



Original article

Lactobacilli species diversity in gut microbiota of renal failure patients

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ABSTRACT

Lactobacillus species and other acid producing bacteria could inhibit aerobic bacteria, normalize the intestinal microbiota and decrease the uremic toxins in patients with chronic kidney disease. Therefore, the aim of this study is assessment of abundance of lactobacillus spp. in fecal flora of End-stage kidney disease (ESKD) and kidney transplanted patients. DNA of 20 fecal samples of ESKD and kidney-transplanted as patients group and 20 fecal samples of non-ESKD and -kidney-transplanted as control group, which were admitted to hospitals, were extracted. Amplified DNA by 16srRNA illumina V3 and V4 primers were sequenced by MiSeq system. Total 651 strains, 37 (5.68%) strains were identified as order Lactobacillales. The strains were classified into six family, nine genus and 37 species. The most abundance genus in both groups are *Streptococcus* spp. and *Lactobacillus* spp. and the lowest abundance genus in both groups are *Pediococcus* and *Leuconostoc*. Comparing the abundance mean of the strains revealed that there is no significant association between control group and disease group, but the abundance mean of the strains in control group increase in compared to disease group. More diversity was exhibited in lactobacillus spp. in patients with chronic kidney disease compared to control group. Some of the species of *Lactobacillaceae* family such as *L. acidophilus* was not found in both patients and control groups. In addition, more of the species of *Lactobacillaceae* were decreased in patients group, whereas certain species were increased in patients group compared to control group.

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1. Introduction

The gastrointestinal tract of human consists of various bacteria including aerobic and anaerobic bacteria. The entire of colon is filled by mostly commensal and obligatory anaerobic bacteria such as lactobacilli (Muyzer et al., 1993; Dahroud et al., 2016; Quagliariello et al., 2016). Lactobacillus genus is a gram-positive, non-sporulating, catalase negative and rod-shaped bacillus that contains more than ninety validly described species and subspecies (Jabbari et al., 2017). Lactobacillus species are included in the division of Firmicutes (Ludwig et al., 2010) and are belonged to the

members of lactic acid bacteria, which are found in human and animals intestinal microbiota (Vaughan et al., 2005; Ludwig et al., 2010). As well as, Lactobacillus species are one of the most commonly used probiotic strains in the wide range of food components in preventing chronic inflammation disease and enhancing health (Pandey et al., 2015). The fermentative characteristics of *lactobacillus* spp. have classified them into three divisions including obligate heterofermentative lactobacilli (OHFL), obligate homofermentative lactobacilli (OHOL) and facultative heterofermentative lactobacilli (FHFL) [24]. The activity of these aerobic and anaerobic bacteria has a major impact on the host health and characteristics such as development of the immune system, host protection against pathogens, and positive effects on the host nutrition and colonic health (Falk et al., 1998; Hooper et al., 2001; Gholizadeh et al., 2019). The protein-bound uremic toxins such as p-cresyl sulphate (PCS), indoxyl sulphate (IS), p-cresylglucuronide, kynurenic acid and indole acetic acid (IAA), which are normally excreted into the urine, could be contributing factor to progression chronic renal disease that have deleterious effects (Vanholder and Glorieux,

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2015). These uremic toxins could lead to damage endothelial cells or renal tubular cells by fibrotic changes, inflammatory responses or enhancing cellular oxidative by activation of Nicotinamide adenine dinucleotide (NADPH) oxidase, P53, nuclear factor- κ B (NF κ B) (Shimizu et al., 2013; Watanabe et al., 2013; Vanholder et al., 2014; Vanholder and Glorieux, 2015). The precursors of these uremic toxins are produced by gut microbiota from tyrosine or tryptophan and absorbed from the colon. Alters in the absorption of the uremic toxins from the colon may affect renal function (Vanholder and Glorieux, 2015) (Fig. 1).

Recent studies suggested that two third of individuals with abnormalities and a disequilibrium in the gastrointestinal and ecosystem have uremia (Kang, 1993). The majority of these abnormalities and disequilibrium happen in the colon and ileum that intestinal microbiota plays critical roles. The increase in aerobic bacteria such as *Escherichia coli* results in a decreasing in the anaerobic bacteria such as bifidobacteria and lactobacilli, as well as, generating toxic substrates, called uremic toxins (Simenhoff and Dunn, 1996; Vaziri et al., 2015). Several studies demonstrated that there is a deteriorated intestinal barrier in individuals with uremia, which is due to the abnormalities and disequilibrium of gut microbiota caused by the increase of pathogenic bacteria (Lenoir-Wijnkoop et al., 2007; Nakabayashi et al., 2010; Vaziri, 2012). Higher concentrations of urea and, consequently, increased ammonium are demonstrated in chronic renal diseases, which resulted in an increase pH that could promote aerobic bacteria in the gastrointestinal and, subsequently, produce uremic toxins. Conversely, carbohydrates fermentation and acetic and lactic acid production by bifidobacteria and lactobacilli acidify the intestinal that could inhibit aerobic bacteria and normalize the intestinal microbiota in patients with chronic renal failure. In uremic individuals the fecal urease activity is increased at high concentrations of plasma urea. Therefore, a beneficial and consideration factor in uremic patients is the increase in colon microbial urease (Chow et al., 2003). Chronic renal disease is a health problem that can lead to end-stage kidney disease (ESKD), which is required kidney transplantation therapy. The modulation of interrelationship between the gut and kidney may be a potential role to slow progression of chronic renal disease (Ritz, 2011). Several studies suggested that alters in the gut microbiota affects the gut environment, which could correlate with kidney disease (Chow et al., 2003; Anders et al., 2013; Ramezani and Raj, 2014). Rossi et al. (2012) demonstrated that the pre- and probiotic therapy could significantly reduce IS and PCS in the chronic renal disease population. In addition, Yoshifuji et al. (2015) demonstrated that *Lactobacillus* spp.

supplementation are improved intestinal changes and renal damage through activation of *Lactobacillus* recognized TLR (toll-like receptor). In another study, Vaziri et al. (2013) demonstrated that the abundance of Lactobacillaceae and Prevotellaceae in the intestinal microbiota flora are significantly decreased in the uremic animals compared to the control animals. Therefore, the aim of this study is assessment of abundance of *Lactobacillus* spp. in fecal flora of ESKD and kidney transplanted patients.

2. Methods and materials

2.1. Sample collection

Fresh fecal samples were directly collected from the anus of 20 ESKD and kidney-transplanted patients, which were admitted to kidney transplantation ward of Emam-Reza teaching and treatment hospital, Tabriz, Iran. 20 fecal samples of admitted patients who had not been admitted for ESKD and kidney transplantation as a control group. The underlying causes of ESKD in the study population included chronic pyelonephritis in one patient, polycystic kidney disease in one, post renal and urolithiasis in two, chronic kidney disease of unknown etiology in three, glomerulonephritis in five and hypertensive nephrosclerosis in eight patients. Patients with gastrointestinal individuals, malignancy, individuals who had been treated with antibiotics within 3 months before the enrollment in the study, infections, active inflammatory disorders, diabetes were excluded. The fecal samples were immediately stored at -80°C until DNA extraction. In addition, all patients were evaluated for blood creatinine test, blood urea-nitrogen test and blood uric-acid test.

2.2. DNA extraction

Approximately 4 g of Fecal samples, which mixed vigorously with a spoon was homogenized aseptically and vortexed for more than 5 min. DNA of the mixture were extracted by the QIAamp Stool Mini Kit (Qia gene, Germany), according to the manufacturer's instruction.

2.3. PCR amplification and next generation sequencing

The PCR amplification for each sample was performed by the V3 and V4 hyper variable region of bacterial 16 s rRNA. The primer sequences of two universal bacterial 16 s rRNA gene were Illumina

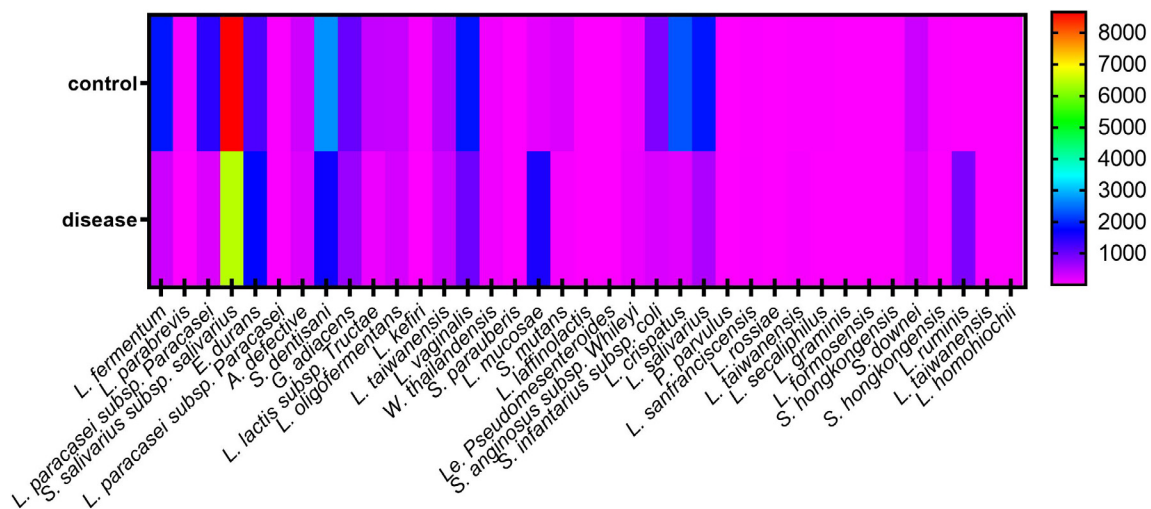


Fig. 1. The heat map generated from abundance mean of various species.

V3 F: TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGCWGCGAG and Illumina V4 R: GTCTCGTGGGCTCGGAGATGTGT A TAAGAGACAGGACTACHVGGGTA TCTAATCC (Klindworth et al., 2013). The amplification reaction was set up as follows: 2X KAPA HiFi Hot Start Ready Mix 25 μ l, 2 μ l aliquots of both amplicon forward and reverse primer (10 μ M), and template DNA 5 μ l in a total 50 μ l volume. Polymerase chain reaction (PCR) reaction was performed in a T100™ thermal (Bio-Rad, USA) using following program: 1 cycle of initial denaturing at 95 °C for 5 min, followed by 35 cycles of denaturing at 95 °C for 1 min, annealing at 55 °C for 45 s and extension at 72 °C for 1 min; and a final extension at 72 °C for 1 min. The PCR products were assessed using electrophoresis in 1% agarose gel in Tris-boric acid-EDTA buffer, stained with ethidium-bromide and visualized under UV light. The PCR products were sent to Omega Bioservices company and sequencing of the PCR products was performed on a MiSeq system (100 k \times 300 bp paired-end reads) (Illumina, USA). Bioinformatics analyses were performed via Illumina's BaseSpace in parallel with Illumina's in-house QIIME 2 pipeline.

2.4. Statistical analysis

Statistical analysis was performed in GraphPad Prism 8. The study data was analyzed by using descriptive statistics including mean and standard deviation (STDEV) and Mann-Whitney

nonparametric test and unpaired *t* test with Welch's correction were applied to compare mean of data.

3. Results

The characteristics of participants between control group did not differ with disease group in age (59.3 ± 7.89 vs. 53.20 ± 12.03), and gender (male: 10, female: 10 vs. male: 14 vs. female: 6). Total 651 strains from 40 fecal samples were identified, which 37 (5.68%) strains were identified as order Lactobacillales. The strains were classified into six family including Lactobacillaceae (18/37), Streptococcaceae (14/37), Enterococcaceae (1/37), Aerococcaceae (1/37), Carnobacteriaceae (1/37) and Leuconostocaceae (2/37). In addition, the strains were classified into nine genus and 37 species including Lactobacillus (17/37), Straptococcus (10/37), Lactococcus (4/37), Abiotrophia (1/37), Granulicatella (1/37), Enterotococcus (1/37), Weisella (1/37), Leuconostoc (1/37), and Pediococcus (1/37). The most abundance genus in both groups are Streptococcus ($4.47\% \pm 3.67\%$ in control group and $3.12\% \pm 2.47\%$ in disease group) and Lactobacillus ($3.41\% \pm 4.15\%$ in control group and $1.78\% \pm 2.88\%$ in disease group) and the lowest abundance genus in both groups are Pediococcus ($0.00005\% \pm 0.00009\%$ in control group and $0.0006\% \pm 0.0009\%$ in disease group) and Leuconostoc ($0.004\% \pm 0.003\%$ in control group and $0.002\% \pm 0.001\%$ in disease group). *S. dentisiani*

Table 1

The abundance of various species of order Lactobacillales isolated from fecal samples of control and disease groups.

Species	Control group mean	Individuals collected	Min	Max	STDEV	Disease group mean	Individuals collected	Min	Max	STDEV	p-value
<i>L. fermentum</i>	1861.5	19	0	28,623	6158.6	334.05	15	0	4887	1079.9	0.497
<i>L. parabrevis</i>	59.95	6	0	1109	235.76	1.7	4	0	12	3.78	0.330
<i>L. paracasei</i> subsp. <i>paracasei</i>	1427.8	18	0	27,153	5775.4	234.75	16	0	3303	742.79	0.350
<i>S. salivarius</i> subsp. <i>salivarius</i>	8650	20	237	29,245	8977.9	6436.2	20	517	32,920	9572.81	0.242
<i>E. durans</i>	1204.2	19	0	15,560	3412.4	1756.85	20	3	13,984	3674.53	0.337
<i>L. paracasei</i> subsp. <i>paracasei</i>	38.1	10	0	425	90.69	27.2	7	0	285	74.97	0.328
<i>A. defecative</i>	316.4	15	0	3012	695.88	220.6	15	0	3474	770.99	0.690
<i>S. dentisiani</i>	2711.9	20	14	17,221	4067.4	1642.05	20	37	16,661	3750.48	0.059
<i>G. adiacens</i>	1031	20	3	7570	1819.1	681.55	19	0	10,394	2291.94	0.139
<i>Lc. lactis</i> subsp. <i>tractae</i>	381.35	10	0	7102	1509.1	119.15	7	0	1814	403.56	0.438
<i>L. oligofermentans</i>	378.45	20	2	2479	633.57	278.45	19	0	1585	473.20	0.147
<i>L. kefiri</i>	60.8	5	0	999	213.78	3.15	4	0	36	8.59	0.511
<i>L. taiwanensis</i>	500.5	17	0	3667	976.43	341.7	17	0	5605	1242.55	0.541
<i>L. vaginalis</i>	1847.35	16	0	15,259	3800.3	977.6	19	0	15,009	3330.58	0.984
<i>W. thailandensis</i>	85.85	7	0	1663	354.06	97.45	5	0	1910	426.66	0.551
<i>S. parauberis</i>	9.1	9	0	119	25.14	6.05	7	0	44	11.98	0.667
<i>L. mucosae</i>	173.35	11	0	3286	698.2	1536.85	10	0	29,149	6506.20	0.837
<i>S. mutans</i>	253.8	12	0	3199	681.86	67.1	14	0	547	136.17	0.810
<i>Lc. raffinolactis</i>	4.15	2	0	79	3479.1	5.25	6	0	40	10.82	0.173
<i>Le. Pseudomesenteroides</i>	12.95	6	0	73	24.34	6.8	9	0	37	11.55	0.624
<i>S. anginosus</i> subsp. <i>whileyi</i>	120.85	18	0	685	6225	135.75	16	0	953	243.39	0.909
<i>S. infantarius</i> subsp. <i>coli</i>	865.25	15	0	15,643	3328.1	254.7	13	0	3724	858.44	0.303
<i>L. crispatus</i>	2289.65	19	0	28,128	6280	199.3	19	0	1614	350.88	0.920
<i>L. salivarius</i>	1859.05	16	0	13,385	3879.2	549.6	18	0	3717	1041.07	0.920
<i>P. parvulus</i>	0.15	1	0	3	0.63	1.85	4	0	28	6.27	0.164
<i>L. sanfranciscensis</i>	26.8	5	0	488	103.53	34	8	0	528	117.25	0.324
<i>L. rossiae</i>	4.35	6	0	45	10.90	3.35	5	0	26	6.90	0.915
<i>L. taiwanensis</i>	15.7	7	0	168	38.55	49.2	5	0	972	217.20	0.399
<i>L. secaliphilus</i>	22.6	5	0	375	79.69	1	2	0	16	3.64	0.181
<i>L. graminis</i>	2.45	4	0	20	5.44	7.3	6	0	78	18.75	0.513
<i>Lc. formosensis</i>	11.9	4	0	205	45.59	4.35	5	0	74	16.45	0.978
<i>S. hongkongensis</i>	9.95	6	0	141	30	1.65	4	0	19	4.47	0.416
<i>S. downei</i>	348.6	7	0	4686	1068	222.4	8	0	4205	937.68	0.771
<i>S. hongkongensis</i>	33.75	7	0	305	78.82	7.4	6	0	101	22.63	0.530
<i>L. ruminis</i>	6.55	4	0	114	24.23	872.35	6	0	17,298	3866.24	0.408
<i>Lc. taiwanensis</i>	7.25	1	0	145	74.57	4.45	1	0	89	19.90	1
<i>L. homohiochii</i>	3.35	1	0	67	14.28	1.5	1	0	30	6.70	1

(2711.9 ± 4067.4) and *L. crispatus* (2289.65 ± 6280) were the most abundant species in control group and *P. parvulus* (0.15 ± 0.63) and *L. graminis* (2.45 ± 5.44) were the lowest species. In disease group, *S. salivarius* subsp. *salivarius* (6436.2 ± 9572.81) and *E. durans* (1756.85 ± 3674.53) were the most abundant species and *L. secaliphilus* (1 ± 3.54) and *S. hongkongensis* (1.65 ± 4.47) were the lowest abundant. The abundance of various species are shown in Table 1. *S. salivarius* subsp. *salivarius*, *S. dentisiani*, *G. adiacens*, and *L. oligofermentans* were found in all control group. As well as, *S. salivarius* subsp. *salivarius*, *E. durans* and *S. dentisiani* were found in all disease group. Comparing the abundance mean of the strains revealed that there is no significant association between control group and disease group (P-value > 0.05), but the abundance mean of the strains in control group increase in compared to disease group (719.9 ± 1534 vs. 462.8 ± 1115, P = 0.275). In addition, the presence of order Lactobacillales in the individuals of control group was not significant difference in compared to the individuals of disease group (10.49 ± 1.071 vs. 10.27 ± 1.044, P = 0.848).

4. Discussion

This study describes the abundance and diversity of order Lactobacillales in two groups including control group and disease group described as ESKD and kidney transplanted patients. In control group, the abundance of Lactobacillales were lower than disease group, and their species composition was different in comprised to disease group.

Marteau et al. (2001) demonstrated that the rRNA of Lactobacillus-Enterococcus group in healthy human is 6.6% of fecal bacterial rRNA, which is more than our study that found 3.8% in control group and 2.38% in disease group. In addition, Stsepetova et al. detected lactobacilli in all fecal samples by real-time PCR and 90% by the culture method in the samples, and we found lactobacilli in all fecal samples, which could suggested that the NGS method is the best for assessment of fecal bacteria.

This study is one of the few research concerning abundance of lactobacilli and their effects on chronic kidney disease patients, specifically comparing in the two groups as control and chronic kidney disease patients groups. However, the importance of this type of study in kidney disease patients lies in the benefits that could be obtained if the decrease of lactobacilli promoted symptoms in chronic kidney disease patients.

It was reported that gut microbiota may be a key role that maintains the gut in patients with chronic kidney disease (Kotanko et al., 2006). In addition, progression of chronic kidney disease may be promoted by uremic toxins, which are made by certain gut microbiota (Satoh et al., 2003). Several studies suggested that early probiotic therapy to remove various uremic toxins could be delayed the onset of ESKD in individuals with progressive chronic kidney disease. Based on the previous study by Alatraste et al. (2014), Yoshifuji et al. (2015) and Vaziri et al. (2013), a decreased of lactobacilli abundance are shown in chronic kidney disease. In addition, the population of butyrate-producing enzymes bacterial families such as Lactobacillaceae and Prevotellaceae are lower in patients with uremia, whereas the population of indole- or p-cresol-producing bacterial families such as Enterobacteriaceae, Clostridiaceae, and Verrococcaceae are higher compared to health individuals (Wong et al., 2014). Similar to previous study, Lactobacillaceae is lower in patients group compared to healthy individuals in the present study. Ranganathan et al. suggested that probiotics with combination of *S. thermophiles*, *B. longum* and *L. acidophilus* are significantly reduced BUN and enhanced well-being in patients with chronic kidney disease (Ranganathan et al., 2010). Whereas, we have not found *L. acidophilus* in both control and patients groups. While,

L. fermentum, *L. paracasei*, *L. kefirii*, *L. vaginalis*, *L. crispatus*, *L. salivarius* and other families of order Lactobacillales were reduced two or more folds in patients group compared to control group. In addition, *L. mucosa*, *L. taiwanensis* and *L. ruminis* were increased two or more folds in patients group compared to control group. Therefore, it has been suggested that future studies are necessary to elucidate more precise mechanisms of different lactobacilli strains in chronic kidney disease, subsequently develop more efficient therapeutic strategies. Our data could provide relevant information for these future studies.

5. Conclusion

More diversity was exhibited in lactobacillus spp. in patients with chronic kidney disease compared to control group. Some of the species of Lactobacillaceae family such as *L. acidophilus* was not found in both patients and control groups. In addition, more of the species of Lactobacillaceae were decreased in patients group, whereas certain species were increased in patients group compared to control group. This diversity could suppose a novel mechanisms and therapeutic strategies to slow the progression of renal damage and delay the onset of ESKD in patients with chronic kidney disease.

Declaration of Competing Interest

The authors declare that they have no conflict of interest.

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