Original Article

Butyrate-Producing Bacteria as Microbiomarkers of Chronic Kidney Disease Progression in Children.

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ABSTRACT

Introduction: The gut microbiota is increasingly recognized as a critical component in the control of host health. Dysbiosis and chronic kidney disease (CKD) have a bidirectional link. Butyrate- producing bacteria have recently gained attention and is a poorly understood faecal state in CKD children.

Aim: to study the variations in butyrate generating species (Roseburia spp. and F. prausnitzii) in the faeces of children with CKD at different stages.

Methods: A case-control study with 52 CKD children and 26 healthy subjects was conducted. To verify the alterations in these species, the quantitative real-time polymerase chain reaction (qPCR) was performed.

Results: Roseburia spp. and F. prausnitzii were considerably lower in CKD children compared to controls and were significantly lower in CKD stage5 ($p < 0.001$). The best cutoff of F. prausnitzii ratio for association with end stage renal disease is ≤15.205 with area under curve 0.928 with ($p<0.001$). Roseburia spp., F. prausnitzii ratio were statistically significantly lower in CKD patients with thrombosis than cases without evidence of thrombosis ($p=0.011$, $p=0.009$) respectively.

Conclusion: The depletion of Roseburia spp. and F. prausnitzii can be considered microbiomarkers of CKD inflammation, thrombosis, and progression in CKD children.

Keywords: Butyrate, Roseburia spp., Faecalibacterium prausnitzii, microbiota, chronic kidney disease.

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INTRODUCTION

One of the most important public health issues is chronic kidney disease (CKD), which affects 10 to 15% of the world's population [1]. Gut microbes play a crucial part in the pathogenesis and progression of CKD, dysbiotic microbiota enhanced the generation of uremic toxins from the gut and changed the intestinal barrier. These modifications, in turn, lead to the accelerated progression of renal damage [2]. Bacterial translocation occurs in individuals with end stage renal disease (ESRD) and has been linked to microinflammation, [3] which was more severe in dialysis patients [4]. Recent findings have shown that there is extensive decomposition of the structure of the intestinal epithelial barrier and profound changes in gut microorganisms in late CKD, implying a role in the pathogenesis of inflammation and uremic toxicity, exacerbated cardiovascular disease, and many CKD related complications [5]**.** The accumulation of uremic toxins damages several organs, including the kidney. Conversely, a quantitative decrease in short chain fatty acids (SCFAs), especially butyrate, aided in the course of CKD [6].

Butyrate has anti-inflammatory properties, helps to maintain gut homeostasis, and influences tissues and organs beyond the gut when absorbed into the bloodstream [7, 8]. Several studies have demonstrated that the etiology of CKD is directly influenced by inflammation [9]**.** Much research suggested the possibility of employing anti-inflammatory agents to treat CKD [10]. Butyrate regulates the differentiation of Treg cells [11]. The depletion and malfunctioning of regulatory T cells exacerbate ESRD [12]. According to cultural and molecular research, the principal butyrate generating bacteria detected in human faeces include phylogenetically diverse (*Roseburia spp*.) and Clostridium coccoides (*F. prausnitzii*) [13]**.** *F. prausnitzii* accounts for more than 5% of the human gut microbiome, making it the most prevalent bacterial species in the gut [14]. *F. prausnitzii* depletion has been proposed as a biomarker for inflammatory bowel disease [15]. However, *F. prausnitzii and Roseburia species* distribution in children with CKD is poorly understood, so the goal of this study was to investigate and estimating variations in these species and their cardiovascular impact in different stages of CKD children.

METHODS

Studied Group: A case control study was conducted at pediatric Nephrology Units in Zagazig Children Hospital from December 2021 to January 2023. The study was performed on 52 CKD children [26 CKD stage 5 at regular dialysis (ESRD), 26 CKD (stages 1–4)] and 26 healthy control children, age and gender matched. In this study, the (2012) kidney disease improving global outcomes (KDIGO) clinical practice guidelines were used to define and classify CKD [16]**.** Children who received antibiotics, probiotics, prebiotics, and laxatives in the four weeks before sample collection, as well as diabetic and hyperlipidemic children, were excluded from the study.

Assessment of clinical and biochemical parameters: Each Patient was subjected to thorough history taking with special focus evidence of the previous episodes of thrombosis (e.g., arteriovenous fistula thrombi that were confirmed with Doppler ultrasonography). Clinical

parameters were assessed including weight, height, body mass index (BMI) as Kg/m^2.Blood urea nitrogen (BUN), serum creatinine, blood glucose level, serum cholesterol, triglyceride (TG), C-reactive protein (CRP) were measured. Estimated glomerular filtration rate (eGFR) was calculated using Schwartz formula.

Quantification of bacteria using quantitative polymerase chain reaction (qPCR): Fresh stool samples were collected from the participants and stored at –80°C until PCR analysis. DNA was extracted from fecal samples using extraction kit (Zymo Research Corp, Irvin, CA, United States) following the directions provided by the manufacturer. To quantify *Roseburia and F. Prausnitzii* in the faecal samples, quantitative real-time PCR was performed. Applied biosystem real time PCR (Step one TM, real-time PCR system, Applied Biosystems Inc, USA) was used to analyze the faecal microbiota. All CKD patients' and controls' DNA extracts were diluted (1:10). The standard curve was created using a pool of healthy children's stool samples and two-fold serial dilution of the extracted bacterial DNA. Total bacteria, *Roseburia, and F. Prausnitzii* standard curves were created. Each bacterial family was represented as a ratio of the total faecal bacteria in each sample. **Table 1** lists the primers used (Willowfort, Birmingham). The final reaction volume for the amplifications was 20 ul, which contained 10 ul of 2x SYBR mix (Willowfort, Birmingham), 1 ul of each primer, and 8 ul of bacterial DNA. The initial phase of the amplification process involved denaturing the DNA for 10 minutes at 95 °C. Then, 35 cycles of denaturation for 15 seconds at 95 °C, annealing for 30 seconds at 60 °C, and elongation for 40 seconds at 72 °C were performed. The process ended with an elongation step at 72°C for 10 minutes.

STATISTICAL ANALYSIS

SPSS (Statistical Program for the Social Sciences) version 26 was used to analyze the data. Depending on the type of data, quantitative variables were described using their means and standard deviations or median and range. The chi square test was used to compare categorical variables. The Mann Whitney test, Kruskal Wallis test and one way ANOVA test were used to compare data. Pairwise comparison and Tukey HSD comparison were performed to determine the difference between each two distinct groups when the difference was significant. Spearman rank correlation coefficients were employed to determine the degree and direction of the association between two continuous variables. Linear regression analysis was used to determine the related independent factors for the dependent factor. The ROC curve was used to determine the best cutoff of *Roseburia and F. Prausnitzii* in diagnosis of ESRD. The level statistical significance was set at $p<0.05$. A highly significant difference was present if $p\leq0.001$.

RESULTS

Patients and controls characteristics: When CKD and ESRD patients were compared to healthy controls, their BUN and creatinine levels were significantly higher, but their eGFR was lower. The CRP level differ significantly between CKD, ESRD, and control individuals ($p<0.001$). Age, sex, BMI, glucose, TG, and cholesterol differences between the analyzed groups are statistically insignificant **Table 2.**

Distribution level of SCFAs producing bacteria: Between CKD children and controls, *Roseburia spp. and F. prausnitzii* showed a significant difference (p<0.001)**.** On doing pairwise comparison test, the difference is significant between each two individual groups. *Roseburia species* in ESRD children decreased in comparison to CKDstage1-4 (median 9.26 vs. 23.76, p = 0.01)**.** *F. prausnitzii* in ESRD children were decreased in comparison to CKD1-4(median 4.24 vs. 12.06, p =0.009) **Table 3**.

Correlation study: Spearman rank correlation analysis demonstrated that both *Roseburia* spp. and *F. prausnitzii* showed statistically significant negative correlation with CRP, creatinine, BUN, and positive correlations with eGFR **Table 4**.

Regression analysis: Among factors significantly correlated to the *F. prausnitzii* ratio, only eGFR (unstandardized β=0.589) and CRP (unstandardized β=-6.606) were significantly independently associated with it **Table 5.** Among factors significantly correlated to the *Roseburia* spp. ratio, only *F. prausnitzii* ratio (unstandardized β=1.324) was significantly independently associated with it **Table 6**.

Receiver operating characteristic (ROC) curve: The best cutoff of the *F. prausnitzii* ratio for association with ESRD is ≤15.205 with area under curve 0.928, sensitivity 96.2%, specificity 71.2%, positive predictive value 62.5%, negative predictive value 97.4% and overall accuracy 79.5% (p<0.001) **Figure 1.** The best cutoff of *Roseburia* spp. ratio for association with ESRD is ≤11.815 with area under curve 0.921, sensitivity 92.3%, specificity 86.5%, positive predictive value 77.4%, negative predictive value 95.7% and overall accuracy 88.5% (p<0.001) **Figure 2.**

Boxplot showing relation between evidence of thrombosis and both *Roseburia and F. Prausnitzii* ratio: We found in 11 out of 52 CKD children had experienced previous episodes of thrombosis in the later period, the median of *Roseburia* spp. Ratio was statistically significantly lower in cases with thrombosis than cases without evidence of thrombosis (9.72, 10.84) respectively with p=0.011. The median of *F.prausnitzii* ratio was statistically significantly lower in cases with thrombosis than cases without evidence of thrombosis (6.7, 10. 86) respectively with p=0.009 **Figure 3**.

Table 1: The primers used in quantitative polymerase chain reaction.

Target Bacteria	Primer	Sequence (5′to 3′)	Product (bp)	Reference
Universal bacteria	Uni-F	ACTCCTACGGGAGGCAGCAGT	200	[35]
	Uni-R	GTATTACCGCGGCTGCTGGCAC		
Roseburia spp	Ros-F	TACTGCATTGGAAACTGTCG	230	[36]
	Ros-R	CGGCACCGAAGAGCAAT		
F. Prausnitzii	Fae-F	GGAGGAAGAAGGTCTTCGG	248	[35]
	Fae-R	AATTCCGCCTACCTCTGCACT		

Table 2: Comparison between the studied group regarding demographic & Laboratory data.

Parameter	ESRD	CKD stages I – IV	Control group	χ^2	p
	N=26 (%)	N=26 (%)	N=26 (%)		
Gender:					
Female	14 (53.8%)	10 (38.5%)	15 (57.5%)	2.154	0.341
Male	12 (46.2%)	16 (61.5%)	11 (42.5%)		
	Mean ± SD	**Mean ± SD**	**Mean ± SD**	**F**	**p**
Age (year)	11.15 ± 2.38	10.38 ± 2.33	10.46 ± 2.08	0.907	0.408
Height (cm)	134.38 ± 12.45	135.42 ± 15.85	128.12 ± 10.44	0.647	0.526
Weight (kg)	33.81 ± 8.35	30.73 ± 4.49	34.62 ± 5.36	1.695	0.191
BMI	18.42 ± 2.45	18.73 ± 2.15	20.87 ± 1.39	0.178	0.837
Urea	50.4 ± 7.13	5.36 ± 1.7	6.76 ± 1.42	916.588	<0.001**
Tukey HSD	P_1 <0.001**	P_2 0.474	P_3 <0.001**		
Creatinine	5.99 ± 1.89	0.84 ± 0.17	0.49 ± 0.06	204.863	<0.001**
Tukey HSD	P_1 <0.001**	P_2 0.494	P_3 <0.001**		
Glucose	76.74 ± 8.69	78.2 ± 10.6	73.41 ± 4.82	2.229	0.115
Triglycerides	63.06 ± 18.2	63.77 ± 16.9	54.34 ± 4.37	2.68	0.075
T.cholesterol	75.4 ± 16.07	77.6 ± 16.46	70.36 ± 7.26	1.845	0.165
CRP	2.33 ±1.41	3.82 ± 1.61	0.87 ± 0.09	36.764	<0.001**
Tukey HSD	P_1 <0.001**	P_2 0.001**	P_3 0.003*		
eGFR	9.5 ± 2.52	68.39 ± 7.56	103.15 ± 7.33	1491.58	<0.001**
Tukey HSD	P_1 <0.001**	P_2 <0.001**	P_3 <0.001**		

F One way ANOVA test χ^2chi square test ****p** ≤0.001 is statistically highly significant *p<0.05 is statistically significant **p1** difference between ESRD group and CKD stages I to IV group **p2** difference between CKD stages I to IV groups and control groups **p3** difference between ESRD and control groups **BMI:** body mass index **eGFR:** estimated glomerular filtration rate.

Table 3: Comparison between the studied groups regarding butyrate-producing bacteria.

Parameter	ESRD	CKD stages I – IV	Control group	KW	p
	Median (IQR)	Median (IQR)	Median (IQR)		
ROS ratio	**9.26 (1.87 – 10.65)**	**23.76(10.06 – 33.35)**	**105.29(73.39 –113.8)**	**57.589**	**<0.001****
Pairwise	**P_1 0.01***	**P_2 <0.001****	**P_3 <0.001****		
FAE ratio	**4.24 (0.5 – 10.92)**	**12.06(10.84 – 25.5)**	**78.05(52.07 – 81.1)**	**59.395**	**<0.001****
Pairwise	**P_1 0.009***	**P_2 0.001****	**P_3 <0.001****		

KW Kruskal Wallis test **p≤0.001 is statistically highly significant *p<0.05 is statistically significant p1 difference between CKD on HD group and CKD stages I to IV group p2 difference between CKD stages I to IV groups and control groups p3 difference between CKD HD and control groups. ROS: *Roseburia* spp. FAE: *Faecalibacterium prausnitzii*

Table 4: Correlation between the ROS, FAE ratio and the studied parameters.

Parameter	ROS ratio		FAE ratio	
	r	p	r	p
Age (year)	-0.111	0.322	-0.086	0.455
Height (cm)	-0.087	0.451	-0.088	0.441
Weight (kg)	0.094	0.415	-0.048	0.679
BMI	0.009	0.938	0.095	0.408
Urea	-0.453	**<0.001****	-0.496	**<0.001****
Creatinine	-0.818	**<0.001****	-0.814	**<0.001****
Glucose	-0.15	0.198	-0.137	0.233
Triglycerides	0.015	0.895	-0.087	0.448
T. cholesterol	-0.048	0.677	-0.138	0.227
CRP	-0.428	**<0.001****	-0.54	**<0.001****
eGFR	0.823	**<0.001****	0.821	**<0.001****
FAE ratio	0.806	**<0.001****		

r Spearman rank correlation coefficient ****p** ≤0.001 is statistically highly significant. **ROS:** Roseburia spp. **FAE:** Faecalibacterium prausnitzii

Table 5: Linear forward regression analysis of factors significantly correlated to FAE ratio.

	Unstandardized Coefficients		Standardized Coefficients			95.0% Confidence Interval	
	β	Std. Error	Beta	t	p	Lower	Upper
(Constant)	12.989	5.108		2.543	0.013*	2.813	23.164
eGFR	0.589	0.052	0.702	11.265	<0.001**	00.485	.694
CRP	-6.606	1.199	-0.343	-5.510	<0.001**	-8.995	-4.218

****p**≤0.001 is statistically highly significant. **FAE:** Faecalibacterium prausnitzii

Table 6: Linear forward regression analysis of factors significantly correlated to ROS ratio.

	Unstandardized Coefficients		Standardized Coefficients			95.0% Confidence Interval	
	β	Std. Error	Beta	t	p	Lower	Upper
Constant	3.541	6.874		0.515	0.608	-10.149	17.231
FAE ratio	1.324	0.147	0.718	8.984	<0.001**	1.031	1.618

****p**≤0.001 is statistically highly significant. **ROS:** Roseburia spp. **FAE:** Faecalibacterium prausnitzii

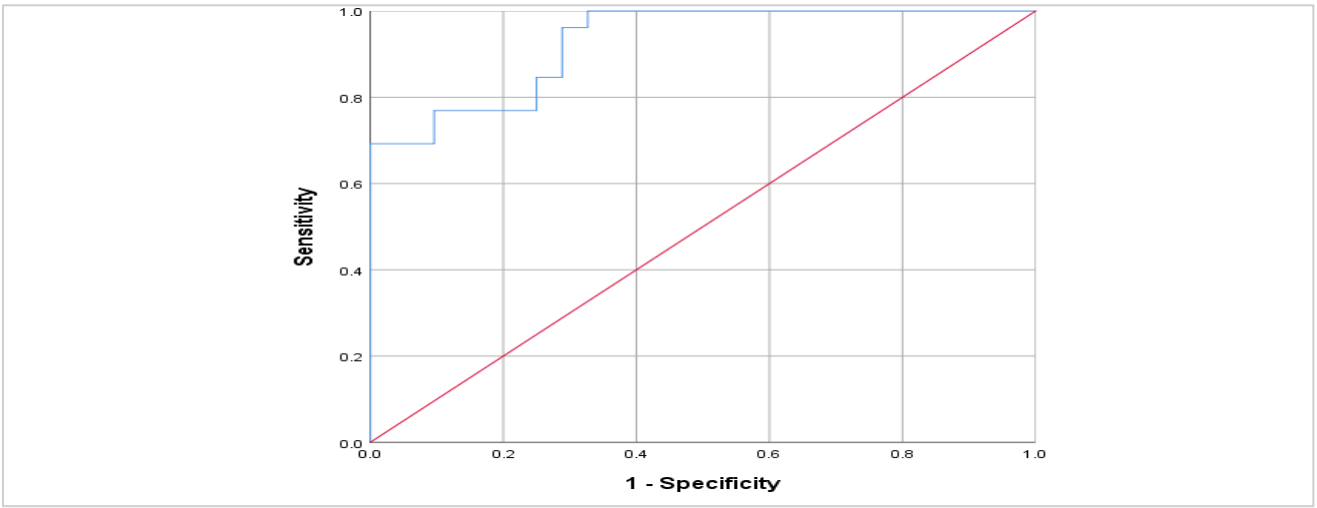

Figure 1: Receiver operating characteristic curve showing Performance of *F. prausnitzii* ratio for association with ESRD.

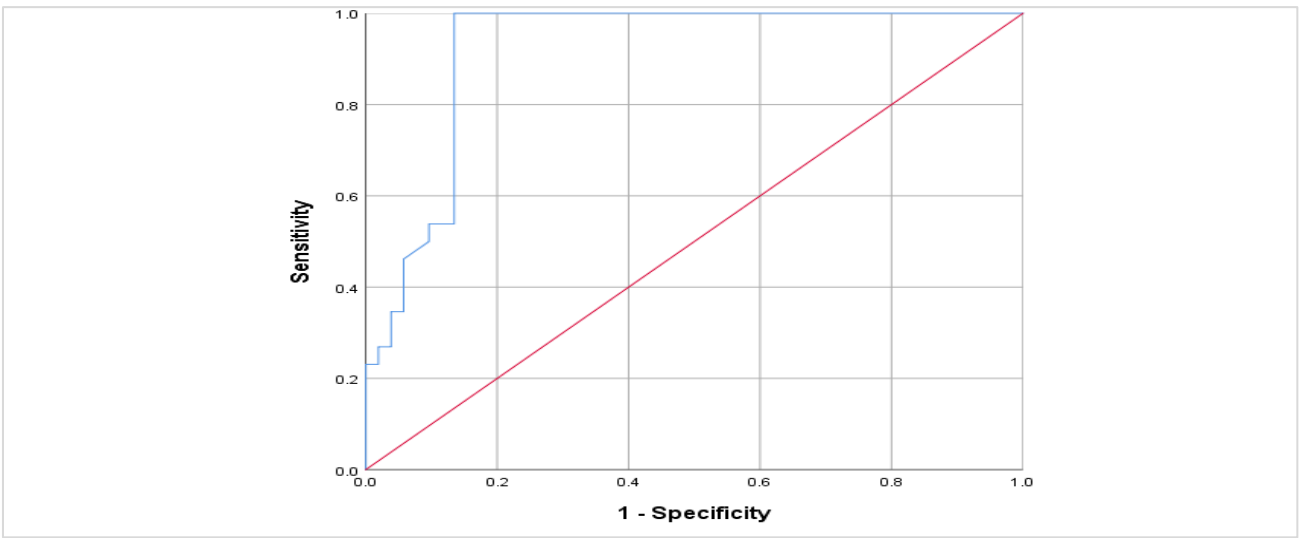

Figure 2: Receiver operating characteristic curve showing Performance of *Roseburia spp.* ratio for association with ESRD.

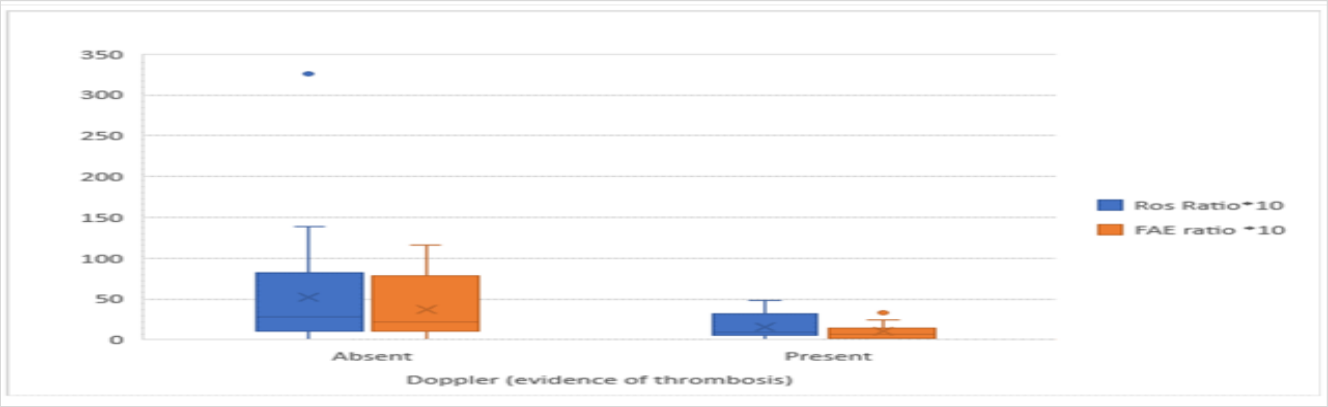

Figure 3: Boxplot showing relation between evidence of thrombosis and both Roseburia spp. And F. prausnitzii ratio.

DISCUSSION

CKD and dysbiosis have bidirectional link, the uremic context influences the microbiota while toxins and metabolites produced in the gut impact the course of CKD. The buildup of microbial metabolites and toxins has been associated with renal function loss and increased mortality risk; however, some protective metabolites, like SCFAs and bile acids, improve kidney function and enhance survival in CKD patients [17, 18]. Evidence suggests that the typical butyrate producing bacteria enhances the equilibrium of the microecology in the host's intestine and has a good influence on nutrient absorption. As a result, new probiotics as therapeutic targets for modifying gut microbiota often aim to improve microbiota dysbiosis [19].

Because of the absence of apparent clinical symptoms in the early stages, most CKD patients progress to renal failure at the time of therapy, with a bad prognosis. As a result, to improve the prognosis of CKD patients, it is critical to look for new diagnostic indicators and treatment targets for CKD [20]. Many efforts have been tried to target gut microbiota or its metabolites for CKD management; therefore, there is a need to discover beneficial commensal potential for CKD.

Our study included 52 CKD child aged [1 to 16 years] and 26 healthy control age and sex matched attending inpatient and outpatient nephrology clinics, aimed to explore and quantify differences in butyrate producing bacteria (F. prausnitzii and Roseburia species) in different stages of CKD. There is no statistically significant difference between the study groups in terms of age or gender, BMI, glucose, TG, or cholesterol, which is consistent with a previous study to detect the features of the gut microbiota on the basis of renal function [21, 22].

Roseburia spp. and F. prausnitzii were found to be significantly more prevalent in health controls than in CKD children in this study, with the reduction being more severe in individuals with advanced renal function deterioration. This agrees with previous studies [21, 23-25]. On the other hand, another study found no significant difference in the abundance of the butyrate producing species between ESRD patients and healthy kidney donors [26]. There are various causes for these conflicting findings; the diversity of the gut

microbiota is known to be altered by a variety of variables including dietary habits, geography, genetc factors, and age which may influence test results [27].

Our study is, to the best of our knowledge, the first study evaluating the levels of key fecal butyrate producing species in the different stages of CKD children. Roseburia spp. and F.prausnitzii levels were negatively correlated with creatinine levels; the opposite tendency was observed regarding eGFR. Supporting our results, another study found that butyrate addition to drinking water reduced BUN and serum creatinine levels in urine as well as renal pathology and macrophage infiltration as well as kidney inflammation [28]. This indicated that a decrease in the butyrate producing species Roseburia spp. and F. prausnitzii played a role in CKD progression.

In the current study, Roseburia spp. and F.prausnitzii were negatively associated with CRP levels. These findings suggest that bacteria that produce butyrate are useful for inflammatory conditions in patients with CKD. Data recently supports the favorable effect of short chain fatty acids in moderating inflammation and oxidative stress, both of which are implicated in the pathogenesis and progression of CKD [29, 30]. Together with our findings, these results support the idea that the decline in butyrate producing species may be involved in Inflammation pathogenesis in CKD patients. In current study, among factors significantly correlated to F.prausnitzii ratio, only eGFR and CRP significantly independently associated with it .The best cutoff of F.prausnitzii and Roseburia spp. ratio for association with ESRD is ≤15.205, ≤11.815 with area under curve 0.928, 0.921 ($p<0.001$) respectively. This agrees with another study that concluded that the loss of the butyrate producing bacteria Roseburia spp., F. prausnitzii probably plays a role in the CKD-related inflammation and progression [21].

In our study we found that Roseburia spp. and F.prausnitzii ratio in CKD children who had experienced previous episodes of thrombosis were statistically significantly lower than cases without thrombosis. This agrees with many studies that found that patients with cardiovascular diseases have lower levels of butyrate generating bacteria in their gut, including Roseburia spp. and F.prausnitzii [31-33]. SCFAs not only decrease interferon- (IFN-) production and protect against mucosal inflammation, but they also slow the progression of atherosclerotic lesions [34].

RECOMMENDATIONS

Treating gut microbiome dysbiosis, reducing bacterial production of uremic toxins, and increasing SCFA production would improve health outcomes for CKD patients.

LIMITATIONS

The sample size is small, so the findings from this study need to be validated in a larger cohort and extended with studies of the whole microbial community linked with CKD.

CONCLUSION

F. prausnitzii and Roseburia spp. depletion may serve as microbiomarkers of CKD inflammation, thrombosis, and progression.

ABBREVIATIONS

BMI	Body mass index
BUN	Blood urea nitrogen
CKD	Chronic kidney disease
CRP	C-reactive protein
ESRD	End stage renal disease
e GFR	Estimated glomerular filtration rate
F.prausnitzii	Faecalibacterium prausnitzii
q PCR	Quantitative real-time polymerase chain reaction
SCFAs	Short chain fatty acids
TG	Triglyceride

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AUTHORS' CONTRIBUTIONS.

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STATEMENTS

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This study protocol and the consents were approved and deemed sufficient by the Ethical Committee of Zagazig University (ZU-IRB#7051) and informed written consent was obtained in every case from their legal guardians.

Consent for publication
The contents and material of the manuscript have not been previously reported at any length or being considered for publishing elsewhere.

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