Original Article

Dietary phosphate disturbs of gut microbiome in mice

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Disorder of phosphate metabolism is a common pathological condition in chronic kidney disease patients. Excessive intake of dietary phosphate deteriorates chronic kidney disease and various complications including cardiovascular and infectious diseases. Recent reports have demonstrated that gut microbiome disturbance is associated with both the etiology and progression of chronic kidney disease. However, the relationship between dietary phosphate and gut microbiome remains unknown. Here, we examined the effects of excessive intake of phosphate on gut microbiome. Five-week-old male C57BL/6J mice were fed either control diet or high phosphate diet for eight weeks. Analysis of the gut microbiota was carried out using MiSeq next generation sequencer, and short-chain fatty acids were determined with GC-MS. In analysis of gut microbiota, significantly increased in *Erysipelotrichaceae* and decreased in *Ruminococcaceae* were observed in high phosphate diet group. Furthermore, high phosphate diet induced reduction of microbial diversity and decreased mRNA levels of colonic tight junction markers. These results suggest that the excessive intake of dietary phosphate disturbs gut microbiota and affects intestinal barrier function.

***Key Words*: hyperphosphatemia, microbiota, chronic kidney disease, tight junction, short chain fatty acids**

Inorganic phosphate (Pi) is an essential nutrient in skeletal and tooth formation, energy metabolism, and intracellular signaling1. Serum Pi levels are maintained by a complex interplay of intestinal absorption, exchange with intracellular and bone storage pools, and renal tubular reabsorption.[1–3] Parathyroid hormone (PTH), fibroblast growth factor (FGF23), and 1α,25-dihydroxyvitamin D_3 are hormones well-known for their role in regulating Pi metabolism.[1–3] Chronic kidney disease (CKD) is a global health problem associated with a significant risk of end-stage renal disease (ESRD), cardiovascular disease (CVD), and death.[4] Hyperphosphatemia is one of the major complications of CKD.[5,6] Hyperphosphatemia is associated with cardiovascular morbidity and mortality.[6,7] Elevated serum Pi level can be involved in both vascular calcification[8] and endothelial dysfunction.[9–11] Elevated serum Pi and FGF23 levels also have been reported to be associated with infectious diseases in hemodialysis patients.[12,13] Dialysis patients are more likely than patients with early-stage CKD to have a higher incidence of bacterial translocation, suggested to cause suppression of immune function and systemic inflammation.[14,15]

Recently, it has been shown that CKD is associated with "dysbiosis", a disorder of gut microbiota.[16] CKD patients and CKD model animals have reported that decreased diversity of gut microbiota, decreased short-chain fatty acid (SCFA)-producing bacteria, and increased urea toxin-producing bacteria. Decreased SCFA and increased urea toxins decrease the intestinal barrier function and increase permeability.[17] It has been speculated that dysbiosis is caused by inadequate dietary fiber intake due to potassium restriction or altered in gut microbiome by medication.[18]

Various dietary factors and patterns can affect both hyperphosphatemia and the environment of microbiota in CKD.[5,19] Excessive consumption of high Pi foods, especially those with Pi-containing food additives, increases intestinal and serum Pi concentrations.[5,20,21] In addition, luminal secretion of Pi in the gastrointestinal tract can increase in CKD patients with hyperphosphatemia,[22] therefore, we hypothesized that the luminal increase in Pi may also affect the gut microbiome. Furthermore, the impact of dietary Pi on gut microbiota has not been clarified yet. In the present study, we examined the effects of dietary Pi on gut microbiota using mice.

Materials and Methods

Animals and experimental design. Five-week-old male C57BL/6J mice were purchased from Japan SLC (Shizuoka, Japan) and individually caged in a climate-controlled room (22 ± 2°C) with a 12 h light-dark cycle. Mice were fed either 0.4% [control Pi (CP)] or 1.2% [high Pi (HP)] Pi diet, respectively, by a pair-feeding procedure for 8 weeks. The diets were based on modified AIN-93G rodent diet, and formulated with KH_2PO_4 (Table 1). The body weight of the mice was checked weekly during the experimental period, and their amount of daily food intake was recorded. All mice were euthanized under anesthesia and blood, urine, colon, and fecal samples were collected.

Table 1. Composition of the experimental diets

	Pi: 0.4% (g)	Pi: 1.2% (g)
AIN-93G	59.5	59.5
Milk casein	20.0	20.0
Sugar	5.3	5.3
Mineral Mix	1.6	1.6
$CaCO_3$	1.5	1.5
Soy bean oil	7.0	7.0
KH_2PO_4	1.7	5.3
KCl	0.4	0.0
Dextrin	3.0	0.0
Total	100.0	100.0

We used a mineral mix and an altered AIN-93G diet derived from casein, $CaCO_3$, and KH_2PO_4.

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The experiments were approved by the Animal Care and Use Committee of Tokushima University and were performed in accordance with the guidelines for the care and handling of laboratory animals.

Biochemical analysis. Plasma, urine and cecum concentrations of Pi, calcium (Ca), and creatinine (Cre) were determined using the Phospha-C test (Wako, Osaka, Japan), the Calcium-E test (Wako), and the LabAssay™ Creatinine (Wako), respectively.

Extraction of genomic DNA. Genomic DNA from fecal samples was isolated using a Favorprep Stool DNA Isolation Mini Kit (FAVORGEN Biotech Corp., Ping-Tung, Taiwan) according to the manufacturer's protocol.

Bacterial 16S rRNA sequencing. Bacterial 16S ribosomal RNA (rRNA) sequencing of fecal samples was carried out by Repertoire Genesis Inc. (Osaka, Japan) using the Miseq Illumina sequencing platform. 16S rRNA gene libraries were constructed using primers specific to the V3–V4 region. Clustering of reads into operational taxonomic units (OTUs) using the Quantitative Insights Into Microbial Ecology (QIIME) software, the merged sequences were aligned to the Greengenes database ver. 13.8 containing the sequences for OTUs devoid of chimeric

Table 2. Sequence of oligonucleotide primers in the quantitative RT-PCR analysis

Gene name	Forward	Reverse
mouse occludin	5'-TGAGCACCTTGGGATTCCG-3'	5'-AAAAGGCCTCACGGACATGG-3'
mouse claudin1	5'-GTTTGCAGAGACCCCATCAC-3'	5'-AGAAGCCAGGATGAAACCCA-3'
mouse ZO-1	5'-CTTCTCTTGCTGGCCCTAAAC-3'	5'-TGGCTTCACTTGAGGTTTCTG-3'
mouse β-actin	5'-CTGGGGTGTTGAAGGTCTCAAACATG-3'	5'-CTGACCCTGAAGTACCCCATTGAACA-3'

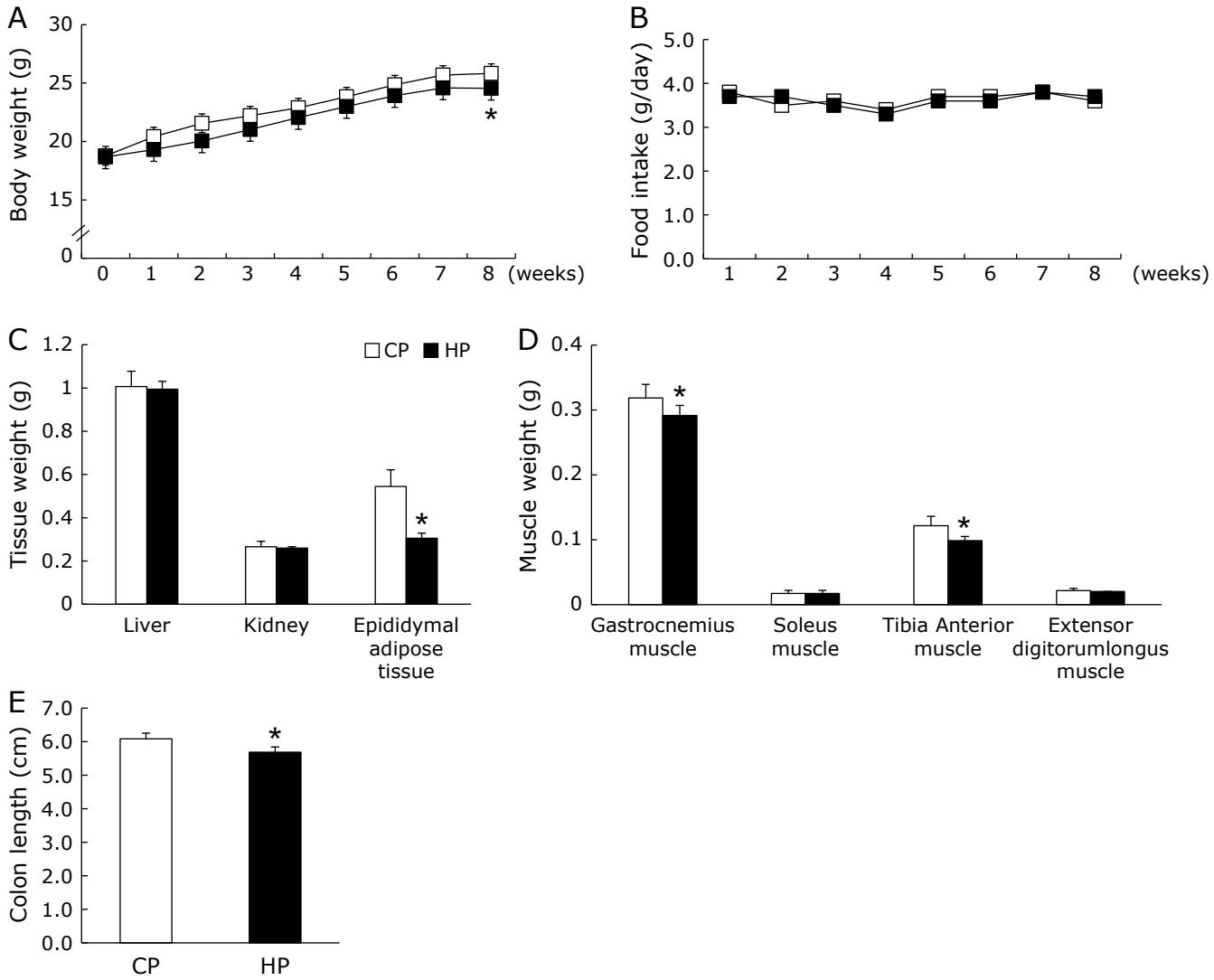

Fig. 1. Effects of dietary Pi on body weight and tissue mass. (A) Changes in body weight during experimental diets. (B) Food intake during experimental diets. (C) Weight of liver, kidney and epididymal adipose tissue. (D) Weight of skeletal muscle. (E) Changes in colon length. Data are expressed as mean ± SEM (n = 6–7). *p<0.05.

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sequences. OTUs were defined as a group of sequences with a similarity of 97% or more. Then, the rarefaction curves were constructed and α-diversity indices of the chao1 was calculated. The LDA Effects Size (LEfSe: Linear Discriminant Analysis Effects Size) algorithm was used with the online interface Galaxy (http://huttenhower.sph.harvard.edu/lefse/, accessed 2017-11-30). LEfSe coupled robust tests for measuring statistical significance (Kruskal-Wallis test) with quantitative tests for biological consistency (Wilcoxon-rank sum test). The differentially abundant and biologically relevant features are ranked by effect size after undergoing LDA. An effect size threshold between 2–3 (on a $\log_{10}$ scale) was used for all biomarkers discussed in this study.

Determination of concentration. Three hundred μl of 2-Ethylbutyric Acid (20 μg/ml, Tokyo Chemical Industry, Tokyo, Japan) solution was added to the cecum contents as an internal standard, stirred, and centrifuged (20,000 × *g*, 20 min, 4°C). Hydrogen chloride and diethyl ether were added to the super-

Table 3. Biochemical data of phosphate metabolism

	CP	HP
Plasma		
Phosphate (mg/dl)	7.66 ± 0.51	8.33 ± 0.49
Calcium (mg/dl)	8.90 ± 0.46	7.38 ± 0.32*
Creatinine (mg/dl)	0.85 ± 0.03	0.90 ± 0.08
Urine		
Pi/Cre (mg/mgCre)	8.38 ± 1.42	17.04 ± 2.63*
Ca/Cre (mg/mgCre)	0.21 ± 0.05	0.24 ± 0.04

Five-week-old male C57BL/6J mice were fed either 0.4% [control Pi (CP)] or 1.2% [high Pi (HP)] Pi diet, respectively, by a pair-feeding procedure for 8 weeks. Mean ± SEM (n = 4–7). *p<0.05 vs CP group.

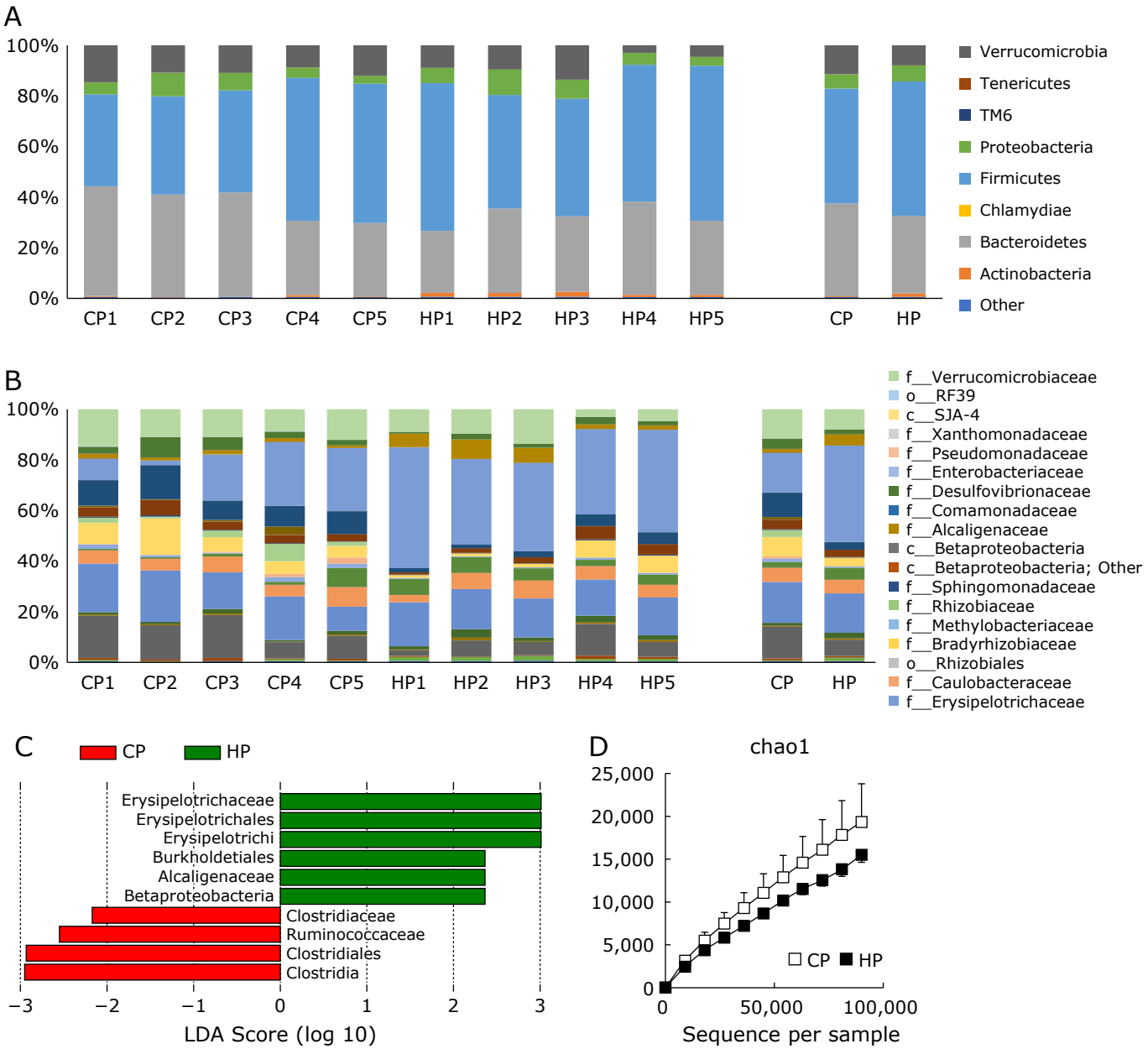

Fig. 2. Effects of dietary Pi on gut microbiome. (A) Relative abundance microbial on the basis of the average number of subfamily at phylum level and (B) class, order, family level. (C) LDA score at family level. (D) chao1 as index of α-diversity (n = 5). See color figure in the on-line version.

natant and stirred, and the organic layer was collected after centrifugation (10,000 × *g*, 3 min, 4°C). The organic layer was used as the sample solution, and the derivatization reagent MTBSTFA (Sigma-Aldrich) was added and allowed to stand for 8 h, after which acetic acid, propionic acid, and butyric acid were determined by gas chromatography. The detector was an Agilent Technologies 7890B, with a program of holding at 40°C for 2 min, rising to 170°C at 15°C/min, rising to 90°C/min, and holding at 300°C for 5 min.

RNA extraction and real-time PCR analysis. Total RNA was extracted from colonic tissue using an RNA iso Plus (TaKaRa Bio. Inc., Shiga, Japan). First strand cDNA was synthesized from total RNA (2.5 μg) using a MMLV-reverse transcriptase (Invitrogen, San Diego, CA) with an oligo-dT primer. After cDNA synthesis, real-time PCR (Applied Biosystems, Carlsbad, CA) was performed using SYBR Green PCR master mix (Thermo Fisher Scientific, Waltham, MA). The amplification programs were set as follows: initial denaturation at 95°C for 20 min, followed by 40 cycles of 95°C for 3 s, and 60°C for 30 s. The PCR products were quantified and the mRNA expression was normalized to β-actin as a housekeeping gene. The primer sequences are listed in Table 2.

Statistical analysis. Data are expressed as mean ± SEM. The significance of differences between two groups was assessed using an unpaired two-tailed *t* test. The association between cecal Pi concentration and SCFA concentration, and SCFA concentration and abundance of *Erysipelotrichaceae* and *Ruminococcaceae* were analyzed using Pearson's correlation coefficient. All data analysis was performed using GraphPad Prism 5 software (Graphpad Software, San Diego, CA). $P<0.05$ was considered to indicate statistical significance.

Results

Effects of dietary Pi on body weight and tissue weight. First, we examined the effects of dietary Pi on body weight and tissue weight of mice. Mice fed with the HP diet showed significantly decreased body weight at 8 weeks after the start of feeding (Fig. 1A), despite of no differences in total food intake among the two groups throughout the experimental period (Fig. 1B). There were no significant differences in the liver and kidney, but the epididymal fat was significantly decreased in the HP group (Fig. 1C). In comparison with skeletal muscle weight, HP group was significantly decreased gastrocnemius muscle and tibialis anterior muscle (Fig. 1D). HP group was significantly shorter colon length than CP group (Fig. 1E).

Effects of dietary Pi on biochemical parameters in plasma and urine. There were no significant differences in plasma Pi and Cre levels between two groups. However, the plasma Ca levels was significantly higher in the HP group than in the CP group (Table 3). Urinary Pi excretion increased in the HP diet group, but there were no significant differences in urinary Ca and Cre levels.

Gut microbiota and α-diversity are altered by dietary Pi. Next, we analyzed the microbial composition using the 16S rRNA sequencing to elucidate the alteration of the gut microbiota by dietary Pi. Compared with the CP group, the HP group showed that a population of gut microbiota was altered (Fig. 2A and B). To investigate the specific differences in gut microbiota between CP and HP groups, LEfSe difference analysis was performed. In the HP group, the relative abundances of *Erisipelotrichaceae* were higher, and *Ruminococcaceae* were lower than the CP group (Fig. 2C). The α-diversity refers to the diversity within samples in terms of species richness. Chao1 was lower in the HP group than in the CP group (Fig. 2D).

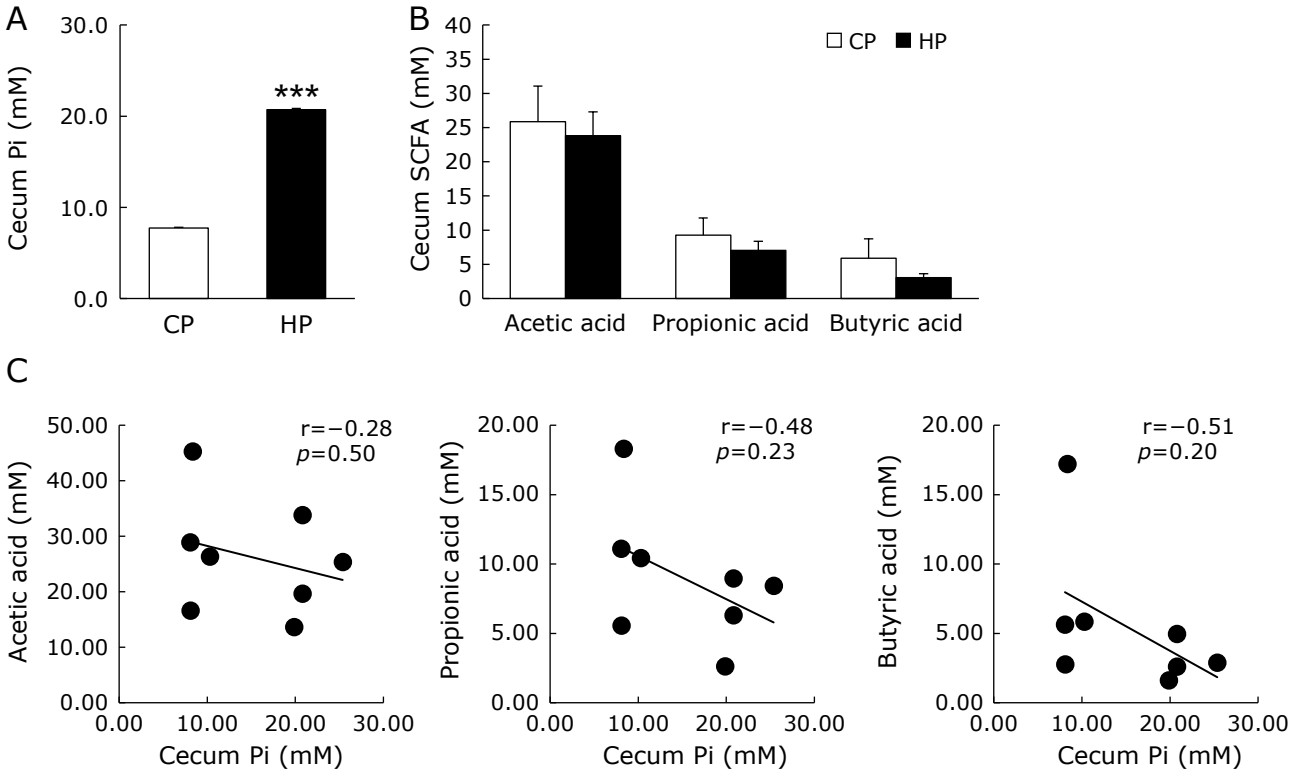

Fig. 3. Effect of dietary Pi on cecum Pi concentration and SCFA concentration. (A) Cecum Pi concentration. (B) Concentration of acetic acid, propionic acid and butyric acid in cecum. (C) Correlation of cecum Pi concentration with concentration of acetic acid, propionic acid and butyric acid. Data are expressed as mean ± SEM (n = 4–5). ***$p<0.0001$.

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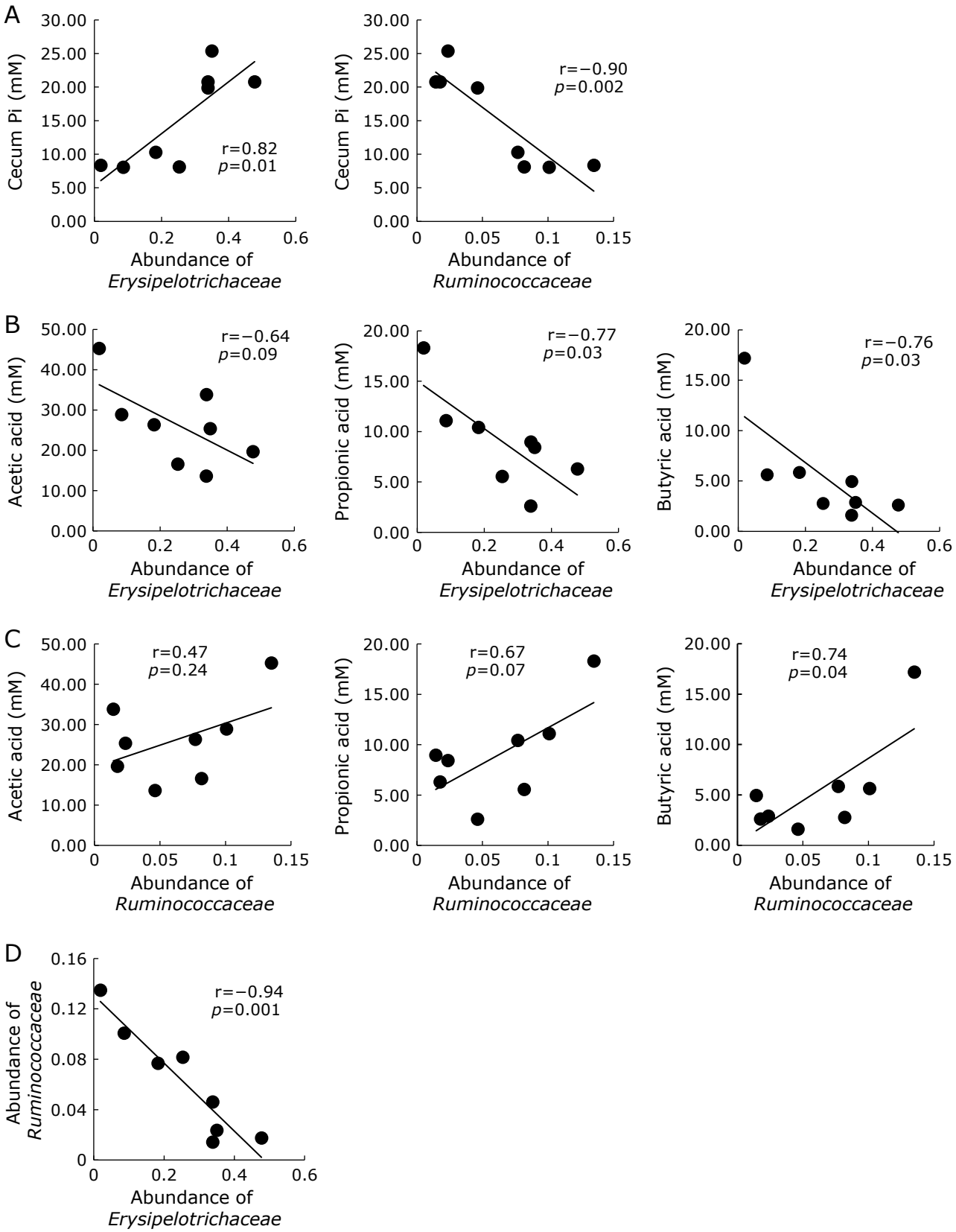

Fig. 4. Abundance of *Erisipelotrichaceae* and *Ruminococcaceae* correlate with SCFA concentration. (A) Correlation of cecum Pi concentration with abundance of *Erisipelotrichaceae* and *Ruminococcaceae* (B) Correlation of abundance of *Erisipelotrichaceae* with concentration of acetic acid, propionic acid and butyric acid. (C) Correlation of abundance of *Ruminococcaceae* with concentration of acetic acid, propionic acid and butyric acid. (D) Correlation of abundance of *Erisipelotrichaceae* with abundance of *Ruminococcaceae.* Data are expressed as mean ± SEM (n = 4–5).

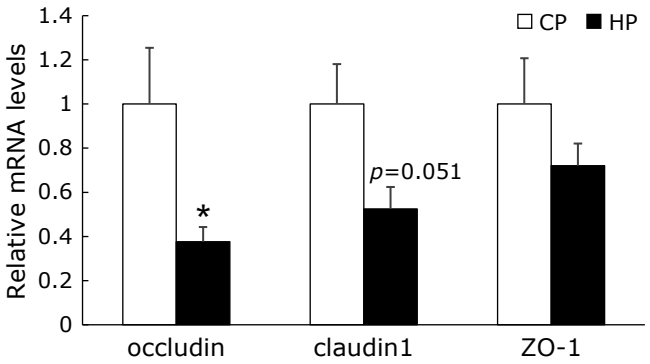

Fig. 5. Effects of dietary Pi on colonic tight junction mRNA levels. (A) occludin, (B) claudin1, (C) ZO-1 mRNA levels of colonic tissue were determined by quantitative real-time RT-PCR analysis. Data are expressed as mean ± SEM (n = 6–7). *p<0.05.

Effect of dietary Pi on cecum Pi concentration and SCFA concentration. To examine the influx of dietary Pi into the colon, we evaluated the cecum Pi concentration. HP group was 3-fold higher than the CP group (Fig. 3A). However, there were no significant differences in acetic acid, propionic acid, butyric acid concentration between the two groups (Fig. 3B).

Abundance of *Erisipelotrichaceae* and *Ruminococcaceae* correlate with SCFA concentration. Excessive intake of dietary Pi significantly altered the abundance of *Erysipelotrichaceae* and *Ruminococcaceae*. The abundance of these bacteria was significantly correlated with cecum Pi concentration (Fig. 4A). Interestingly, *Erysipelotrichaceae* which are known to be involved in inflammation,[23] was negatively correlated with cecum SCFA concentrations (Fig. 4B). In contrast, *Ruminococcaceae* known to be involved in butyric acid production,[24] correlated positively with cecum SCFA concentration (Fig. 4C). These bacteria in contrast showed a significantly negatively correlation (Fig. 4D).

Effects of dietary Pi on colonic tight junction mRNA levels. The tight junction (TJ) is one of the intercellular connections that connect adjacent epithelial cells and prevent various molecules from passing between cells. We measured colonic occludin, claudin1, and zonula occludens-1 (ZO-1) mRNA expression. As shown in Fig. 5, occludin mRNA levels in the HP group were significantly decreased than that in the CP group. In addition, claudin1 and ZO-1 mRNA levels tended to be decreased by HP diet.

Discussion

In the present study, we investigated the effects of dietary Pi on the gut microbiome. The HP group showed the altered composition of the gut microbiota, including the increased *Erysipelotrichaceae* and the decreased *Ruminococcaceae*. Furthermore, ingestion of HP diet decreased microbial diversity and mRNA levels of TJs markers in the colon.

HP group mice were tended to decrease in body weight compared with CP group. Our previous study showed that HP diet negatively regulate lipid synthesis in the liver and increase mRNA expression related to lipid oxidation and UCP1 in BAT, thereby preventing visceral fat accumulation.[25,26] In fact, HP group significantly decreased fat mass in this study, as consistent with previous study. Plasma Pi levels were no differences between CP and HP group, but urinary Pi excretion was significantly increased in the HP group. Urinary Pi excretion is equivalent to Pi absorption in gastrointestinal tract, we confirmed that HP group absorbed more Pi than CP group. Dietary Pi is absorbed in the proximal small intestine by type 2b sodium dependent cotransporters (NaPi-2b) expressed on the apical membranes of enterocytes; an estimated 70% of all dietary Pi content is absorbed via the small intestine.[27,28] Sugihara *et al.*[29] reported that fed a HP diet to colitis model mice exacerbated the condition, and that the expression of NaPi-2b, which is responsible for phosphate absorption, was decreased in the colon. Although it is widely recognized the small intestine is responsible for most Pi absorption, cecum Pi was 3-hold higher than the HP group compared with the CP group. This result suggested that excessive dietary Pi can also load Pi into the colon, consequently affect the local environment for gut microbiome.

Erysipelotrichaceae was significantly increased in the HP group. This bacterial family belongs to the Firmicutes phylum, and various report for the host physiology and/or disease are on the rise. The relative abundance of *Erysipelotrichi* positively correlated with TNF-α levels in the studies investigating patients who had chronic HIV infection and were receiving suppressive antiretroviral therapy and HIV-uninfected controls.[30,31] In addition, *Erysipelotrichaceae* is enriched in colorectal cancer patients, and animal models of colon cancer and inflammatory bowel diseases (IBD).[23,32,33] These reports suggest that *Erysipelotrichaceae* is related to inflammation in the gastrointestinal tract. More interestingly, although *Erysipelotrichaceae* was increased in the adenine-induced CKD mice, it was decreased with improvement of renal failure.[34] *Erysipelotrichaceae* may also affect the deterioration of CKD. Thus, *Erysipelotrichaceae* is associated with various diseases, but its physiological effects remain unclear. In the present results, abundance of *Erysipelotrichaceae* was significantly negatively correlated with SCFA concentrations. In addition, the abundance of *Erysipelotrichaceae* showed a significant negative correlation with the abundance of *Ruminococcaceae*. Therefore, it is suggested that *Erysipelotrichaceae* correlated with SCFA concentrations because the HP diet altered the gut microbiome, which *Ruminococcaceae* was inferior.

In contrast, *Ruminococcaceae* was significantly decreased in the HP group. This bacterial family produces SCFA, especially butyrate, which is an important energy source for intestinal epithelial cells and thus plays a key role in maintaining gut homeostasis.[35] Mariadason *et al.*[36] have reported that SCFA enhances the paracellular permeability in Caco-2 cell. In addition, intestinal barrier functions are impaired in CKD.[17] This impairment leads to a high intestinal epithelial permeability, resulting in bacterial translocations from the gut lumen into the bloodstream, and this increased bacterial translocation is reported to be a source of microinflammation in CKD.[7] SCFA are important for intestinal barrier function maintaining TJ. HP diet was decreased in mRNA expression of components of TJ in the colon, this is presumed to be related to the reduction of *Ruminococcaceae*. However, the present results that SCFA concentrations did not differ between the two groups. The SCFA concentration in the intestinal lumen is determined by the difference between the rate of SCFA production by the intestinal microflora and the absorption by bacteria and epithelial cells.[37] It is possible that the difference was not observed because the rate of absorption was not taken into account in this evaluation.

This study had some limitations. The first issue was the analysis of the intestinal microflora could only be performed down to the family level. For example, *Clostridia* contains both pathogenic and SCFA-producing bacteria, and it was not possible to determine which one decreased in this study. More detailed analysis will be needed in the future. Another limitation was the composition of the gut microbiota differs between mice and humans, so future analysis on humans is also necessary.

We conclude that dietary Pi alter the composition of gut microbiota and can decrease the microbial diversity. Furthermore, these results suggest that dietary Pi may decrease the TJ in gastrointestinal tract. Such a disturbance may be related to the progres-

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sion of CKD. Adequate management of dietary Pi would be need to keep the gut environment in good state, and be another favorable effect of dietary Pi restriction in CKD patients.

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Conflict of Interest

YT received grant from Bayer Yakuhin, Ltd. The other authors have no conflicts of interest.

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