Role of intestinal microbiota in cardiovascular disease risk in end stage renal disease patients

Chronic inflammation is considered as a non-traditional risk factor for cardiovascular mortality in the chronic kidney disease (CKD) population. Recent studies have revealed that alterations in gut microbiota composition and intestinal barrier have been associated with inflammation and oxidative stress in CKD patients which in turn promotes adverse cardiovascular outcomes and CKD progression. The purpose of our study was to assess the alteration of beneficial gut microbiota mainly Lactobacillus acidophilus in fecal samples of patients with end stage renal disease (ESRD) and the extent of its effect by the co-existence of cardiovascular complications. Forty patients with ESRD, another forty ESRD with CVD and 20 healthy adults participated in the study. The fecal composition of Lactobacillus acidophilus was identified using de Man Rogosa Sharp agar followed by further confirmation using polymerase chain reaction technique. A significant abundance of Lactobacillus acidophilus in ESRD patients compared to controls was noticed, and this increase was detected mainly in ESRD patients with CVD. Furthermore, Lactobacillus acidophilus was not correlated significantly with serum cholesterol, triglycerides or urinary protein creatinine ratio. In conclusion, Lactobacillus acidophilus as a beneficial microbiome has no protective role against progression of CKD and CVD risks. Thus, the exact role of gut microbiota in CKD progression has not been yet fully elucidated and further investigation will be needed.

**Keywords:** gut microbiota, Lactobacillus acidophilus, ESRD, CKD, CVD
access, can significantly alter the biochemical milieu of the intestinal tract and thus may alter the structure, composition, and function of microbial flora [11].

The loss of kidney function in CKD leads to urea secretion into the gastrointestinal tract. Subsequent hydrolysis of urea by urease expressed by some gut microbes, leads to the formation of large quantities of ammonia that could affect the growth of commensal bacteria [12]. Subsequently, these events can contribute to uremic toxicity, inflammation, and cardiovascular, nutritional, and other complications of CKD. Other contributing factors that could alter the growth of intestinal microbiota include slow colonic transit [13], metabolic acidosis [14], intestinal wall edema [15] and possibly oral iron intake [16].

Lactic acid bacteria (LAB) such as Lactobacillus acidophilus are considered to be beneficial microorganisms and have been associated with multiple potential health effects in both humans and animals [17]. Among these effects, the hypocholesteremic effect of Lactobacillus acidophilus has recently gained interest due to its potential role in the prevention of atherosclerosis.

Moreover, the use of Lactobacillus acidophilus strain LB as probiotic was associated with reduced bacterial translocation in the uremic rats and increased macrophage phagocytosis. This strain is not only considered as antibacterial [18], but also reduces epithelial barrier loss induced by pathogenic bacteria such as the Escherichia coli strain C1845 [19].

For this purpose, the present study was designed to assess the alteration of beneficial gut microbiota mainly Lactobacillus acidophilus in fecal samples of Egyptian patients with ESRD and the extent of its effect by co-existence of cardiovascular complications in comparison to healthy controls.

**Material and methods**

This case control study included 100 adult subjects; being categorized into 3 groups; first group of 20 healthy subjects as controls, second group of 40 ESRD patients with no cardiovascular diseases, and third group of 40 ESRD patients with established cardiovascular diseases were recruited in the period from May 2014 to May 2016 from the hemodialysis unit of the Ain Shams University hospitals, Cairo, Egypt. The healthy subjects were not on any medications and had no established medical problems. The Human Research Ethics Committee of Ain Shams University hospital approved this study and all participants gave informed consent.

According to KDIGO CKD classification 2012, ESRD subjects were eligible for the study inclusion if the estimated glomerular filtration rate (eGFR) <15 mL/min/1.72 m², and they were on hemodialysis [20,21].

CVD was diagnosed if the participant had one or more of the following: echocardiographic abnormalities (systolic dysfunction, diastolic dysfunction or left ventricular hypertrophy) and past history of myocardial infarction, or heart failure. Exclusion criteria included: a) known chronic gut-related diseases, (b) patients who underwent bariatric surgeries, (c) patients with end-stage liver disease, and (f) patients who were treated with antibiotics, prebiotics or probiotics for at least three consecutive days for any reason during one month prior to the study enrollment.

The subjects’ demographics, body mass index (BMI), history of hypertension, mean arterial blood pressure (MABP), and drug history of either antihypertensive medication or statins were recorded. In addition all patients were investigated before their hemodialysis session for the following: hemoglobin levels (Hb), cholesterol, triglycerides, low density lipoprotein cholesterol (LDL-C), high density lipoprotein cholesterol (HDL-C), uric acid, ferritin, urinary protein creatinine ratio (UPCR), and eGFR. Also, echocardiography was done for the patients.

**Stool culture for Lactobacillus acidophilus**

Stool culture on de Man Rogosa Sharp agar (MRS), after sample collection from the patients. Quantitative culture for Lactobacillus acidophilus (in colony forming unit per gram [CFU/g] of stools) was done by weighing 1 gram of the stool specimen diluted in 2 ml of sterile saline.

Serial dilutions of this suspension were prepared in sterile saline solution; 1 ml of each dilution was inoculated into MRS agar plates.
using a standard loop. Incubation of the plate was done anaerobically at 37°C for 2–3 days. Colony forming unit per gram was detected for each stool specimen using the following formula:

\[ \text{CFU/Gm} = \frac{N \times D \times W}{2} \]

Where CFU: colony forming unit, Gm: gram, N: the number of colonies on the plate, D: dilution, W: the weight of the stool specimen in Gm, and 2: the original dilution of the stool specimen.

### DNA extraction and sequencing for Lactobacillus acidophilus from stools

Stool samples were collected from ESRD patients and healthy subjects. All samples were processed for DNA extraction within 24 h after receipt.

#### DNA isolation

DNA from Lactobacillus acidophilus in the stool samples was extracted using proteinase K protocol after centrifugation of the broth for 2 min. Briefly, bacterial cells were re-suspended in 467 l of freshly prepared (50 mM Tris–HCl, 10 Mm EDTA, PH 7.5) 30 UL of 10% SDS and 3 l of 20 mg/ml proteinase-k, Sigma Aldrich. Following incubation at 65°C for 45 min with shaking, the DNA was obtained by phenol/chloroform/isoamyl alcohol (25:24:1) precipitated with isopropanol washed with ethanol (70%) and dried under vacuum [22]. DNA pellet was resuspended in 25 l of sterile distilled water and stored at -20°C.

#### PCR primer

DNA pellet was confirmed to be of bacterial origin using the universal 16-S primer. F (5-AGA GTT TCC TGG CTC TTA AG-3) and R (5-ACG ACC TTG TTA GMT CGA CTT3) that amplify a 1500 bp fragment. The genus specific primers of identification of Lactobacilli were designed on 16SrRNA as described by Dubernet et al [23].

The genus specific primers, (Sigma, USA) for Lactobacilli amplifies a 123 bp fragment. LbL MA1-rev (5-CTC AAA ACT AAA CAA AGT TTC-3) and R16-1 (50-CTT GTA CAC ACC GCC CGT CA-3).

#### PCR conditions and procedure

PCR reaction mixture(25 l) was composed of 25 pmol of each primer, 0.2 mM of each dNTP, 1xPCR buffer 50 mM Tris–HCl, PH 8.8, 2.5 mM MgCl₂, 15 Mm (NH₄)₂SO₄ 0.45% Triton X-100, 10 ng of bacterial DNA and 1U ofTaq DNA Polymerase (Biotech International, Australia).

The PCR was carried out in a ROTOR Gene Q (Berlin, Germany) following the manufacturer's instruction. The amplification program is as follows: initial denaturation at 95°C for 5 min, followed by 30 cycles consisting of denaturation at 95°C for 30 s, annealing at 55°C for 30 s, extension at 72°C for 30 s, and a 7 min final extension step at 72°C. Amplification products were subjected to electrophoresis in 1% agarose gels (Electrophoresis grade, Invitrogen) in TAE buffer (40 mM Tris acetate, 1 mM EDTA, pH 8.2) followed by ethidium bromide staining (5 g/ml) and visualized under UV light. The strain Lactobacillus Acidophilus ATCC 4356 was used as positive control.

### Statistical analysis

Data were analyzed using SPSS (Statistical Program for Social Sciences) version 19. Biomarker values were expressed as mean ± standard deviation (SD), median, and interquartile ranges. Comparison of two groups was analyzed using Student’s t-test and Wilcoxon Rank Sum test for parametric and non-parametric data respectively. One-way analysis of variance (ANOVA) with post Hoc analysis (Tukey’s test) was used for the comparison of three groups. Chi-square test was used to compare categorical data. Ranked Spearman correlation was used for measuring the correlation between the different variables. A p-value of less than 0.05 was considered significant.

### Results

Demographic and clinical characteristics of the studied groups are shown in **TABLE 1**.

The review of antihypertensive medication in both ESRD groups showed that almost 70-75% were on either mono therapy or combined treatment of antihypertensive drugs. Approximately 70% used statins in their treatment regimen.

ESRD patients with CVD exhibited significant elevation of HDL-C, triglycerides while UPCR was significantly increased in those
Table 1. Demographic and clinical characteristic of the studied groups.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Control group N=20</th>
<th>ESRD without CVD N=40</th>
<th>ESRD with CVD N=40</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>45.4 ± 3.5154</td>
<td>47.2 ± 11.3051</td>
<td>44.225 ± 10.4648</td>
</tr>
<tr>
<td>Gender, n (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>14 (70%)</td>
<td>26 (65%)</td>
<td>31 (77.5%)</td>
</tr>
<tr>
<td>Female</td>
<td>6 (30%)</td>
<td>14 (33%)</td>
<td>9 (22.5%)</td>
</tr>
<tr>
<td>BMI (kg/m²)*</td>
<td>28.36 ± 1.3508</td>
<td>29.32 ± 1.8747</td>
<td>29.673 ± 1.838</td>
</tr>
<tr>
<td>MABP**(mmHg)</td>
<td>78.875 ± 11.3503</td>
<td>80.8 ± 10.2887</td>
<td>86 ± 9.4032</td>
</tr>
</tbody>
</table>

All values are expressed as mean ± SD except gender which is expressed as no. (%).

*BMI: controls vs. ESRD without CVD, p<0.001; controls vs. ESRD with CVD, p<0.001; ESRD without CVD vs. ESRD with CVD, p=0.952

**MABP: controls vs. ESRD without CVD, p<0.001; controls vs. ESRD with CVD, p<0.001; ESRD without CVD vs. ESRD with CVD, p<0.001

Table 2. Laboratory parameters of the ESRD studied groups.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>ESRD without CVD N=40</th>
<th>ESRD with CVD N=40</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hb (gm/dl)</td>
<td>9.805 ± 1.5147</td>
<td>9.785 ± 1.4181</td>
<td>0.744</td>
</tr>
<tr>
<td>Triglycerides (mg/dl)</td>
<td>155.5 (79.5-193)</td>
<td>211 (176.5-231)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>T.Cholesterol (mg/dl)</td>
<td>185.5 (143-226)</td>
<td>211 (178-231)</td>
<td>0.02</td>
</tr>
<tr>
<td>HDL-C (mg/dl)</td>
<td>54 (43-65)</td>
<td>71 (54-100)</td>
<td>0.001</td>
</tr>
<tr>
<td>LDL-C (mg/dl)</td>
<td>108.5 (64.25-122.75)</td>
<td>111.5 (76-132)</td>
<td>0.114</td>
</tr>
<tr>
<td>Ferritin (ng/ml)</td>
<td>239.5 (126.75-557.75)</td>
<td>243.5 (205-537.25)</td>
<td>0.61</td>
</tr>
<tr>
<td>Serum uric acid (mmol/L)</td>
<td>5.85 (5-6)</td>
<td>5 (4-6)</td>
<td>0.029</td>
</tr>
<tr>
<td>UPCR (mg/mg)</td>
<td>4388 (3326.25, 5619)</td>
<td>561 (449.25, 2261)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>eGFR (ml/min)</td>
<td>6 (5,8)</td>
<td>5 (5,6)</td>
<td>0.952</td>
</tr>
</tbody>
</table>

All values are expressed as median, interquartile except for Hb which is expressed as mean ± SD

Hb, hemoglobin; HDL, high density lipoprotein; LDL, low density lipoprotein; UPCR, urinary protein creatinine ratio; eGFR, estimated glomerular filtration rate.

Table 3. Lactobacillus acidophilus (CFU/gm) count among the study groups.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control group N=20</th>
<th>ESRD without CVD N=40</th>
<th>ESRD with CVD N=40</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lactobacillus acidophilus (CFU/gm)</td>
<td>24.66 ± 3.13</td>
<td>27.57 ± 3.77</td>
<td>30.47 ± 2.72</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

Tukey's test

Healthy Control vs. ESRD without CVD P<0.001**
Healthy Control vs. ESRD with CVD P<0.001**
CKD without vs. ESRD without CVD P<0.001**

Table 4. Correlation of Lactobacillus acidophilus count and the various studied parameters among ESRD without and with CVD complications

<table>
<thead>
<tr>
<th>Parameters</th>
<th>ESRD without CVD</th>
<th>ESRD with CVD</th>
<th>r</th>
<th>P-value</th>
<th>r</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>-0.005</td>
<td>0.046</td>
<td>0.776</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BMI (kg/m2)</td>
<td>-0.252</td>
<td>-0.193</td>
<td>0.234</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MABP (mmHg)</td>
<td>-0.228</td>
<td>-0.123</td>
<td>0.448</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Triglycerides (mg/dl)</td>
<td>0.313</td>
<td>-0.159</td>
<td>0.328</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T.Cholesterol (mg/dl)</td>
<td>-0.177</td>
<td>-0.201</td>
<td>0.897</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HDL-C (mg/dl)</td>
<td>0.016</td>
<td>0.05</td>
<td>0.76</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LDL-C (mg/dl)</td>
<td>-0.252</td>
<td>-0.155</td>
<td>0.338</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>eGFR (ml/min)</td>
<td>-0.168</td>
<td>-0.114</td>
<td>0.482</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>U-PCR (mg/m)</td>
<td>-0.025</td>
<td>-0.153</td>
<td>0.347</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

BMI, body mass index; MABP, mean arterial blood pressure; HDL-C, high density lipoprotein cholesterol; LDL-C, low density lipoprotein cholesterol; UPCR, urinary protein creatinine ratio; eGFR, estimated glomerular filtration rate.

The difference in the incidence of detected Lactobacillus acidophilus in stool samples of patients ESRD without CVD complications as shown in TABLE 2.
ESRD versus healthy subjects was statistically significant at p<0.001. Further analysis revealed that Lactobacillus acidophilus count was higher among ESRD patients with CVD complications than ESRD without CVD complications (TABLE 3).

On assessing the correlation between Lactobacillus acidophilus and various studied parameters among ESRD patients, Lactobacillus acidophilus count did not differ significantly with age, BMI, MABP as well as various metabolic parameters (TABLE 4), but a weak non-significant negative correlation was observed with triglycerides, total cholesterol, LDL-C as well as eGFR and U-PCR.

Discussion

It has been strongly suggested that the gut microbiome is an important factor in the maintenance of health [24], and intestinal dysbiosis that accompanies CKD allowed the development of oxidative stress and inflammation, which is a contributing factor, among other factors, to CVD risk in CKD patients [25].

To best of our knowledge, this study represent the first one in Egypt that assess the alteration of Lactobacillus acidophilus as a representative of beneficial gut microbiota in fecal samples of ESRD patients on hemodialysis and study the extent of its effect especially on those with CVD. Surprisingly, we found that there is abundance of Lactobacillus acidophilus in ESRD patients compared to healthy control. Moreover, there was relative abundance of Lactobacillus acidophilus in ESRD patients with CVD rather than ESRD patients without CVD. Our findings may be accounted for by the fact that all ESRD patients included in the study were on regular hemodialysis, which may have a positive effect on restoring gut symbiosis, thus introducing a novel therapeutic strategy that may ameliorate the harmful effect of dysbiosis. In addition, we supposed that, differences in the underlying systemic diseases, medications and, particularly, dietary restrictions as opposed to uremia could have modified the structure and function of the gut microbiome in those patients with ESRD. Furthermore, little is known about the genetic basis of the microbiome and how many genetic differences exist between enterotypes of gut microbiota within or between populations. A recent Chinese study concluded that considerable differentiation was observed between enterotypes and among healthy Chinese populations that was reflected in both the taxonomic composition as well as the genetic makeup of their functional genes, which could have been influenced by a variety of factors, such as diet and host genetic variation [26].

Alteration of colon microbiota is observed under uremic conditions. In contrast to our findings, early in 1996, Hida et al., using traditional plating methods for the analysis of fecal samples, did not detect any significant differences in the total number of colonic bacteria in hemodialysis patients compared to healthy individuals [27]. Moreover, it has been recently demonstrated that there is no change in level of Lactobacillus acidophilus in the stomach of CKD patients [25].

Furthermore, Wang et al. were the first to analyse the gut microbiota by real-time PCR and found that different Bifidobacterium species as B. catenulatum, B. longum, B. bifidum, Lactobacillus plantarum and Lactobacillus paracasei were found at lower rates in peritoneal dialysis patients versus healthy individuals [28].

Vaziri et al. demonstrated via microarray analysis that there was a significant difference in the abundance of 190 microbial operational taxonomic units (OTUs) between the ESRD and the normal control individuals, who presented an increased abundance of Enterobacteriaceae, particularly certain clusters of Escherichia coli sequences [11].

Another study by Wong et al supported the hypothesis that CKD patients may have an abundance of bacterial families possessing urease, uricase, and indole- and p-cresol forming enzymes such as Clostridiaceae, Enterobacteriaceae and Verrucomicrobiacea, which lead to the ureamic toxicity and systemic inflammation in these patients and a reduction of bacterial families possessing butyrate-forming enzymes, including Lactobacillus and Prevotellae [29].

Another data from a Brazilian study confirmed an alteration of the gut microbiota composition in patients with stage III–IV CKD, where a reduction of fecal microbial diversity was observed upon the PCR-denaturing gradient gel electrophoresis of 16S rRNA gene fragments in CKD patients when compared with healthy
individuals [30]. Similarly, an experimental study in Japan analyzing the species of gut microbiota and found that the Lactobacillus population was reduced under CKD conditions [31].

Therefore, recent findings demonstrated that accumulation of metabolites of gut microbiota secondary to imbalance of gut microbiota together with changes in colonic epithelium may contribute to the progression of CKD and associated cardiovascular disease [32,33]. Toxic gases, indoxyl sulphate, p-cresyl sulphate, amines, ammonia and trimethylamine n-oxide (TMAO) as well as precursors for lipopolysaccharides (LPS) may be absorbed into the bloodstream and be responsible for activation of innate immunity, promotes the inflammatory state associated with CKD and, increases the incidence of cardiovascular disease [8,30]. Indeed, several studies have shown that these toxins could be reliable markers of cardiovascular disease and mortality in CKD patients [34,35]. Moreover, Celik et al. [36] who focused on the alterations of the small intestine in patients with chronic heart failure (CHF), stated that increased bowel wall permeability and reduced intestinal resorption function secondary to the bowel wall edema and disturbed microcirculation in the gut are thought to be responsible for malabsorption of nutrients in patients with chronic heart failure and contribute to malnutrition and cardiac cachexia as well as it may lead to the translocation of bacteria and/or endotoxin, which may be an important stimulus for inflammatory cytokine activation in CHF. In addition, Sandek et al. detected a lack of immunological defense with an increased in bacterial biofilm and inflammatory response in CHF [37].

In recent years, there is a growing interest in restoring the symbiosis of intestinal microflora in CKD aiming to reduce the generation of uraemic toxins, oxidative stress, and inflammation. The investigation on efficacy of probiotics to decrease production of uremic toxin and to improve renal function has been performed in some human CKD studies [38]. Ranganathan et al. [39] observed an improvement in quality of life and a reduction in serum uric acid and creatinine levels in non-dialysed CKD patients after a probiotic supplementation. In contrast, Hyun et al. [40] showed that there was no significant effect of probiotics on the reduction of uraemic toxins in paediatric dialysis patients. Wang et al. [41] reported that after 6 months of probiotic supplementation in peritoneal dialysis patients, a reduction on serum levels of pro-inflammatory cytokines was observed. Studies with prebiotics are limited. Meijers et al. [42] observed significant reduction of p-cresyl sulphate levels in haemodialysis patients after a 4-week prebiotic oligofructose-inulin supplementation. Recently, Vaziri et al. [43] showed that prebiotic supplementation as a high resistant starch diet retards CKD progression and attenuates oxidative stress and inflammation in CKD rats.

Concerning blood lipid in the present, we couldn’t find any significant correlation between Lactobacillus acidophilus composition and blood lipid in ESRD patients. However, unexpectedly, we observed increase levels of HDL-cholesterol in ESRD patients with CVD compared to ESRD patients without CVD. This finding could be explained by a Japanese study that excess oxidative stress may have gave rise of dysfunctional HDL in patients with ESRD and these patients with high HDL-cholesterol under these conditions may have enriched oxidized HDL which can result in increased CVD morbidity and mortality [44].

This study has several limitations, namely a relatively small study group, not considering other gut microbiota composition with limitation to lactobacillus acidophilus only. In addition to that, strict dietary restrictions that our ESRD patients were followed must have contributed to the observed changes in their microbial flora. Patients with CKD are polymedicated, other drugs rather than antibiotics, prebiotic and probiotic should be considered as proton pump inhibitor, antidepressant, statins may be associated with distinct gut microbiota changes [45]. Other medication that may also slow intestinal transit, phosphorus binders, ion exchange resins [13], or iron supplements that may have an effect on microflora but it is not well defined [46,47].

In summary, it is known that the imbalance in the composition of the intestinal microbiota in CKD/ESRD patients may represent another non-traditional risk factor because this imbalance can contribute to an increase in chronic inflammation and oxidative stress and CVD complications. Unfortunately, our
present findings cannot support the findings of considering Lactobacillus acidophilus as having a reno-protective effect in ESRD patients. Further studies performed on a larger study population in Egypt, and including a wide diversity of microbiomes, are necessary to confirm this association. Also the requirements of studies that correlate the existence of Lactobacillus acidophilus to various inflammatory markers in CKD are mandatory. Accordingly, interventions aiming to restore and maintain the balance and function of the intestinal microbiota are extremely important.
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