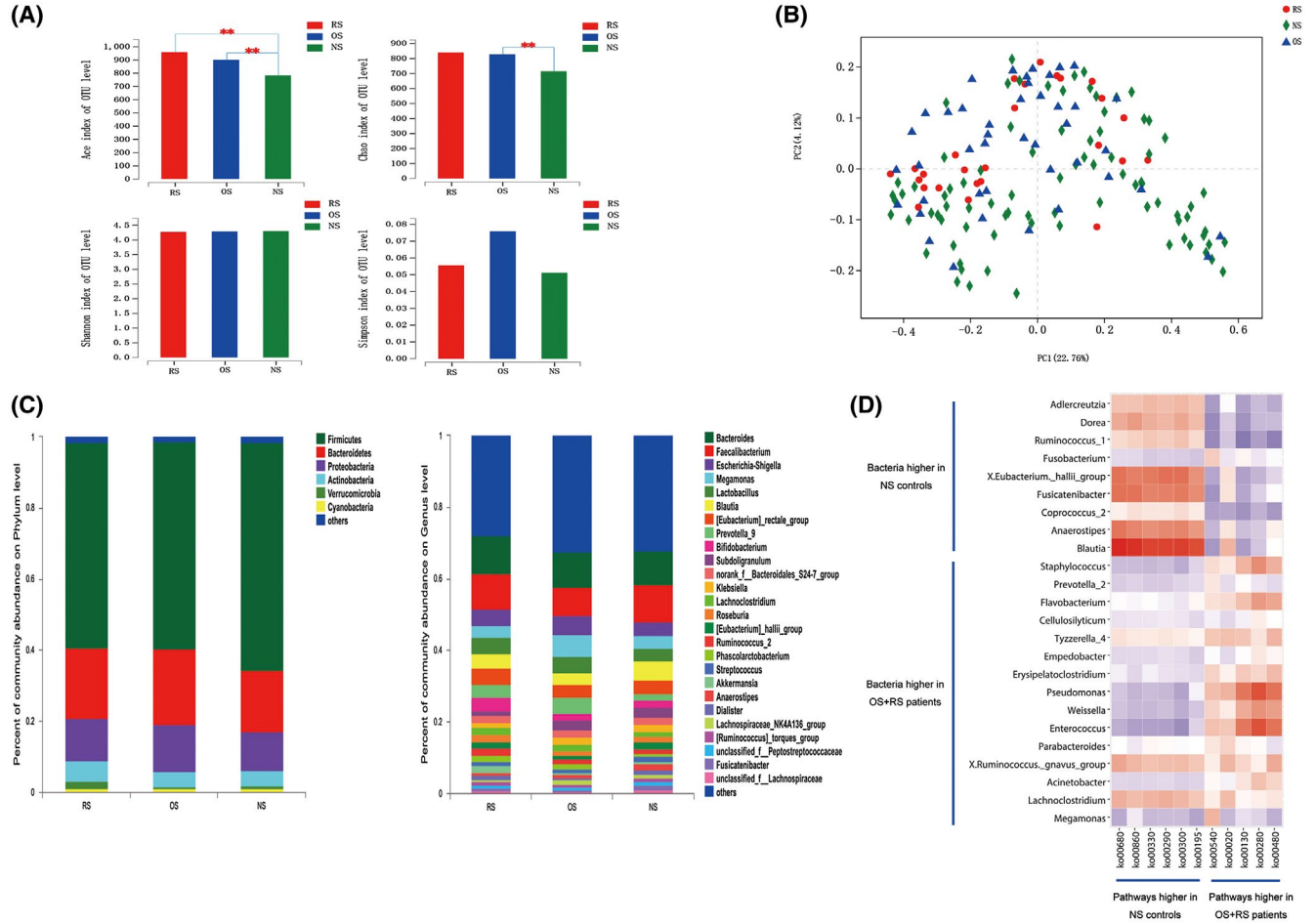

FIGURE 2 Analysis of fecal microbiota among non-kidney stone (NS) controls (n = 84), occasional stone (OS) patients (n = 43) and recurrent stone (RS) patients (n = 26) by using 16s rRNA. A, Comparison of alpha diversity of gut microbiota between NS controls, OS and RS patients. Ace, Chao, Shannon, and Simpson indices at operational taxonomic units (OTUs) level were compared between NS controls (green), OS (blue), and RS patients (red) by the Wilcoxon rank-sum test (**$P < .01$). B, Comparison of beta diversity of gut microbiota between NS controls, OS and RS patients. PCoA score plot based on binary pearson distance at OTUs level revealed classification of NS controls, OS and RS patients. Green diamond represented NS controls. Blue triangle represented OS patients. Red points represented RS patients. C, The composition of gut microbiota in NS controls, OS and RS patients at phylum or genus level. D, The associations of differential bacteria with differential metabolic pathways in the NS controls and kidney stone (KS) patients (OS + RS)

Salsuginibacillus_kocurii, and *Sebaldella_termitidis*) were found at the species level (Figure 3B).

We further determined changes in functional composition by using the KEGG pathway database ($P < .05$). Pearson correlations between different gut microbiota and different KEGG Orthology (KO) or gene pathways among NS controls, OS patients and RS patients were shown in Figures 4 and 5. Predominant *Lactobacillus_diolivorans* in OS patients was positively correlated with biofilm formation. Predominant *Fictibacillus_phosphorivorans* in OS patients were positively correlated with benzoate degradation, phenylalanine metabolism, biofilm formation, beta-Alanine metabolism and caprolactam degradation.

Moreover, predominant *Enterococcus_faecium* in OS patients were positive correlated with isoquinoline alkaloid biosynthesis, limonene and pinene degradation, benzoate degradation, phenylalanine metabolism, biofilm formation, propanoate metabolism, tropane, piperidine and pyridine alkaloid biosynthesis, beta-Alanine metabolism, butanoate metabolism, and caprolactam degradation.

However, predominant *Xanthobacter*_sp__126 in RS patients was positive correlated with carotenoid biosynthesis. Predominant *Lentisphaera_araneosa*, *Arenitalea_lutea*, and *Akkermansia*_sp__KLE1605 in RS patients were all positive correlated with sesquiterpenoid and triterpenoid biosynthesis and carotenoid biosynthesis (Figure 5).

3.8 | Oxalate degradation genes in gut microbiota and oxalate levels in urine

RS patients presented a higher median urinary oxalate (18.10 μg/mL) than OS patients (8.53 μg/mL) and NS controls (4.60 μg/mL). The Kruskal-Wallis test showed that the

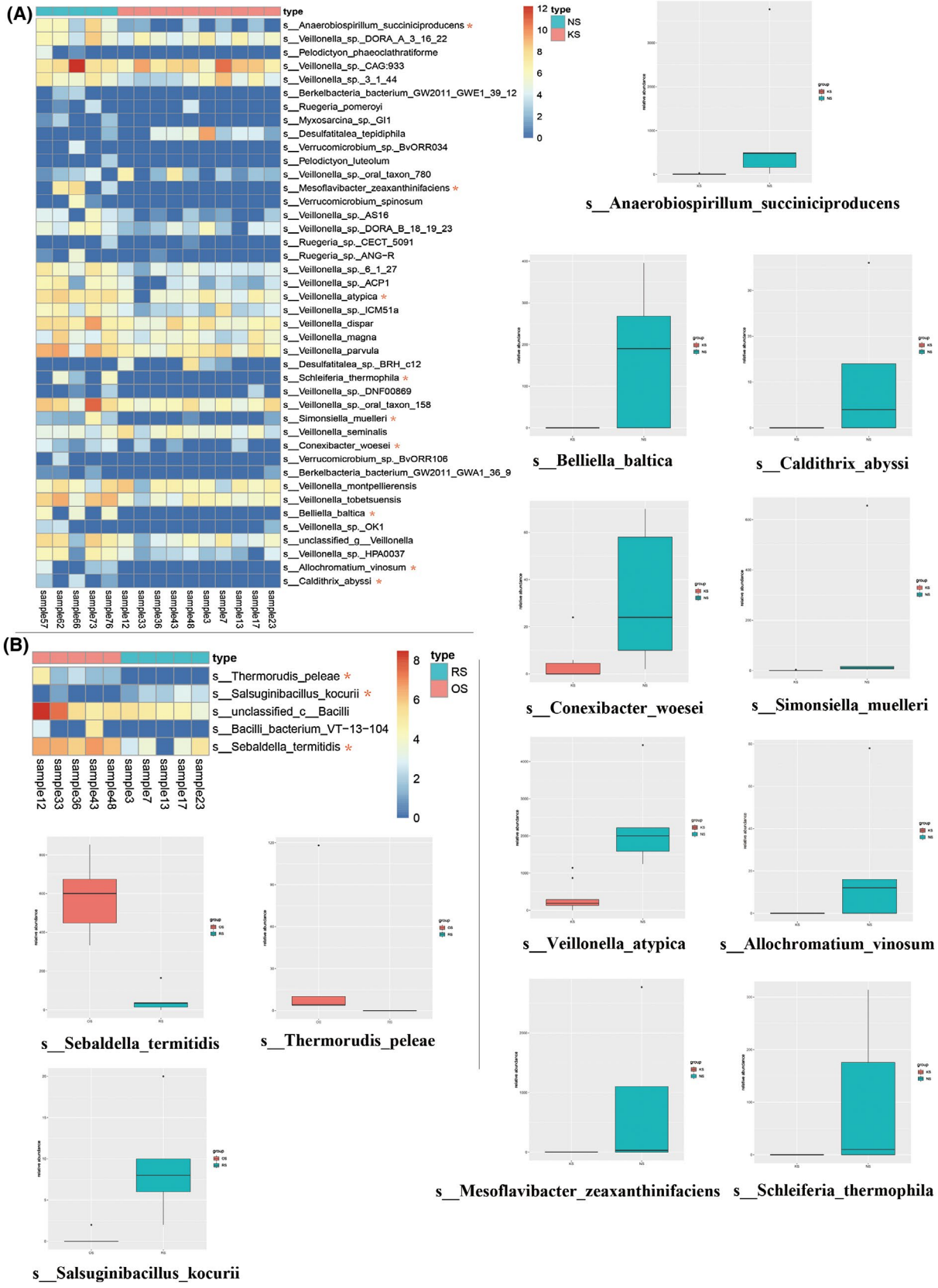

FIGURE 3 Analysis of fecal microbiota among non-kidney stone (NS) controls (n = 5), occasional stone (OS) patients (n = 5) and recurrent stone (RS) patients (n = 5) by using 16s rRNA at species level using metagenomics. A, The different bacteria between NS controls and kidney stone (KS) patients (OS + RS) ($P < .05$). B, The different bacteria between OS patients and RS patients ($P < .05$). (* $P < .01$)

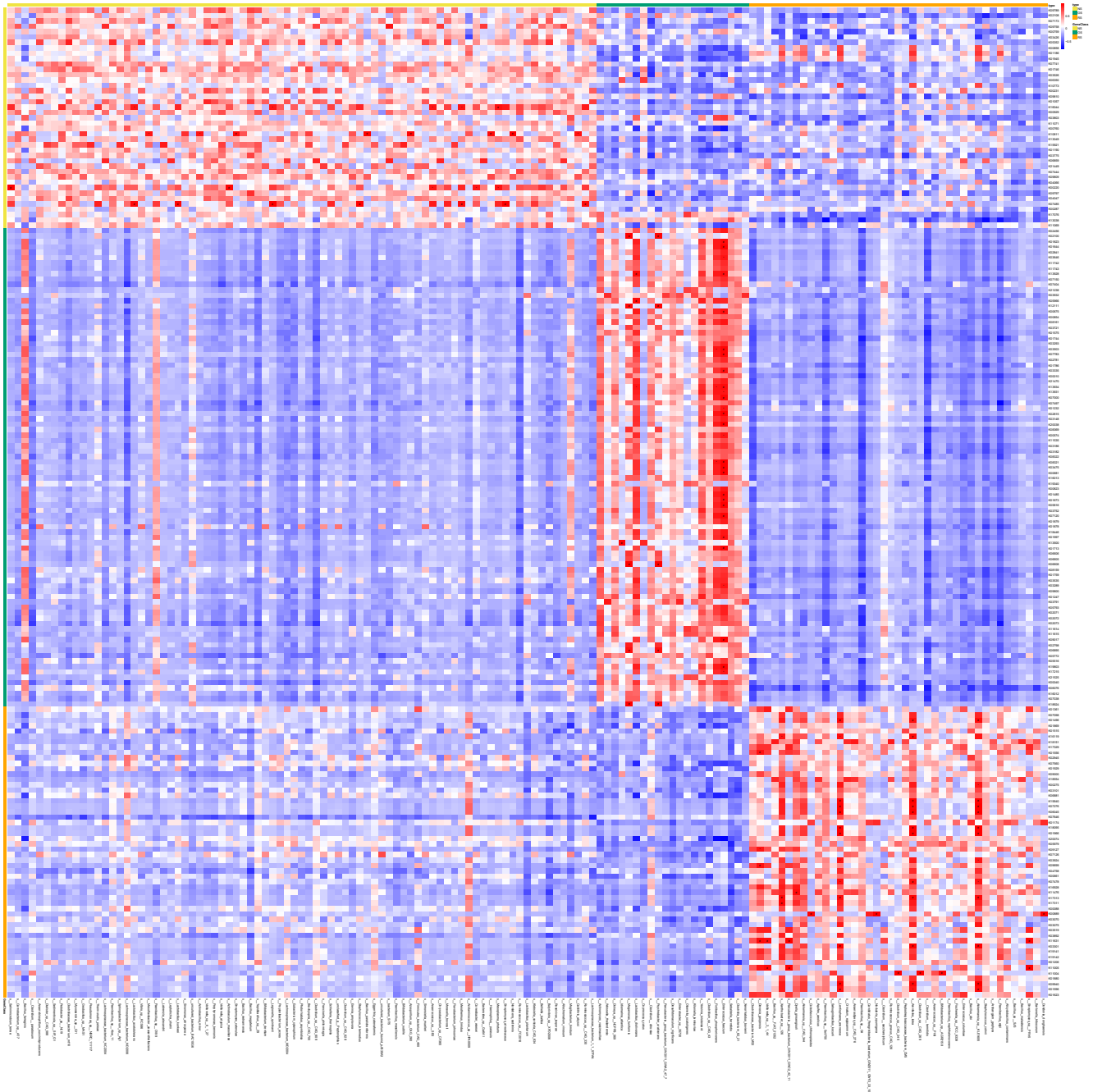

FIGURE 4 Heatmap showing pearson correlation between different KEGG Orthology (KO) and different microbial taxa identified by metagenome sequence-based analysis in non-kidney stone (NS) controls (n = 5), occasional stone (OS) patients (n = 5) and recurrent stone (RS) patients (n = 5)

median urinary oxalate was significantly different across the groups ($P = .004$). However, based on metagenomics analysis, the fecal microbiota of metagenomics data presented no significant differences in the abundance of *O formigenes* ($P = .744$), genes involved in oxalate degradation (formyl-CoA transferase ($P = .254$) and oxalyl-CoA decarboxylase ($P = .216$)) between KS patients and NS controls (Figure S1E-G).

3.9 | Fecal SCFAs concentrations correlate with abundance of distinct bacterial taxonomic groups

Pearson correlation was used to quantify the associations of different bacteria and the abundance of metabolic pathways in NS patients and KS patients, separately. Interestingly, using the 16S data, the abundance of pathways involved in

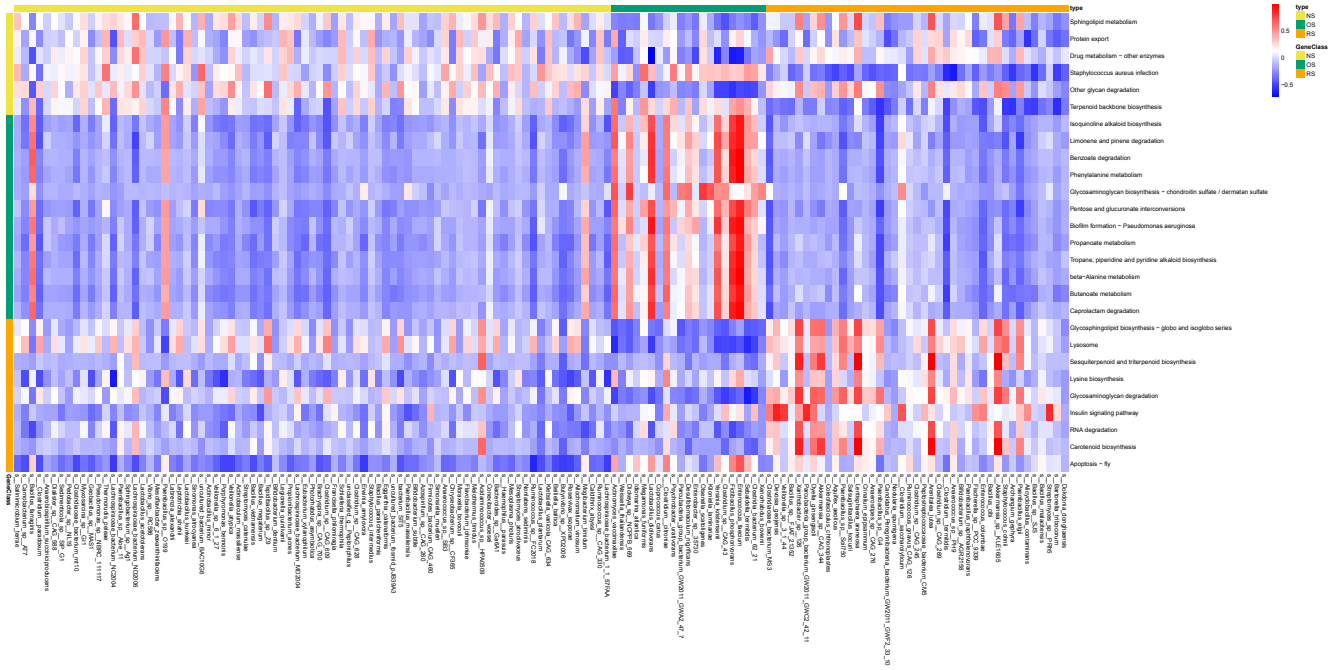

FIGURE 5 Heatmap showing pearson correlation between different KEGG pathways and different microbial taxa identified by metagenome sequence-based analysis in non-kidney stone (NS) controls (n = 5), occasional stone (OS) patients (n = 5), and recurrent stone (RS) patients (n = 5)

SCFAs production was positively correlated with the level of bacteria higher in the gut microbiota of NS controls, but was negatively correlated with that of KS patients (Figure 6A), which indicated that the gut microbiota of NS controls may tend to produce more SCFAs than KS patients.

To validate the role of SCFAs in renal calcium oxalate stones, we evaluated the fecal SCFAs concentration in NS controls, OS and RS patients. The concentration of acetic acid in the feces was higher in RS patients (median = 53.20 μg/mg, IQR = 28.17 μg/mg) than that in OS patients (45.63 μg/mg, IQR = 37.41 μg/mg) and NS controls (median = 21.44 μg/mg, IQR = 10.22 μg/mg) ($P < .001$) (Figure 6B, Table S6).

We explored the relationship between the fecal SCFAs concentrations and the abundance of bacterial taxonomic groups. Using 16S data, we found that butanoic acid-3-methyl, pentanoic acid, and hexanoic acid were highly correlated with taxa belonging to Ruminococcaceae_UCG_005 in NS controls (Figure 6C). To investigate the relationship between the fecal SCFAs and function of gut microbiota among groups, metagenomics data was analyzed by Pearson correlation analysis. Propionic acid was highly correlated with genes for *araE* (K02100) and *lysE* (K06895) in OS patients (Figure 6D). In RS patients, acetic acid was positively correlated with genes for *licA* (K16119), *shc* (K06045), *PCCB* (K01966), *pckA* (K01596), *treS* (K17311), *treU* (K17313), *hisI* (K01496), *pdxH* (K00275), *IMPDH* (K00088), *mexY* (K18095), and *secG* (K03075), which were involved in the pathways of metabolism of terpenoids and polyketides, carbohydrate metabolism, membrane transport, amino acid metabolism, metabolism of cofactors and vitamins, nucleotide metabolism, signal transduction, and genetic information processing, respectively (Figure 6D).

We found high fecal concentration of acetic acid was highly positively correlated with glyoxylate and dicarboxylate metabolism (*PCCB,* K01966) in RS patients with high level of urinary oxalate. We further investigated the bacterial taxonomic groups related to glyoxylate and dicarboxylate metabolism (*PCCB,* K01966) among NS controls, OS and RS patients. The results showed that the abundance of s__*Ruminococcaceae_bacterium*_AE2021 association with *PCCB* was significant higher in KS patients than that in NS controls ($P < .05$) (Figure 6E).

3.10 | Effect of SCFAs on renal calcium oxalate crystals

To verify the effect of SCFAs on the formation of renal calcium oxalate crystals, we gave different SCFAs to renal calcium oxalate stone model rats. HE and VK staining showed that renal crystals reduced after four weeks' administration of acetate, propionate, or butyrate (Figure 7). The results demonstrated that SCFAs could effectively prevent the formation of renal calcium oxalate crystals.

4 | DISCUSSION

Our study found that depletion of gut microbiota with antibiotics could promote the formation of renal crystals in rats,

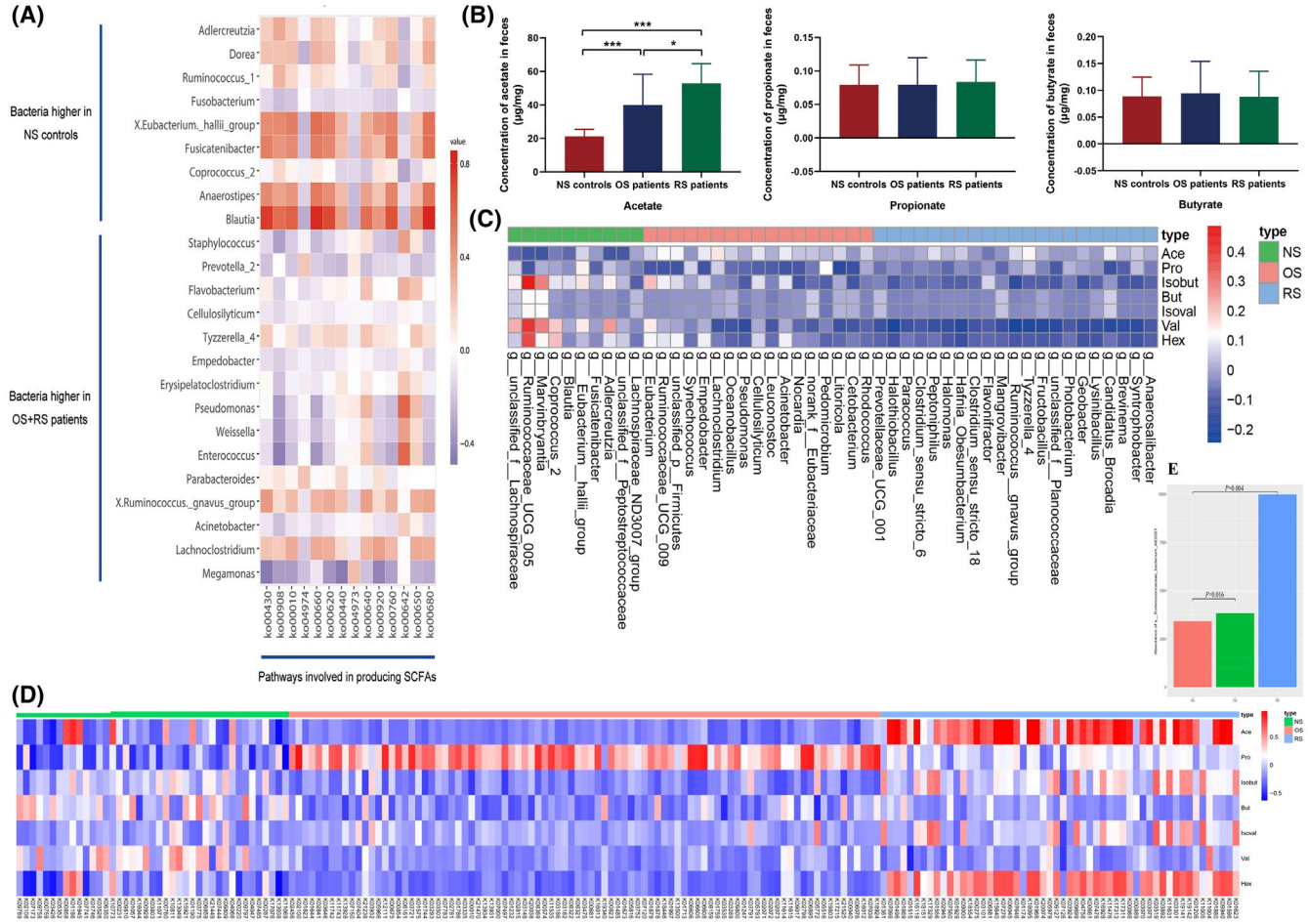

FIGURE 6 Analysis of fecal short chain fatty acids (SCFAs) and gut microbiota among non-kidney stone (NS) controls (n = 84), occasional stone (OS) patients (n = 43) and recurrent stone (RS) patients (n = 26). A, The associations of differential bacteria in NS controls and KS patients with pathways involved in SCFAs production. B, The levels of acetate, propionate, and butyrate among NS controls, OS and RS patients. The levels of acetic acid, propionic acid, and butyric acid among NS controls, OS and RS patients by Mann-Whitney test (*$P < .05$, ***$P < .001$). C, The correlation between SCFAs and gut microbiota. D, The correlation between SCFAs and function of gut microbiota by Pearson correlation analysis. E, The abundance of s__Ruminococcaceae_bacterium_AE2021 association with *PCCB* between healthy controls and patients with kidney stone

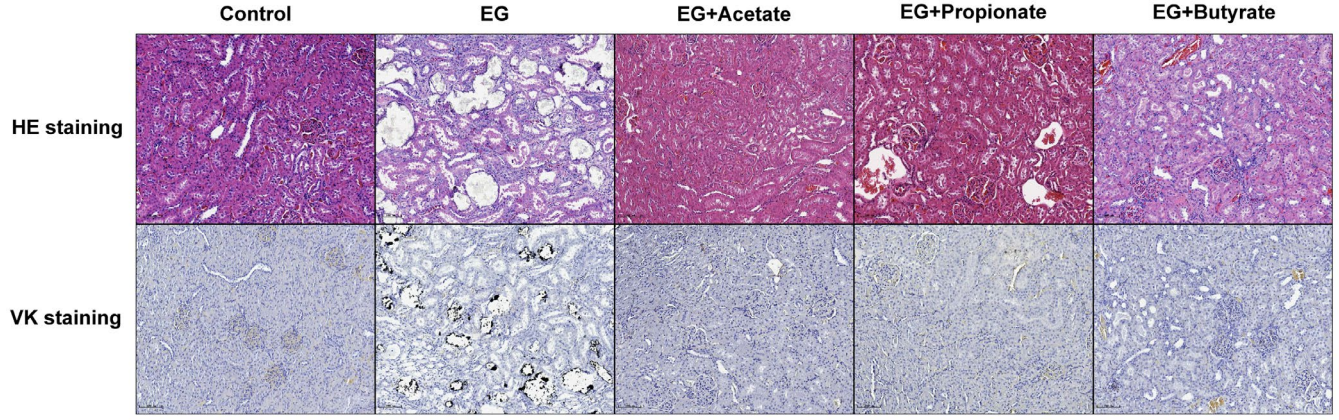

FIGURE 7 Effect of SCFAs on renal calcium oxalate crystals (n = 5/group). Rats received drinking water with EG plus sodium acetate, sodium propionate, and sodium butyrate, respectively, for 4 weeks. Hematoxylin-Eosin (HE) and Von Kossa (VK) staining showed that three kind of SCFAs all could reduce renal calcium oxalate crystals

and then, we investigated gut microbiota profiles and fecal SCFAs in relation to patients with occasional and recurrent renal calcium oxalate stones.

We found that several bacteria were higher in NS controls' gut microbiota compared with those in the KS patients. They were *Blautia*, *Anaerostipes*, *Coprococcus*, *Fusobacterium*, *Ruminococcus*, and *Lachnospiraceae*, of which the common abundant metabolic products were SCFAs.[14,15] SCFAs were known to be the energy sources for enterocytes, which can maintain gut barrier function and in turn decrease permeability of gut, circulating LPS, and systemic inflammation.[16-18] SCFAs can also enter the circulation of the host and reach kidneys to ameliorate inflammatory responses and fibrotic progress in chronic kidney disease.[9,19] Our i*n vivo* study also demonstrated that SCFAs like acetate, propionate, and butyrate could reduce renal calcium oxalate stones in model rats.

This study identified s_*Anaerobiospirillum succiniciproducens*, s_*Veillonella atypica*, and s_*Conexibacter woesei* associated with SCFAs, exhibited significantly higher abundance in NS controls than those in KS patients. *Anaerobiospirillum succiniciproducens* was reported to produce succinic acid,[20] through which propionate can be formed by lactate.[21] *Veillonella atypica* was found to utilize lactate as their sole carbon source into propionate.[22] Hugenholtz et al found *C woesei* preferred acetic acid and propionic acid for utilization.[23] However, in this study fecal acetic acid was higher in KS patients than in NS controls, suggesting that less acetic acid was absorbed and utilized by gut, bacteria, or other organs (kidney) in renal stone patients.

Pseudomonas, *Staphylococcus*, *Megamonas*, *Synechococcus*, *Acinetobacter*, *Cetobacterium*, and *Prevotellaceae*_UCG_001 were found in KS patients, which were associated with inflammatory diseases,[24] indicating that inflammation may be involved in formation of renal stones. In addition, we found that the abundance of metabolic pathways associated with LPS biosynthesis was higher in KS patients. Excessive LPS may aggravate systematic inflammation and contribute to the injury of vascular endothelial cell or even renal tubular epithelial cell.[25] *Halomonas*, abundant in the gut microbiota of the RS patients tend to consume more red meat, was found to be more abundant in omnivores than strict vegetarians, and was associated with higher interleukin-1.[26,27]

Previous studies on gut-kidney axis in nephrolithiasis were often based on the analysis of *O formigenes* and genes involved in oxalate degradation.[28-30] *Oxalobacter formigenes* may degrade oxalate to reduce urinary oxalate excretion, and the bacteria enriched in healthy individuals tended to have more abundance of *O formigenes*.[29] The results showed that the abundance of *O formigenes* and oxalate-degrading genes were not different in NS controls and KS patients, which suggested that *O formigenes* may not play an important role in renal stone formation with our study. Based on 16S data, previous studies demonstrated different results that KS patients had higher, lower, or similar level of formyl-CoA transferase and oxalyl-CoA decarboxylase in the gut microbiota.[30-33] A recent randomized clinical study reported that there was no difference in blood oxalate concentration and stone events between primary hyperoxaluria patients with or without probiotics containing *O formigenes*.[7] The discrepancy between the studies may be attributed to several reasons. First, the included population of these studies had different lifestyle, dietary habits, living environment, ethnic backgrounds, and disease states, all of which would affect the gut microbiota. Second, the relative abundance of *O formigenes* was relatively low in the gut microbiota.

Our study showed that shotgun metagenomics sequencing may identify bacterial species with previously unknown oxalate synthesis properties, such as high SCFAs excretion. We found a highly expressed bacterial gene that was involved in the metabolism of glyoxylate and dicarboxylate (*PCCB,* K01966) among RS patients, and was associated with high levels of urinary oxalate and acetic acid excretion. The results revealed that gut-kidney axis in nephrolithiasis may be related to *O formigenes* and the genes involved in oxalate degradation, as well as other bacteria and genes involved in oxalate synthesis.

The present study was performed on a limited number of people residing in West China, who may not be representative of the general population. However, our study has several strengths as well. First, to our knowledge, this study, which focused on the associations between gut microbiota and renal calcium oxalate stones, has recruited the largest number of Chinese participants among the similar studies. Second, as opposed to the studies that did not distinguish occasional and recurrent episodes, our study further grouped the KS patients into the OS and RS patients. Our refined analyses have shed more light on the biological mechanisms underlying the development of KS. Finally, we have found, perhaps for the first time, that reducing SCFAs absorption and utilization may prevent the formation of nephrolithiasis.

In conclusion, the study showed that the depletion of gut microbiota promoted the formation of renal crystals. Fecal SCFAs dominant patients, with altered gut microbiota composition and the functional metagenome, were associated with oxalate synthesis. These results shed new light on bacteria and genes involved in oxalate synthesis, which may help the development of treatment strategy by regulating gut microbiota.

ACKNOWLEDGMENTS

This work was supported by the National Natural Science Foundation of China [81770703, 81970602]; the Foundation of Science & Technology Department of Sichuan Province

[2018SZ0118]; and the 1·3·5 Project for Disciplines of Excellence, West China Hospital, Sichuan University [ZYGD18011, ZYJC18015, ZY2016104].

CONFLICT OF INTEREST

The authors have stated explicitly that there are no conflicts of interest in connection with this article.

AUTHOR CONTRIBUTIONS

Y. Liu, X. Jin, and K. Wang designed research; Y. Liu, Q. Jiang, Z. Chen, L. Cheng, Y. Ma, Z. Jian, Z. Wei, and J. Ai performed research; Y. Li and H. G. Hong contributed new analytic tools; Y. Liu, X. Jin, H. G. Hong, L. Xiang, Z. Jian, Z. Wei, J. Ai, S. Qi, Q. Sun, and Y. Li analyzed data; Y. Liu and X. Jin wrote the paper; H. G. Hong, S. Qi, Q. Sun, H. Li, Y. Li, and K. Wang reviewed the paper.

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the Supporting Information section.

How to cite this article: Liu Y, Jin X, Hong HG, et al. The relationship between gut microbiota and short chain fatty acids in the renal calcium oxalate stones disease. *The FASEB Journal*. 2020;34:11200–11214. https://doi.org/10.1096/fj.202000786R