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Clostridium butyricum affects nutrition and immunology by modulating gut microbiota

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The gut microbiota has nutritional and protective functions. In patients with end-stage renal disease, changes in the gut microbiota disrupt their protective functions. Probiotics help maintain normal bowel function. However, their role in patients with end-stage renal disease is controversial. We investigated whether *Clostridium butyricum* affects the nutrition and immune function of patients with end-stage renal disease undergoing maintenance dialysis between 2014 and 2015; thirty-seven patients were included. The patients were divided into two groups: one in which *C. butyricum* was administered and one in which it was not. One tablet of the probiotics, which contained 20 mg of *C. butyricum*, was administered orally three times daily for 2 years in the *C. butyricum* group. The 16S rRNA genes were sequenced from stool samples of 14 (37.8%) patients in the *C. butyricum* group and 23 (62.2%) patients in the control group. The differences in the gut microbiota of the two groups were analyzed. The α-diversity index indicated that the *C. butyricum* group had significantly more operational taxonomic units and higher albumin and transferrin levels than the control group. The effector to target cell ratio was significantly higher in the *C. butyricum* group. In addition, interleukin-6 levels were significantly lower in the *C. butyricum* group, and inflammation was less severe in this group. The patients undergoing maintenance dialysis with *C. butyricum* had abundant gut microbiota. They also had a good nutritional status, low systemic inflammation, and a good immunological status.

Key words: *Clostridium butyricum*, end stage renal disease, gut microbiota, hemodialysis, 16S rDNA

INTRODUCTION

The bacteria found in the human intestinal tract form the gut microbiota [1]. The gut microbiota is a major part of the host microbial flora and contains approximately 10^{14} bacterial cells that are symbiotic with the host [2]. They interact with the metabolism of the host to perform various beneficial functions, including control of nutritional and immunologic functions [3, 4]. However, changes in the intestinal ecosystem can lead to an increase in various pathogenic bacterial species, which can have some detrimental effects on the host's health, such as carcinogenesis [5, 6].

End-stage renal disease (ESRD) is associated with chronic systemic inflammation and plays a central role in cachexia, anemia, and cardiovascular disease [7, 8]. Uremia, the most common complication of ESRD, occurs when waste products are not excreted by the kidneys and instead accumulate in the body.

It has been shown that general systemic inflammation in patients with uremia may be partly due to an impaired intestinal barrier function [9]. Chronic inflammation of the entire gastrointestinal tract and elevated blood levels of intestinal microbial DNA and endotoxin have been reported in hemodialyzed patients [10, 11]. Thus, it has been suggested that patients with uremia have impaired intestinal barrier function, presumably causing endotoxinemia and systemic inflammation. However, the underlying mechanism of intestinal barrier dysfunction in patients with ESRD remains unclear.

Gut microbiota and microbial metabolism by-products play a role in intestinal barrier function [12]. Some intestinal pathogens disrupt the epithelial barrier by internalizing tight junction proteins [13]. Cytokines, produced as a result of intestinal mucosal damage, such as interleukin 6 (IL-6), mediate the systemic inflammatory response by stimulating the production of C-reactive protein, thereby worsening the prognosis of patients

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with ESRD [14]. Compared with healthy individuals, patients with ESRD have significantly different compositions of gut microbiota and colonic microbial metabolomes [15]. Therefore, modification of the gut microbiota may be a promising treatment for patients with ESRD.

Probiotics are microbes that are beneficial to the host's health: they modify the gut microbiota. They are often used in the treatment of dyslipidemia, intestinal infections, inflammatory bowel syndrome, and cancer, due to their metabolic, immunomodulatory, and anti-tumoral properties [4, 16]. Probiotic microorganisms suppress the growth of harmful microorganisms, inhibit carcinogenic enzymes, activate the immune system of host cells, activate immune cells, and produce cytokines [17]. Furthermore, the use of probiotics has been shown to reduce uremia and improve cardiovascular disease [18]. *Clostridium butyricum* (CB) is probiotic that produces short-chain fatty acids (SCFAs), such as butyric acid, acetic acid, and propionic acid. Short-chain fatty acids play a role in suppressing intestinal inflammation and maintaining normal intestinal function [19]. CB has been used to prevent or treat intestinal disorders in animals [20]. In clinical practice, CB is used for the treatment of gastrointestinal disorders associated with the destruction of the gastrointestinal microbiota, such as diarrhea and constipation [21]. The administration of CB has recently been reported to result in changes in the bacterial flora [22].

However, the effect of probiotic products on patients with ESRD remains unknown. Therefore, the aim of our study was to evaluate the effect of CB on the nutritional and immunological statuses of patients with ESRD.

MATERIALS AND METHODS

Participant information

This retrospective study included 37 patients who underwent maintenance hemodialysis (HD) at our institution between April 2014 and March 2015. The patients were divided into two groups: the CB group, which received probiotics, ad the control group, which did not. Baseline demographic and clinical data were retrieved from the hospital database. The inclusion criteria were as follows: HD three times weekly for at least 6 months and vascular access through an arteriovenous fistula or prosthesis. Patients were excluded if they had liver disease, malignant tumors, active collagen disease, chronic hemorrhage, infection, or missing data. The study protocol followed the principles of the Declaration of Helsinki. This study was approved by the Institutional Review Board of the National Hospital Organization Yanai Medical Center (Provided ID Number: Y-1-9). Written informed consent was obtained from each participating patient.

Treatment procedure

One tablet of MIYA-BM® (Miyarisan Pharmaceutical Co., Ltd., Tokyo, Japan), which contained 20 mg of CB, was administered orally 3 times per day. Patients received the probiotics for 2 years.

Data collection

Blood samples were collected at the start of each dialysis session, with an interval of 2 days between sessions. Beta-2 microglobulin (β2MG) was measured using the latex agglutination method. Iron and transferrin were measured using Nitro so PSAP reagent. The serum copper, selenium, and zinc

levels were measured by atomic absorption spectrophotometry. Measurement of immunoglobulin, complements, and cluster of differentiation (CD)4/CD8 was entrusted to Bio-Medical Laboratories, Inc. (Tokyo, Japan). Measurements of natural killer (NK) cell activity and soluble interleukin-2 receptor (sIL-2R) were obtained as previously reported [23]. Serum IL-6 was determined with a Quantikine ELISA kit (R&D Systems, Inc., Minneapolis, MO, USA). Kt/V was calculated using a single-pool kinetic model [24].

Fecal sample collection and DNA extraction

A fresh fecal sample was obtained from each patient and immediately frozen at −80°C. Sample collection and DNA extraction were conducted according to previously described methods [25]. The DNA extraction was performed using an automated DNA isolation system (GENE PREP STAR PI-480, Kurabo, Osaka, Japan).

Polymerase chain reaction amplification, Miseq sequencing, and sequence data process

To amplify the V3-V4 regions of bacterial 16S rRNA, the primer set 341f/R806 [26, 27] was used. Polymerase chain reaction (PCR) was performed under the conditions described previously [25, 28]. The PCR-amplified fragments were paired-end sequenced with 2 × 284-bp cycles using the MiSeq system with MiSeq Reagent Kit version 3 (600 cycle) chemistry. Paired-end sequencing reads were merged using the fastq-join tool with the default settings [29]. The FASTX-Toolkit was used to extract only joined reads with a quality value score of ≥20 for more than 99% of the sequence [30]. The chimeric sequences were deleted with usearch6.1 [31, 32].

Operational taxonomic units clustering

Identification of sequence reads in sequence analyses was performed manually using the Ribosomal Database Project (RDP) Multiclassifier tool ver 2.11, which is available on the RDP website (http://rdp.cme.msu.edu/classifier/) [33]. Identification of bacterial species from sequences was performed using the Metagenome@KIN Ver 2.2.1 analysis software (World Fusion, Japan) and the TechnoSuruga Lab Microbial Identification database DB-BA 13.0 (TechnoSuruga Laboratory, Shizuoka, Japan), with homology for ≥97% [34].

Statistical analysis

Continuous variables are expressed as means and standard deviations and categorical variables are expressed as numbers and percentages. The Mann-Whitney U test was used to compare continuous variables, and Fisher's exact test was used to compare categorical variables. QIIME2 packages were used to calculate α-diversities and perform principal component analysis (PCA). An R package was used to draw the figures. Statistical analyses were performed using JMP Pro (version 14; SAS Institute, Cary, NC, USA). Statistical significance was set at p<0.05.

RESULTS

Baseline characteristics of the study population

This study included 37 patients with an average age of 73.9 years old. There were 21 (56.8%) males and 16 (43.2%) females. Twenty-three (62.2%) patients had hypertension, 23

(62.2%) had diabetes, and 14 (37.8%) had cerebral infarction. The CB group included 14 (37.8%) patients who received the probiotic. The control group consisted of 23 (62.2%) patients who did not receive the probiotic. The patient demographic data are shown in Table 1. There were no differences in age (p=0.381), sex (p=0.733), or body mass index (p=0.984) between the two groups. Serum albumin levels were significantly higher in the CB group than in the control group (p=0.006). There are no differences in Kt/V (p=0.087) or β2MG (p=0.355) between the two groups.

Clostridium butyricum *related to gut microbial diversity in ESRD*

According to the α-diversity index, the CB group had a significantly higher number of operational taxonomic units (OTUs) than the control group (p=0.04; shown in Fig. 1A, B). A PCA, based on the distribution of OTUs, was performed to characterize the microbiome space in various samples (shown in Fig. 1C). The relative abundances and distributions of the OTUs at the phylum levels are shown for the two groups in Fig. 2.

The gut microbiota included five main phyla: Firmicutes, Actinobacteria, Bacteroidetes, Proteobacteria, and Verrucomicrobia. The relative abundance of the Firmicutes phylum was 69.8% in the CB group and 66.6% in the control group. The relative abundance of the Bacteroidetes phylum was 8.5% in the CB group and 9.8% in the control group. The Firmicutes/Bacteroidetes (F/B) ratio of the CB group was 11.7, and that of the control group was 11.1; there was no difference between the two groups (p=0.731; Fig. 3).

Table 1. Baseline characteristics

	Control, N=23 (62.2)	CB, N=14 (37.8)	p-value
Age	74.93 ± 9.33	72.24 ± 9.78	0.381
Sex M/F	14/9 (60.9/39.1)	7/7 (50.0/50.0)	0.733
BMI	19.06 ± 2.37	19.27 ± 2.71	0.984
HT	14 (60.8)	9 (64.3)	0.709
DM	14 (60.8)	9 (64.3)	0.709
Stroke	9 (39.1)	5 (35.7)	1
TP	5.96 ± 0.71	6.12 ± 0.61	0.626
Alb	2.82 ± 0.53	3.31 ± 0.38	0.006
T. Bil	0.39 ± 0.23	0.38 ± 0.17	0.572
AST	19.65 ± 13.79	15.01 ± 5.42	0.396
ALT	16.43 ± 10.27	12.71 ± 6.01	0.329
CK	82.86 ± 115.69	38.57 ± 26.48	0.204
UN	47.35 ± 18.34	41.93 ± 16.24	0.372
Cr	5.77 ± 2.19	5.74 ± 1.75	0.707
UA	5.36 ± 1.56	4.79 ± 1.19	0.355
β2MG	26.83 ± 4.61	27.85 ± 5.39	0.259
Kt/V	1.69 ± 0.41	1.99 ± 0.48	0.097
TG	102.73 ± 53.21	96.85 ± 46.99	0.742
HDL	47.04 ± 13.43	49.92 ± 10.95	0.339
LDL	79.82 ± 28.01	79.64 ± 25.62	0.975

CB: *Clostridium butyricum*; Alb: albumin; AST: aspartate aminotransferase; ALT: alanine aminotransferase; β2MG: beta-2 microglobulin; BFR: blood flow rate; BMI: body mass index; CK: creatin kinase; Cr: creatin; DM: diabetes mellitus; HDL: high-density lipoprotein; HT: hypertension; LDL: low-density lipoprotein; T. Bil: total bilirubin; TG: triglyceride; TP: total protein; UN: urea nitrogen.
Data are presented as means and standard deviations for continuous variables and as numbers and percentages for categorical variables.

Correlation between gut microbiota and systemic inflammation

As shown in Table 2, there were no differences in trace elements between the two groups. The rapid turnover protein transferrin was significantly more abundant in the CB group (p=0.043). The effector to target cell (E/T) ratio was higher in the CB group (p=0.021). IL-6 levels were significantly lower in the CB group (p=0.001).

DISCUSSION

The α-diversity index indicated that the gut microbiota was more abundant in patients who received CB than in patients who did not receive CB. In addition, in the CB group, the albumin level, transferrin level, and E/T ratio were higher, while the IL-6 level was lower, indicating better nutritional and immunological statuses.

Systemic edema due to fluid overload, which is a common complication in patients with ESRD, leads to edema of the intestinal wall, and aggressive ultrafiltration by hemodialysis results in intestinal ischemia due to hypotension [35]. The mechanical mechanism of ESRD is the impairment of the intestinal epithelial barrier as a result of intestinal edema and intestinal ischemia, causing endotoxinemia and systemic inflammation. In addition, indoxyl sulfate and p-cresol sulfate have been identified as colon-derived uremic toxins produced in response to changes in the composition and function of the gut microbiota and are reported to be an independent predictor of cardiovascular events [36]. A strong association between ESRD and uremia has been reprted, and changes in the intestinal biochemical environment specific to patients with ESRD contribute to systemic inflammation, malnutrition, and other complications.

Compared with healthy controls, the diversity of the gut microbiota of patients with ESRD was significantly reduced, indicating that the human gut microbiota changes significantly as patients leavs from a healthy state in ESRD [37]. The current study found that the gut microbiota was more abundant in the CB group, and changes in the diversity of the gut microbiota induced by probiotics may have beneficial effects on the kidneys [18]. It was confirmed that the administration of probiotics to patients with stage III-IV chronic kidney disease altered the composition of the microbial flora and resulted in a decrease in serum urea levels [38]. Probiotics favor urea hydrolysis, and they have been shown to be urea-targeted drugs that carry out "intestinal dialysis" [39]. In addition, biomarkers of inflammation are inversely correlated with renal function [40], and the assistance of renal function with probiotics helps to control chronic inflammation by reducing urea levels [41]. Therefore, the administration of probiotics alters the environment of the gut microbiota and replaces the renal function by eliminating uremic retention solutes that are associated with increased ESRD mortality [42, 43].
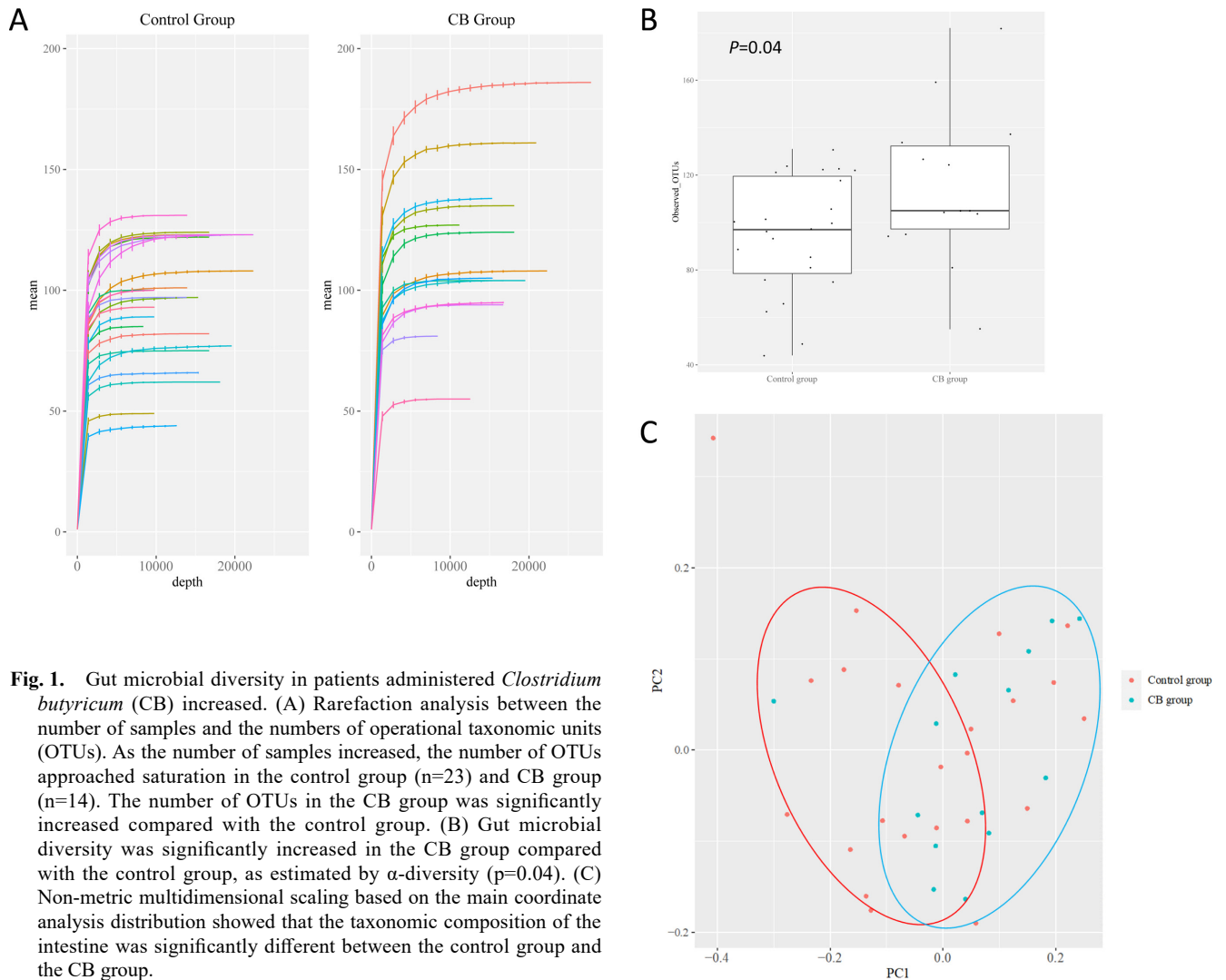
Probiotics are thought to exert anti-inflammatory effects by increasing the level of SCFAs [44]. Patients with ESRD have been shown to have low serum and fecal short chain fatty acid (SCFA) levels [45]. It has also been shown that the administration of CB increases butyric acid levels and restores the composition of the gut microbiota, thereby suppressing the production of uremic toxins [46]. Butyric acid has been shown to slow the progression of disease into ESRD [45], and acetic acid has been shown to reduce the incidence of acute kidney injury [47]. After the administration of probiotics, single-cell RNA sequencing

Fig. 1. Gut microbial diversity in patients administered *Clostridium butyricum* (CB) increased. (A) Rarefaction analysis between the number of samples and the numbers of operational taxonomic units (OTUs). As the number of samples increased, the number of OTUs approached saturation in the control group (n=23) and CB group (n=14). The number of OTUs in the CB group was significantly increased compared with the control group. (B) Gut microbial diversity was significantly increased in the CB group compared with the control group, as estimated by α-diversity (p=0.04). (C) Non-metric multidimensional scaling based on the main coordinate analysis distribution showed that the taxonomic composition of the intestine was significantly different between the control group and the CB group.

at SCFA-related receptors showed a higher expression of anti-inflammatory and regeneration-related genes [44], indicating that the protective effect of probiotics is associated with the SCFA-mediated regulation of inflammatory responses.

Uremic inflammation has been shown to be associated with protein-energy wasting (PEW) [48]. PEW is found in many patients with ESRD and causes skeletal muscle depletion [49]. Inflammation promotes PEW, and an overproduction of inflammatory factors is associated with increased metabolism and decreased body mass [50]. IL-6 is involved in the sarcopenic process by inhibiting the secretion of insulin-like growth factor-1, leading to the development of PEW and muscle catabolism [51]. In addition, diarrhea is one of the causes of malnutrition due to impaired intestinal barrier function in patients with ESRD [35, 52]. CB can be used clinically for diarrhea, constipation, and other symptoms associated with disruption of the intestinal microflora [53]. Furthermore, it enhances enterocyte integrity [18]. In the CB group in this study, IL-6 levels were significantly lower and albumin and transferrin levels were significantly higher. The administration of CB may restore the diversity of the gut microbiota in patients with ESRD, which would allow for the metabolism of urea, leading to less inflammation. It also

protects the mucosal epithelium by alleviating nutrient absorption disorders, resulting in an improved nutritional status.

Patients in both groups in this study exceeded the Kt/V value recommended by the Kidney Disease Outcomes Quality Initiative Guidelines [54], and there was no difference in β2MG between the two groups, thus dialysis efficiency did not affect uremia. Indoxyl sulphate and p-cresyl sulphate, which are other intestinal uremic toxins that are positively correlated with IL-6 [55], may have been reduced in the CB group. However, further investigations are required to determine the levels of these toxins in patients with ESRD receiving CB.

Probiotics have been shown to enhance both innate and adaptive immunity of the host immune system by reducing the presence of pathogens [56]. Some probiotic strains promote B cell differentiation, and thereby increase the production of immunoglobulin A, which is useful in preventing the invasion of pathogenic microorganisms [18]. In this study as well, the E/T ratio was higher in the CB group, indicating that innate immunity was activated.

Next-generation sequencing technology has shown that the human gut microbiota is mainly composed of seven phyla: Firmicutes, Bacteroides, Proteobacteria, Actinobacteria,
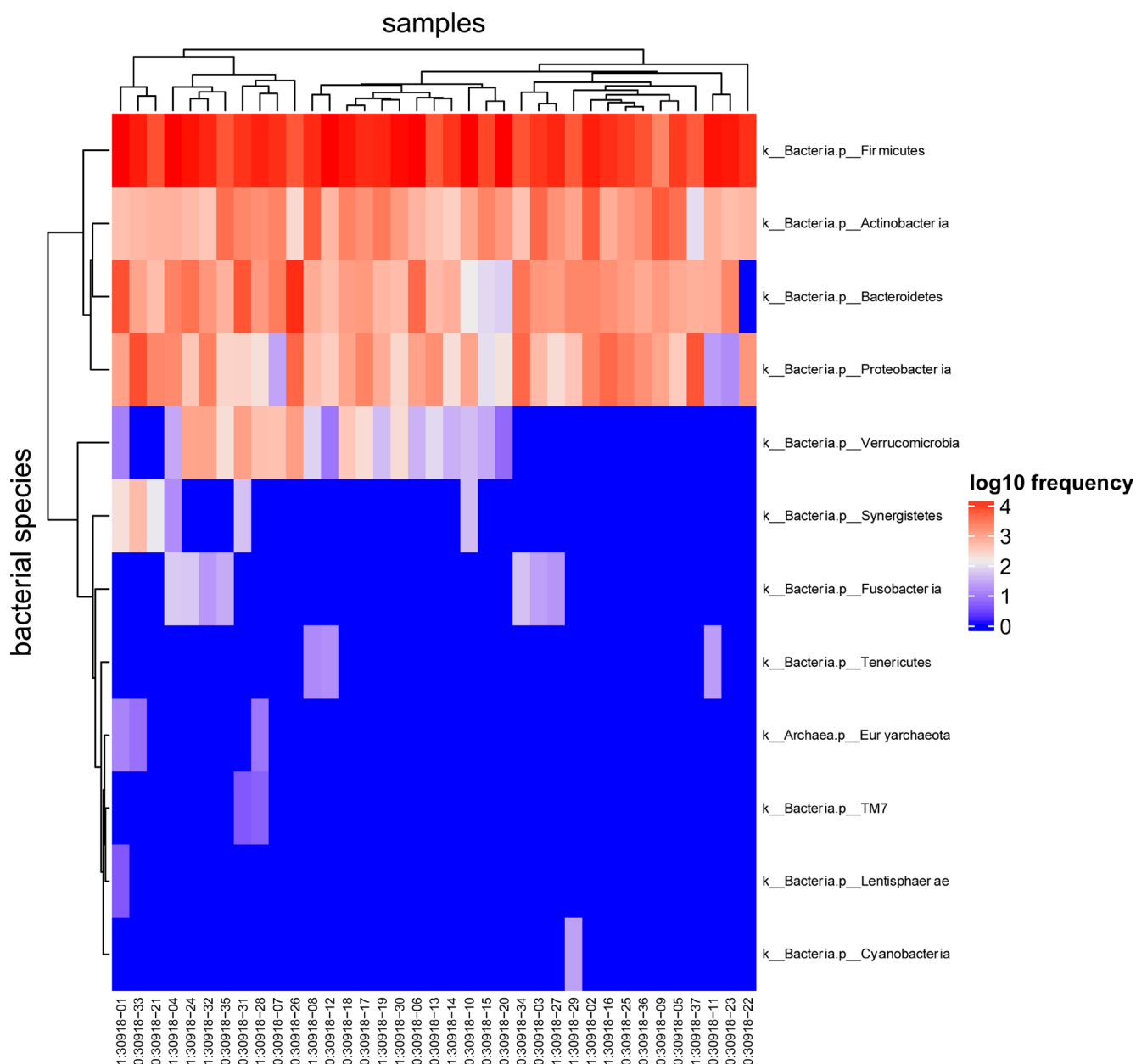
Fig. 2.   Phylogenetic profiles of the gut microbiome. The relative abundance of the bacterial community at the phylum level.

Fusobacteria, Verrucomicrobia, and Cyanobacteria [57]. The two most predominant phyla in the human gut are Firmicutes and Bacteroides [58]; however, in this study, Actinobacteria and Firmicutes were the two most predominant phyla. The increase in Firmicutes with CB administration is consistent with previous reports [59]. Antibiotics, such as streptomycin and vancomycin, have been discovered in Actinobacteria [60]. Since microbiota that survive and compete for nutrients and living space produce antibacterial compounds [61], changes in the gut microbiota in patients with ESRD may have an effect on the increase in Actinobacteria. The increased abundance of Firmicutes and Bacteroides indicates microbial imbalance [62]. The administration of CB did not alter the gut microbiota composition of Firmicutes and Bacteroides, and the F/B ratio was not significantly different between the two groups. CB increased the amount of gut microbiota, but did not appear to affect the proportions.

This study was not without limitations. It was a retrospective, observational study that included a small number of patients. A prospective, randomized controlled trial with a large number of patients is needed to determine the relationships between ESRD and the gut microbiota.

In conclusion, the α-diversity index suggests that the gut microbiota was more abundant in patients undergoing maintenance dialysis who received CB than in those who did not receive CB. CB positively affects the gut microbiota and nutritional and immunological statuses of patients with ESRD undergoing HD.
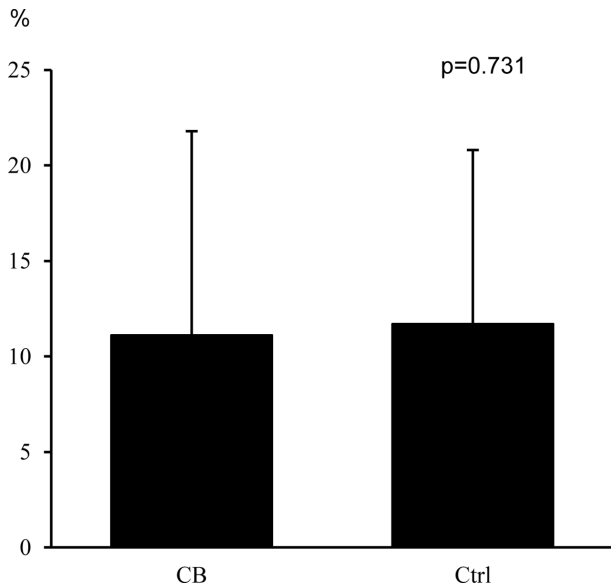
Fig. 3. The Firmicutes/Bacteroidetes ratio in the *Clostridium butyricum* (CB) group and control group.

Table 2. Comparison of the trace elements between CB and control group

	Control, N=23 (62.2)	CB, N=14 (37.8)	p-value
WBC	5.73 ± 2.18	5.49 ± 2.85	0.511
Ly	21.41 ± 9.31	26.81 ± 11.53	0.106
Hb	9.67 ± 1.55	9.95 ± 1.35	0.471
Sodium	136.73 ± 2.76	137.57 ± 4.16	0.603
Karium	4.11 ± 0.66	3.95 ± 0.53	0.626
Calcium	8.34 ± 0.91	8.51 ± 0.69	0.405
Phosphorus	3.55 ± 1.31	3.31 ± 1.19	0.481
Ferrum	48.21 ± 23.61	55.35 ± 29.75	0.481
Copper	89.56 ± 29.61	79.01 ± 35.02	0.331
Selenium	87.34 ± 21.23	86.42 ± 15.06	0.826
Zinc	52.13 ± 13.62	50.14 ± 6.65	0.937
Transferrin	158.91 ± 47.01	190.64 ± 46.94	0.043
Prealbumin	19.84 ± 8.01	23.87 ± 8.31	0.158
IgG	1420.78 ± 543.45	1608.28 ± 538.99	0.246
IgA	328.01 ± 127.59	303.85 ± 171.41	0.323
C3	79.73 ± 20.29	86.57 ± 25.32	0.433
C4	25.73 ± 6.28	26.37 ± 5.76	0.863
CD4	42.34 ± 8.44	36.13 ± 11.27	0.058
CD8	34.58 ± 10.75	33.21 ± 7.97	0.695
CD4/8	1.23 ± 0.53	1.42 ± 0.65	0.398
IL-6	23.35 ± 17.48	8.89 ± 7.91	0.001
E/T ratio			
10:1	10.35 ± 6.16	14.59 ± 3.54	0.033
20:1	19.75 ± 13.56	27.71 ± 7.59	0.021

CB: *Clostridium butyricum*; C: complement; CD: cluster of differentiation; E/T ratio: effector cells/target cells ratio; Hb: hemoglobin; Ig: immunoglobulin; IL-6: interleukin-6; Ly: lymphocyte; NK cell: natural killer cell; sIL-2R: soluble interleukin-2 receptor; WBC: white blood cell.
Data are presented as means and standard deviations for continuous variables and as numbers and percentages for categorical variables.

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